INTERACTION BETWEEN RESPIRATORY CARBON FLOW AND
PHOTOSYNTHETIC LIGHT HARVESTING IN THE GREEN ALGA,

SELENASTRUM MINUTUM

by

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ABSTRACT

Although the regulation of respiration by photosynthesis has been extensively studied, very little is known about the regulation of photosynthesis by respiration. In this thesis, it was proposed that changes in respiratory carbon flow would affect the ratio of reduced/oxidized pyridine nucleotides and affect reduction of the PQ(cyt b$_6$f) pool via a thylakoid-bound NAD(P)H-PQ oxidoreductase. In turn, reduction of the PQ(cyt b$_6$f) pool would result in a state 1 to 2 transition which could poise the photosynthetic electron transport chain for a decrease in NADPH production. The purpose of the present study was to rigorously test the hypothesis that increased respiratory carbon flow, which increased reduced/oxidized pyridine nucleotide ratios, would increase PQ reduction and result in a state transition. The corollary of this hypothesis was that increased respiratory carbon flow, which did not affect the ratio of reduced/oxidized pyridine nucleotides, would not affect PQ(cyt b$_6$f) reduction or result in a state transition. In the green alga, *Selenastrum minutum*, five treatments were shown to increase dark respiratory carbon flow as measured by CO$_2$ release and/or starch degradation. These treatments were further subdivided on the basis of their ability to cause a state transition. Class 1 treatments included NH$_4^+$ assimilation by N-limited cells, anaerobic treatment, and uncoupling with CCCP and all resulted in large perturbations in room temperature and 77K fluorescence emission indicative of a state 1 to 2 transition. These changes were correlated with reduction of the PQ pool as measured by changes in the kinetics of time-resolved fluorescence decay and induction. Class 2 treatments included NO$_3^-$ assimilation by N-limited cells and Pi assimilation by Pi-limited cells. Both of these treatments resulted in only small changes in fluorescence emission suggesting that a state 1 to 2 transition had not occurred. Both NO$_3^-$ and Pi treatment had only minor effects on PQ(cyt b$_6$f) reduction as measured by fluorescence decay kinetics. However, in cells treated with NO$_3^-$, measurement of PQ reduction made using fluorescence induction kinetics was not consistent with the original hypothesis. This inconsistency was proposed to result from actinic effects of signal averaging. Increases in the
NADH/NAD but not NADPH/NADP ratio were correlated with class 1 treatments while class 2 treatments resulted in small (Pi) or intermediate (NO₃⁻) changes in NADH/NAD ratios. Key respiratory metabolites were examined for each of the five treatments. An examination of the combined mass action ratio for TP to PGA conversion indicated that carbon flow, via NAD-GAPDH and PGA kinase, was significantly enhanced by class 1 treatments while no significant change was noted for class 2 treatments. NAD-GAPDH has been shown to be 86% localized in the chloroplast in *Chlamydomonas reinhardtii* (Klein, 1986 Planta 167:81). It was proposed that chloroplastic NAD-GAPDH activity was responsible for an increase in chloroplastic NADH/NAD ratios, reduction of PQ, and a state transition. The results of the present study suggest that respiratory carbon flow can regulate the poising of the photosynthetic electron transport chain for the NADPH/ATP production ratio via the state transition. This may have ramifications for interactions between respiration and photosynthesis in both the dark and the light. The physiological significance of this interaction was discussed.
# TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. ii

TABLE OF CONTENTS ................................................................................................................ iv

LIST OF FIGURES ....................................................................................................................... vii

LIST OF TABLES .......................................................................................................................... ix

LIST OF SYMBOLS AND ABBREVIATIONS ................................................................................. x

ACKNOWLEDGMENTS .................................................................................................................. xii

PROLOGUE ..................................................................................................................................... xiii

CHAPTER 1: THEORETICAL CONSIDERATIONS
GENERAL INTRODUCTION AND LITERATURE REVIEW .......................................................... 1
  Photosynthesis .......................................................................................................................... 1
  Respiration ............................................................................................................................... 6
  Interaction between photosynthesis and respiration .............................................................. 8
  A mechanism to regulate the ratio of linear to cyclic electron transport ................................ 14
  Can respiratory carbon flow influence the redox state of the PQ(cyt b$_{6}f$) pool
  and poise the ratio of linear to cyclic electron flow?: ..................................................... 20
  The hypothesis to be tested: ................................................................................................. 31

CHAPTER 2: EXPERIMENTAL RATIONALE: A FRAMEWORK TO TEST
THE HYPOTHESIS
INTRODUCTION ............................................................................................................................ 33
  Rationale for the hypothesis to be tested .............................................................................. 33
  Methods which can be used to measure state transitions ..................................................... 34
  Rationale for working with Selenastrum minutum ............................................................... 37

MATERIALS AND METHODS .................................................................................................... 38
  Cell culture (chemostats) ....................................................................................................... 38
  Treatments .............................................................................................................................. 39
  Experimental ......................................................................................................................... 40
  Fluorescence measurements ............................................................................................... 40
  Other measurements ............................................................................................................ 41

RESULTS ....................................................................................................................................... 42
  Effect of treatments on CO$_{2}$ efflux and starch degradation ................................................ 42
  Effect of treatments on fluorescence emission ...................................................................... 42

DISCUSSION ............................................................................................................................... 47
  Effect of treatments on respiratory carbon flow ................................................................. 47
  Effect of treatments on fluorescence emission .................................................................... 51
SUMMARY........................................................................................................................................... 55

CHAPTER 3: TESTING THE MODEL: THE EFFECTS OF CLASS 1 and CLASS 2 TREATMENTS ON REDUCTION OF THE PQ(CYT b6f) POOL
INTRODUCTION ........................................................................................................................................ 57
Methods which can be used to infer the redox state of the PQ pool ................................................. 58
MATERIALS AND METHODS................................................................................................................. 60
Experimental conditions ....................................................................................................................... 60
Fluorescence ......................................................................................................................................... 60
RESULTS............................................................................................................................................... 63
Time-resolved fluorescence decays ....................................................................................................... 63
Time-resolved fluorescence inductions ................................................................................................ 65
DISCUSSION .......................................................................................................................................... 69
DCMU and Class 1 treatments: ........................................................................................................... 69
Class 2 treatments (Pi and NO3– assimilation) .................................................................................... 71
SUMMARY............................................................................................................................................. 73

CHAPTER 4: THE EFFECTS OF CLASS 1 AND CLASS 2 TREATMENTS ON THE REDOX STATE OF THE PYRIDINE NUCLEOTIDE POOL
INTRODUCTION ........................................................................................................................................ 75
Techniques to measure pyridine nucleotides ....................................................................................... 77
MATERIALS AND METHODS................................................................................................................. 78
Experimental ......................................................................................................................................... 78
Pyridine nucleotide determinations .................................................................................................... 78
Fluorescence ......................................................................................................................................... 80
RESULTS............................................................................................................................................... 80
NADP, NADPH, and the NADPH/NADP ratio ..................................................................................... 81
NAD, NADH, and the NADH/NAD ratio .............................................................................................. 83
Fluorescence ......................................................................................................................................... 87
DISCUSSION .......................................................................................................................................... 90
NADP, NADPH, and the NADPH/NADP ratio ..................................................................................... 90
NAD, NADH, and the NADH/NAD ratio .............................................................................................. 92
SUMMARY............................................................................................................................................. 96

CHAPTER 5: INTERACTION BETWEEN RESPIRATORY CARBON FLOW AND THE STATE TRANSITION AFTER UNCOUPLING WITH CCCP
INTRODUCTION ........................................................................................................................................ 98
MATERIALS AND METHODS................................................................................................................. 99
Experimental........................................................................................................................................ 99
Starch degradation ............................................................................................................................... 100
Metabolites .......................................................................................................................................... 100
Gas exchange ...................................................................................................................................... 100
Fluorescence ....................................................................................................................................... 101
RESULTS............................................................................................................................................... 101
The effect of CCCP on key respiratory metabolites ........................................................................... 101
CHAPTER 6: DEVELOPMENT OF A COMPREHENSIVE MODEL FOR THE INTERACTION BETWEEN RESPIRATORY CARBON FLOW AND POISING OF THE STATE TRANSITION

INTRODUCTION .................................................................................................................. 124

RESULTS ........................................................................................................................... 126
Kinetics of changes in ADP, Pyr/PEP and FBP/F6P ......................................................... 126
PGA/TP, NADH/NAD, ATP/ADP and $\Gamma \left( \frac{[PGA][NAD][ATP]}{[TP][NAD][ADP]} \right)$ ......................................................... 129

DISCUSSION ..................................................................................................................... 131
Activation of respiratory carbon flow: the effect of class 1 and 2 treatments on ADP, Pyr/PEP, FBP/F6P and PGA/TP .......................................................... 131
The effect of class 1 and 2 treatments on the combined mass action ratio for NAD-GAPDH and PGA kinase ................................................................. 132
The physiological significance of interaction between respiratory carbon flow and photosynthesis via the CRETC in vivo .......................................................... 136

CHAPTER 7: GENERAL SUMMARY AND CONCLUSIONS ............................................. 139

LITERATURE CITED ........................................................................................................ 143

APPENDIX 1: APPROXIMATION OF $q_{NP}$ AND $q_p$ ..................................................... 163

APPENDIX 2: CORRECTION OF TIME-RESOLVED FLUORESCENCE DECAYS FOR THE ACTINIC EFFECTS OF THE MEASURING BEAM
INTRODUCTION .................................................................................................................. 166
MATERIALS AND METHODS .......................................................................................... 167
RESULTS ........................................................................................................................... 167
DISCUSSION ..................................................................................................................... 173

APPENDIX 3: METABOLITE ASSAYS ............................................................................ 177

CURRICULUM VITAE ....................................................................................................... 179
LIST OF FIGURES

Figure 1: A diagrammatic representation of the photosynthetic electron transport chain and carbon fixation in the chloroplast .......................................................... 2

Figure 2: Pathways of respiratory carbon flow and mitochondrial respiratory electron transport in photosynthetic organisms.............................................................. 7

Figure 3: The phosphorylation mobile antennae model for the state transition .......... 16

Figure 4: Thylakoid electron transport pathways which share plastoquinone in green algae ...................................................................................................................... 23

Figure 5: The effects of treatments which increase respiratory carbon flow on steady-state saturation pulse fluorescence analysis .................................................. 44

Figure 6: The effect of class 1 treatments on absolute fluorescence emission at 77K ..... 48

Figure 7: The effect of class 1, class 2 and DCMU treatments on corrected time-resolved fluorescence decay kinetics ................................................................. 64

Figure 8: The effect of class 1, class 2 and DCMU treatments on time-resolved fluorescence induction kinetics ................................................................. 67

Figure 9: The effect of class 1 treatments on NADP, NADPH and the NADPH/NADP ratio ................................................................. 82

Figure 10: The effect of class 2 treatments on NADP, NADPH and the NADPH/NADP ratio ................................................................. 84

Figure 11: The effect of class 1 treatments on NAD, NADH and the NADH/NAD ratio ................................................................. 85

Figure 12: The effect of class 2 treatments on NAD, NADH and the NADH/NAD ratio ................................................................. 88

Figure 13: The effect of oxygen re-addition on room temperature fluorescence from cells which were anaerobically adapted for 20 minutes ......................................... 89

Figure 14: The effect of CCCP treatment on the long term rate of starch degradation ... 103

Figure 15: The effect of CCCP treatment on the cellular concentration of adenylates ...... 104
**Figure 16:** The effect of CCCP treatment on the cellular concentration of pyruvate and phospho *enol* pyruvate .......................................................... 105

**Figure 17:** The effect of CCCP treatment on cellular levels of fructose bisphosphate, fructose-6-phosphate and triose-phosphate .......................................................... 106

**Figure 18:** The effect of CCCP treatment on cellular levels of glucose-6-phosphate and glucose-1-phosphate .......................................................... 108

**Figure 19:** The effect of CCCP treatment on the rate of dark CO₂ efflux as measured by an open gas exchange IRGA system .......................................................... 110

**Figure 20:** The effect of CCCP treatment on the rate of dark O₂ consumption .......... 112

**Figure 21:** The effect of CCCP treatment on room temperature and 77K fluorescence parameters .......................................................... 113

**Figure 22:** The proposed mechanism for interaction between respiratory carbon flow and poising of the PETC by the state transition after uncoupling with CCCP .......... 115

**Figure 23:** The effect of light intensity on the measurement of room temperature fluorescence parameters .......................................................... 165

**Figure 24:** The effect of DCMU and class 1 and 2 treatments on uncorrected time-resolved fluorescence decay kinetics .......................................................... 169

**Figure 25:** The effect of DCMU and class 1 and 2 treatments on the level of fluorescence induced by the 100 kHz measuring beam .......................................................... 172
LIST OF TABLES

Table 1: The effect of a variety of treatments on the rate of respiratory carbon flow in *S. minutum* as measured by the rate of CO$_2$ efflux and starch degradation ......................... 43

Table 2: A summary of the effect of treatments which increased respiratory carbon flow on room temperature and 77K fluorescence parameters in *S. minutum* ......................... 46

Table 3: The effect of DCMU and class 1 and 2 treatments on the amplitude and half-times of the fast and medium components of time-resolved fluorescence decays which were corrected for the actinic effect of the 100 kHz measuring beam................................................. 66

Table 4: The effect of DCMU and class 1 and 2 treatments on $A_{\text{max}}$, the area above time-resolved fluorescence induction curves .................................................................................. 68

Table 5: The effect of CCCP on the PEP/TP, ATP/ADP, NADH/NAD and combined mass action ratio for NAD-GAPDH and PGA kinase compared to the dark aerobic control ............................................................................................................. 109

Table 6: A comparison of the effects of class 1 and class 2 treatments on changes in key respiratory intermediates and their ratios ......................................................................................... 127

Table 7: The effect of illumination and treatments which increase the rate of respiratory carbon flow on the PGA/TP, ATP/ADP, NADH/NAD and combined mass action ratio compared to the dark aerobic control ................................................................. 130

Table 8: The effect of DCMU and treatments which increase respiratory carbon flow on the amplitude and half times of the fast and medium components of time-resolved fluorescence decays which were not corrected for the actinic effect of the 100 kHz measuring beam ......................................................................................... 170
LIST OF SYMBOLS AND ABBREVIATIONS:

$\alpha_i$ amplitude of the $i$th component of a time-resolved fluorescence decay
ADP adenosine diphosphate
ADRY Accelerator of the Deactivation Reaction of Y, the $\text{H}_2\text{O}$ oxidizing complex of PS2
$A_{\text{max}}$ area between $F_M$ and the time-resolved fluorescence induction curve
AMP adenosine monophosphate
ATP adenosine triphosphate
CCCP carbonyl cyanide-$m$-chlorophenyl-hydrazone
chl chlorophyll
class 1 treatments which increase respiratory carbon flow and cause a state transition
class 2 treatments which increase respiratory carbon flow but do not cause a state transition
CRETC chloroplastic respiratory electron transport chain
cyt cytochrome
DCMU 3-(3,4-dichlorophenyl)-1,1 methyl urea
DBMIB dibromothymoquinone
e$^-$ electron
$F$ the level of fluorescence induced by the measuring beam after treatment
FADH$_2$ flavin adenine dinucleotide
FBP fructose 1,6, bisphosphate
FBPase fructose 1,6 bisphosphatase
$F_0$ minimal level of fluorescence induced by the "non-actinic" measuring beam in dark-adapted cells
$\Phi_p$ quantum yield of PS2 upon illumination
Fd ferredoxin
$F_M$ maximal level of fluorescence induced by a saturating flash
FNR ferredoxin NADP reductase
F6P fructose 6-phosphate
GAPDH glyceraldehyde 3-phosphate dehydrogenase
G6P glucose-6-phosphate
G6PDH glucose-6-phosphate dehydrogenase
G6P glucose 6-phosphate
G1P glucose 1-phosphate
H$_2$ase hydrogenase
KCN potassium cyanide
LHC2 major light harvesting complex of PS2 in higher plants and green algae
LHC1 major light harvesting complex of PS1 in higher plants and green algae
METC mitochondria electron transport chain
NADH nicotinamide adenosine dinucleotide
NADPH nicotinamide adenosine dinucleotide phosphate
NH$_4^+$ ammonium
NO$_3^-$/NO$_2^-$ nitrate/nitrite
OPP  oxidative pentose phosphate (pathway)
P_{680}, P_{700}  reactive chlorophylls of photosystem 2 and 1, respectively
PC  plastocyanin
PEP  phospho enol pyruvate
PETC  photosynthetic electron transport chain
PFK  phosphofructokinase
PGA  phosphoglycerate
Pi  inorganic phosphate
6PG  6-phosphogluconate
6PGDH  6-phosphogluconate dehydrogenase
PK  pyruvate kinase
PRK  phosphoribulokinase
PQ/PQH$_2$  plastoquinone/plastoquinol
PS2  photosystem 2
PS1  photosystem 1
Pyr  pyruvate
QA  primary quinone acceptor of PS2
QB  secondary quinone acceptor of PS2
 qE  energized quenching of fluorescence due to $\Delta$pH
qI  quenching of fluorescence due to photoinhibition
qNP  non photochemical quenching
qp  photochemical quenching
q(t)  fraction of reduced PS2 reaction centres at time, t
QT  quenching of fluorescence due to a state transition
RUBISCO  ribulose bisphosphate carboxylase/oxygenase
RuBP  ribulose bisphosphate
SHAM  salicylhydroxamic acid
TCA  tricarboxylic acid (cycle)
T$_i$  the half-time of the i$^{th}$ component of a time-resolved fluorescence decay
TP  triose phosphate (refers to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate)
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PROLOGUE

This thesis is an integrative work and covers topics ranging in diversity from physiology to biophysics. The challenge in engaging these different disciplines is to introduce each concept in context and to provide enough theory for the uninitiated reader. To this end, I have written an extremely general literature review to introduce the larger concepts on which the experimental rationale for this thesis is based. Each subsequent chapter deals with a subset of the larger, integrative hypothesis developed in the first chapter. A more specific review of the pertinent theory and techniques utilized is provided in the introductory section of each chapter. For added clarity, each chapter is briefly set into the context of conclusions from the preceding chapters.
CHAPTER 1: THEORETICAL CONSIDERATIONS

GENERAL INTRODUCTION AND LITERATURE REVIEW

From an anthropocentric viewpoint, human survival depends upon the use of energy initially fixed by photosynthetic organisms. Photosynthesis is an anabolic process which converts light energy into chemical energy in the form of ATP and NADPH which are used as the energetic currency of the living world. In the plant cell, ATP and NADPH equivalents are stored in reduced carbon compounds via photosynthetic carbon fixation. In turn, the energy stored in these compounds can be released for cellular metabolism by respiration which is catabolic in nature. Since cellular metabolism, including respiration, is not completely efficient, some of the energy stored in reduced carbon compounds is lost as heat. Without photosynthesis to continually replace energy depleted by the respiratory and metabolic needs of heterotrophic and photosynthetic organisms, the free energy level of the biosphere would rapidly decrease. Despite the importance of photosynthesis to life on this planet, relatively little is known about its regulation or interaction with other metabolic processes in the photosynthetic cell. Even less is known about the interaction between photosynthesis and respiration.

The following discussion will review the pertinent concepts of photosynthesis and respiration and move on to explore what is known about interactions between photosynthesis and respiration. Then, a theoretical mechanism will be developed whereby respiration may regulate photosynthetic light harvesting to alter the poising of ATP and NADPH production by the light reactions of photosynthesis. This will provide an explanation of the experimental rationale for this research.

Photosynthesis:

Photosynthesis consists of three distinct but intimately related processes which include light harvesting, the photosynthetic electron transport chain (PETC, see list of abbreviations) and the production of reduced carbon compounds (Calvin cycle) (Figure 1).
**Figure 1:** A diagrammatic representation of the photosynthetic electron transport and carbon fixation in the chloroplast. For an explanation see text. Enzymes: 1, Ribulose bisphosphate carboxylase/oxygenase; 2, phosphoglycerate kinase; 3, glyceraldehyde phosphate dehydrogenase; 4, Triose-phosphate isomerase; 5,8, aldolase; 6, Fructose bisphosphatase; 7,10,12, transketolase; 9, sedoheptulose bisphosphatase; 11, Ribose-5-phosphate isomerase; 13, Ribulose-5-phosphate 3-epimerase; 14, phosphoribulokinase. Abbreviations: Ru5P, ribulose 5-phosphate; RuBP, ribulose bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; TPP, thiamine pyrophosphate; C2, 2 carbon compound; CF₀/CF₁, coupling factor or chloroplastic ATP synthase; hv, light. For rest of abbreviations, see list of abbreviations.
Photosynthetic light harvesting and electron transport: Light harvesting by accessory antenna pigments is required to provide excitation to the reaction center chlorophylls of photosystem 2 (PS2) and photosystem 1 (PS1), P680 and P700, respectively. The efficiency of electron transport, and hence carbon fixation, is dependent on the light harvesting capacity of the two photosystems. In a process known as primary charge separation excitation of the reaction center chlorophyll allows reduction and oxidation of the primary electron (e\textsuperscript{-}) acceptor and donor of each photosystem. With the help of a series of electron transfer components such as plastoquinone (PQ), cytochrome (cyt) b\textsubscript{6}f, and plastocyanin (PC), electrons are transferred in a coordinated light dependent fashion from water to ferredoxin via PS2 and PS1 in a manner which has been formalized in the Z scheme (Hill and Bendal, 1960). The components of the light reactions of photosynthesis, known as the photosynthetic electron transport chain (PETC), are located in the thylakoid membrane such that electrons are transported from the thylakoid lumen to the thylakoid exterior and protons are transported in the opposite direction; the result is the production of a transthylakoid electrochemical gradient. Dissipation of this gradient is accomplished by the ATP synthase which converts the free energy of the transthylakoid electrochemical gradient into ATP.

During linear electron transport, electrons from Fd\textsubscript{red} are utilized to produce NADPH via Fd/NADP reductase (FNR). Although the regulation of electron flow pathways is not well understood, electrons from Fd\textsubscript{red} can also be utilized to reduce O\textsubscript{2} (pseudocyclic e\textsuperscript{-} transport or Mehler reaction), PQ(cyt b\textsubscript{6}f) (cyclic electron transport) or NO\textsubscript{3}/NO\textsubscript{2}. The relative ratio of these types of electron transport can affect the stoichiometry of the ATP/NADPH production ratio via the light reactions. For more detailed reviews see Lawlor (1987), Murphy (1986) and Nelson and Prezelin (1990).

Thylakoid lateral heterogeneity: In green algae and higher plants, improvements in thylakoid sub-fractionation techniques and immunocytochemical electron microscopy have shown that intrinsic thylakoid protein complexes exhibit heterogeneous lateral distribution.
(Anderson, 1992, 1989; Vallon et al., 1986; Olive et al., 1986). The two photosystems are observed to be unequally distributed; PS2 is found almost exclusively in the stacked granal thylakoids while PS1 is found in the unstacked stromal thylakoids (Allen, 1992). Some PS1 is found in the margins of the grana thylakoids and is thought to contribute to linear electron transport. In addition, PS2β, a photochemically inactive form of PS2, is found in the stromal regions (Albertsson et al., 1990; Anderson, 1989, 1992). Immunogold labeling shows that cyt b$_6$f complexes are distributed laterally between the grana, stroma and grana margins (Olive et al., 1986) although distribution is dynamic and appears to be under redox control (Vallon et al., 1991). Similarly, PQ and PC are distributed throughout the three thylakoid domains. Steric considerations appear to govern the distribution of ATP synthase which has a bulky head group. ATP synthase is found in both the stromal thylakoids and the granal margins but not the stacked granal thylakoids (Svensson et al., 1991). 84% of FNR is located in the stroma and granal margins while 16% is located in the grana (Vallon et al., 1986).

Lateral heterogeneity has several implications. First, mobile electron carriers are required for transfer of electrons from PS2 to PS1. Although PQ was initially favoured as the mobile carrier, recent work by Joliot and coworkers has suggested that rapid diffusion of PQ is limited to small domains of less than 8 connected PS2 centres (Joliot et al., 1992; Joliot and Joliot, 1992; Lavergne et al., 1992). On the other hand, immunolocalization of PC has shown dramatic light to dark differences in the location of PC indicating that PC may well be capable of facilitating rapid long range $e^-$ transfer between PS2 and PS1 (Haehnel et al., 1989). Another important consequence of lateral heterogeneity of PETC components is that it may provide separate domains for cyclic and linear electron transport (Svensson et al., 1991). At high light intensity, it is unlikely that long range transport of $e^-$ would occur between the grana and the stroma. Since PS2β is not photochemically functional, Anderson (1992) suggests that under these circumstances linear $e^-$ flow would be restricted to the grana and the grana margins while PS1 in the stroma would be restricted to cyclic photophosphorylation. Although in vivo evidence for this theory is scarce, Anderson (1989) reported that, in in vitro preparations, the
rate of linear photophosphorylation in prestacked thylakoids was 2 fold higher than in artificially unstacked thylakoids at saturating light while the opposite was true for the rate of cyclic e- flow.

**Photosynthetic carbon fixation:** ATP and NADPH produced by the light reactions of photosynthesis are utilized in the chloroplast stroma for carbon fixation and reduction in a process known as the Calvin cycle (or the reductive pentose phosphate pathway) (see Figure 1). CO2 is fixed via ribulose bisphosphate carboxylase/oxygenase (RUBISCO) which carboxylates RuBP to form 2 molecules of PGA. ATP and NADPH are used to phosphorylate and reduce PGA to triose phosphate (DHAP and GAP) in reactions catalyzed by PGA kinase and GAPDH. Triose phosphate can be utilized to regenerate RuBP, produce starch, or exported from the plastid to form sucrose. The ratios of these three processes depends on the tissue type and the relative source and sink demands outside the tissue (Macdonald and Buchanan, 1990).

Although tight coupling exists between the light reactions and CO2 fixation, reductant and ATP produced by the light reactions can also be utilized for assimilation of inorganic N, P or S and for biosynthetic reactions in the stroma such as amino acid and protein biosynthesis (Lawlor, 1987; Turpin and Weger, 1990). Fixation of CO2 via the reductive pentose phosphate pathway in C3 plants requires approximately 1.5 ATP per NADPH (Heber and Walker, 1992). Assuming that the H+/e- ratio of linear electron transport is 2, however, the ATP/NADPH production ratio of linear electron transport is 1.3. This implies that some other form of electron transport is necessary to supplement ATP production (Heber and Walker, 1992). Moreover, metabolism which had a different ATP/NADPH requirement ratio occurring simultaneously with, or instead of CO2 fixation, could also result in ATP requirements in excess of those provided by linear electron transport alone. It has been proposed that increased cellular ATP requirements in the light are met either by pseudocyclic or cyclic electron transport (Horton, 1985; Heber and Walker, 1992; Lawlor, 1987).
Respiration:

Respiration involves 4 major related pathways including glycolysis, the oxidative pentose phosphate (OPP) pathway, the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain (METC). Respiratory carbon flow (OPP pathway, glycolysis and TCA cycle) is compartmentalized in the stroma of the chloroplast, the cytosol and in the matrix of the mitochondrion (Figure 2). Starch is initially degraded to glucose-6-P (G6P) via starch phosphorylase and amylase and further oxidized to triose phosphates (TP) and/or phosphoglycerate (PGA) in the chloroplast. Oxidation of G6P to TP and PGA can occur either via glycolysis or via the oxidative pentose phosphate pathway (Stitt, 1990). The ratio of flow through these two pathways usually indicates the metabolic status of a tissue with high OPP pathway activity generally indicative of high levels of biosynthesis (Miernyk, 1990). TP or PGA is then transported from the chloroplast to the cytosol via a TP(PGA)/Pi translocator where it is further oxidized to pyruvate via the "lower half" of glycolysis. Pyruvate is transported into the mitochondria where it is oxidized and decarboxylated by the tricarboxylic acid (TCA) cycle. Oxidation of carbon via respiratory carbon flow is accompanied by the production of reductant (NADH, FADH2). Reductant can be oxidized either by the METC with concomitant chemiosmotic production of ATP and consumption of O2 or by other processes such as NO3-/NO2-reduction (Turpin and Weger, 1990).

The components of the METC are membrane-bound and exhibit remarkable similarity to the components of the PETC. Both electron transport systems contain mobile quinone carriers (ubiquinone in the METC, plastoquinone in the PETC) and a membrane-bound quinone/cytochrome bc-type oxidoreductase (Scherer et al., 1990). Electron transport is coupled to proton transport across the membrane and, in both cases, a membrane-specific ATP synthase (chloroplastic: CF0/CF1, mitochondrial: F0/F1) converts the free energy of the resultant electrochemical gradient into ATP. The major difference between the two is that the METC oxidizes NADH to produce ATP whereas the PETC utilizes light energy to reduce NADP+ and produce ATP.
Figure 2: Pathways of respiratory carbon flow and mitochondrial respiratory electron transport in photosynthetic organisms. See text for discussion. Abbreviations: PGM, phosphoglucomutase; HPI, hexose phosphate isomerase; PDHC, pyruvate dehydrogenase complex; DH, dehydrogenase; 2OGDH, 2-oxoglutarate dehydrogenase; G6PDH, glucose-6 phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; cyt bc₁, cytochrome bc₁; CoA, coenzyme A; UQ, ubiquinone; Fₒ/F₁, mitochondrial ATP synthase. See list of abbreviations for further information.
Interaction between photosynthesis and respiration:

In photosynthetic organisms, the interaction between respiration and photosynthesis is not well understood. Despite reductionist attempts to separate photosynthesis from respiration, we are beginning to understand that these two processes must interact at a fundamental level. It is important to keep in mind that photosynthesis and respiration are opposing processes. Photosynthesis converts light energy into ATP, reductant, and ultimately into reduced carbohydrate while CO₂ is consumed and O₂ produced. Respiration, on the other hand, involves the oxidation of reduced carbon from photosynthesis to produce reductant which can then be used in the production of ATP with concomitant consumption of O₂ and production of CO₂. To prevent futile cycling, it is extremely important that these two processes be coordinately regulated.

The most likely mediators of interaction between photosynthesis and respiration are the adenine nucleotides (ATP, ADP, AMP) and the pyridine nucleotides (NADH, NAD, NADPH, NADP), effectors which have been shown to regulate many key regulatory enzymes of carbon flow (Graham, 1980). Respiration and photosynthesis involve processes which consume and produce ATP and NAD(P)H in a dynamic fashion and with potentially different stoichiometries requiring an intimate co-regulation between the individual components of both photosynthesis and respiration. Our understanding of the interaction between photosynthesis and respiration is still frustratingly limited and much of the information available at present is conflicting. Much of the present understanding of this interaction has been derived from biochemical studies of key regulatory enzymes and studies of the effects of the induction of photosynthetic carbon fixation on respiration. Although the effects of light-induced changes in pyridine and adenine nucleotides on mitochondrial respiration has been studied in some depth, little is known about the effect respiration has on photosynthesis. This is particularly important as respiration in plants has a major biosynthetic function.
Direct regulation of respiration by photosynthetic activity in the light:

Much confusion about the interaction between photosynthesis and respiration has arisen from measurements of gas exchange. This is due to the fact that measurement, in the light, of respiration in photosynthetic cells is hampered by the superimposition of other gas exchange processes such as photosynthesis, photorespiration and the Mehler reaction (Turpin and Weger, 1990). Measurement of respiratory O₂ consumption in the light has resulted in conflicting observations of the effect of photosynthesis on respiration. For example, some authors report complete inhibition of respiration upon illumination (Mehler, 1951; Radmer and Kok, 1976; Shiraiwa et al., 1988; Avelange and Rebeille, 1991) while others observe little or no effect (Gerbaud and Andre, 1980; Peltier and Thibault, 1985; Weger et al., 1988). Similarly, measurements of respiratory CO₂ efflux in the light have shown values ranging from 20 to 200% of dark CO₂ release rates (Piesker and Apel, 1980; Sharp et al., 1984; Azcon-Bieto and Osmond, 1983; Brooks and Farquhar, 1985; Avelange and Rebeille, 1991; Weger et al., 1989). Because no biochemical measurements have accompanied gas exchange measurements in most cases, it is difficult to resolve these discrepancies.

Classic work on the effect of illumination on pyridine and adenine nucleotides was done by Heber and Santarius who showed that, in the first few minutes of a dark-to-light transition, energy charge (Heber and Santarius, 1965) and the ratio of reduced/oxidized pyridine nucleotides both increased (Santarius and Heber, 1965). Levels of cofactors were measured in the chloroplastic and cytosolic compartments by means of non-aqueous fractionation; these cofactor levels showed that pyridine nucleotides were strictly compartmentalized since chloroplastic NADP was observed to become rapidly reduced while cytosolic NADP was much less affected (Heber, 1974). Longer-term experiments with chloroplasts confirmed increases in the redox state of the chloroplast upon illumination (Harvey and Brown, 1969). In contrast, these same experiments suggested that the ATP/ADP ratio returned to levels close to dark levels in the light after a few minutes (Hampp et al., 1982; Stitt et al., 1982).
It is generally accepted that the METC is under adenylate control (Lambers, 1990; Day et al., 1987; Wiskich, 1980). This control may function directly at the level of ADP-limitation of the ATP synthase or by ADP-limitation of respiratory carbon flow via pyruvate kinase or PGA kinase which would, in turn, limit reductant availability for METC oxidation (Lambers, 1990). It has been proposed that the high ATP/ADP ratio observed during illumination in the chloroplast stroma is transferred to the cytosol via a TP/PGA shuttle and that the consequent transient increase in the cytosolic ATP/ADP ratio causes ADP-limitation of glycolysis and the rate of METC activity (Douce, 1985; Santarius and Heber, 1965). Importantly, the increase in the cytosolic ATP/ADP ratio is transient and returns to dark levels within approximately one minute; this makes it unlikely that ADP-limitation, via photosynthesis, is a large factor under normal photosynthetic conditions (Hampp et al., 1982; Stitt et al., 1982). The recovery of the cytosolic ATP/ADP ratio during illumination may result from the induction of photosynthetic CO₂ fixation and sucrose synthesis which would decrease stromal ATP/ADP ratio (Hanson, 1992).

Photosynthetic control of respiration could also function at the level of high reducing power generated by the light reactions since transfer of ATP to the cytosol via a TP/PGA shuttle would also involve transfer of reductant. It is likely that at least some of the decrease in respiratory CO₂ efflux observed upon illumination is a result of inhibition of substrate decarboxylation via the OPP pathway since isolated G6P dehydrogenase is inhibited by Fd/thioredoxin_red and by high NADPH/NADP ratios (Lendzian and Bassham, 1975). The dehydrogenase reactions of the TCA cycle, in particular malate and isocitrate dehydrogenase, are also thought to be sensitive to increases in the NADH/NAD ratio (Wiskich, 1980; Graham, 1980) which might occur if reductant were shuttled from the chloroplast to the cytosol.

In a RUBISCO-deficient Chlamydomonas reinhardtii mutant, illumination resulted in a 65% inhibition of CO₂ efflux corresponding to a large increase in the NADPH/NADP ratio while O₂ consumption was stimulated. ADP levels were extremely low and unaffected by a light to dark transition. Light-induced inhibition of CO₂ efflux and stimulation of O₂
consumption could be reversed by DCMU leading Gans and Rebeille (1985) to propose that the observed O₂ consumption was due to the Mehler reaction and that respiratory O₂ consumption was inhibited. Uncoupling with CCCP could reverse inhibition of CO₂ efflux suggesting, in this case, that at least a portion of the inhibition was due to competition between the chloroplasts and mitochondria for ADP (Gans and Rebeille, 1985). It is also possible that some O₂ consumption was due to reductant shuttling to the mitochondria resulting in the inhibition of CO₂ efflux by means of an increase in mitochondrial NADH/NAD ratios (Gans and Rebeille, 1988). In contrast, illumination of wild-type *Chlamydomonas*, which is capable of CO₂ fixation, results in DCMU-insensitive O₂ consumption (Peltier and Thibault, 1985). The fact that the Calvin cycle provides an efficient sink for both ATP and reductant suggests that the degree of inhibition of respiration in the light depends on a subtle balance between Calvin cycle activity and the PETC. This may explain why a greater inhibition of CO₂ efflux is observed when cells are illuminated at the CO₂ compensation point or during water stress (Azcon-Bieto and Osmond, 1983; Canvin et al., 1980; Stuhlfauth et al., 1991) and why only a transient inhibition of respiration is seen upon illumination under high CO₂ conditions (Hampp et al., 1982). Illumination also inhibits both respiratory CO₂ efflux and O₂ consumption in photoautotrophic higher plant cell suspensions (Avelange and Rebeille, 1991; Avelange et al., 1991). It appears, then, that changes in photosynthetic the ATP/NADPH production ratio, resulting from changes in coupling between photosynthetic electron transport and carbon fixation, can regulate both respiratory carbon flow and METC activity.

**Indirect regulation of respiration by photosynthesis:**

Photosynthesis in the preceding light period has been observed to affect dark respiratory activity in the light and the subsequent dark period (Weger et al., 1989; Stokes et al., 1990). In spinach leaf discs after prolonged illumination, respiratory O₂ consumption in the ensuing dark period was transiently enhanced by up to 3-fold over steady-state dark levels and this increase was referred to as light enhanced dark respiration (LEDR) (Stokes et al., 1990; Reddy et al.,
Comparison of LEDR under high and low CO$_2$ conditions, sensitivity to light intensity, and DCMU all suggested that the substrate for LEDR was photosynthetically generated (Stokes et al., 1990). Although it has been suggested that LEDR is due to increased METC consumption of reductant generated by glycolytic metabolism of enhanced levels of photosynthetic metabolites (Azcon-Bieto and Osmond, 1983), the lack of CO$_2$ efflux enhancement makes this unlikely (Stokes et al., 1990). Stokes et al. (1990) have suggested that LEDR results either from alternative pathway activity or chloroplastic respiratory electron transport activity (Stokes et al., 1900). On the other hand, measurements of gross gas exchange in a marine diatom have led Weger et al. (1989) to suggest that LEDR is, in fact, a misnomer. These authors suggest that respiratory O$_2$ consumption increases upon illumination as a consequence of increased anabolic biosynthesis during photosynthesis. In turn, the enhanced respiratory rate occurring during the light period can be observed for a brief period after exposure to the dark. In either case, photosynthesis appears to affect the rate of respiratory e$^-$ transport and carbon flow whether directly in the light or indirectly in the subsequent dark period.

**Regulation of photosynthesis by respiratory activity:**

Even if ATP production via METC activity is superfluous in light-saturated conditions, respiratory carbon flow and/or METC activity is necessary because of the requirement for keto acids from the TCA cycle for light-dependent biosynthesis of porphyrins and amino acids (Graham, 1980; Turpin and Weger, 1990). In general, very little is known about the direct regulation of photosynthesis by respiratory activity.

Limited evidence suggests that changes in the relative rates of respiratory carbon flow and/or METC affects the rate of photosynthetic electron transport and/or carbon fixation by changes in ATP/NADPH production and utilization ratios. In barley protoplasts and leaves, selective inhibition of mitochondrial oxidative phosphorylation by oligomycin results in a 40 to 60% inhibition of photosynthetic carbon fixation (Kromer et al., 1988; Kromer and Heldt,
1991). This inhibition suggests that excess reductant from the light reactions could be shuttled to the METC via a malate/oxaloacetate shuttle. This, in turn, would prevent over-reduction of the PETC and could potentially result in up to a 3-fold increase in quantum efficiency for ATP production compared to that provided by linear electron transport (Ebbighausen et al., 1987). Consistent with this role is the fact that oligomycin can greatly increase photoinhibitory damage of the PETC at saturating light intensities (Saradadevi and Raghavendra, 1992). It is also possible that METC activity in the light may provide the ATP required for cytosolic sucrose synthesis (Kromer and Heldt, 1991; Hanson, 1992) or, in extreme cases, to support photosynthetic carbon fixation (Lemaire et al., 1988).

A potential interaction between respiratory carbon flow and poising of photosynthetic electron transport (for ATP and NADPH production)

Although relatively little research has investigated the question of the interaction between respiratory carbon flow and poising of photosynthetic electron transport, an examination of what is known about regulation of photosynthetic light harvesting and electron transport can help in proposing a potential mechanism for regulatory interaction between respiration and photosynthetic electron transport.

When high levels of TCA cycle activity are required for amino acid biosynthesis, it has been shown that carbon flow from glycolysis is required to replenish TCA cycle intermediates used in biosynthesis (Turpin, 1991). This, in turn, could affect the rate of respiratory reductant production in both the chloroplast and the mitochondria. In order to maintain high rates of respiratory carbon flow required for biosynthesis it would be necessary for the reduced pyridine nucleotides to be recycled. In addition, if the chloroplastic pyridine nucleotide pool were to become reduced, this might limit linear electron transport and decrease the efficiency of carbon fixation. Although the METC can recycle reductant in the mitochondria, it is also possible that down-regulation of reductant production by the PETC would make more oxidized pyridine nucleotide available for respiratory carbon flow. Down-regulation of reductant production
could be accomplished by changing the ratio of linear to cyclic electron flow. Although regulation of the ratio of linear to cyclic electron transport is poorly understood, one mechanism which has been proposed to regulate this ratio is the state transition.

A mechanism to regulate the ratio of linear to cyclic electron transport:

Molecular mechanism of the state transition:

Until 1969, scientists were puzzled by what was referred to as the "quantum yield anomaly". The steady state quantum yield of photosynthesis (CO₂ fixed or O₂ evolved per quantum of absorbed light) remains the same over a broad spectrum of light wavelengths although the two photosystems have quite different absorption spectra (Myers, 1971; Barber, 1986). The existence of a regulatory mechanism to explain the quantum yield anomaly was first demonstrated in the green alga *Chlorella pyrenoidosa* (Bonaventura and Myers, 1969) and the red alga, *Porphyridium cruentum* (Murata, 1969) by examining the effects of differential excitation of the two photosystems using monochromatic light and was termed the "light" state transition. Subsequent research has suggested that a number of methods, in addition to differential excitation of the two photosystems, can result in a state transition as defined below.

A model for the molecular basis for state transitions in green algae and higher plants has developed from the discovery of a) reversible phosphorylation of the LHC2 *in vitro* (Bennett, 1979), b) correlation between phosphorylation of LHC2 and fluorescence changes associated with the state transition (Bennett et al., 1980; Horton and Black, 1981; Chow et al., 1981; Steinback et al., 1982; Black et al., 1984; Farchaus et al., 1985) c) evidence for lateral heterogeneity and localization of PS2 and PS1 in appressed and non-appressed thylakoids respectively (Anderson and Andersson, 1982) d) evidence for lateral movement of a mobile subpopulation of phospho-LHC2 from appressed to non-appressed thylakoids (Chow et al., 1981; Staehelin et al., 1982; Anderson et al., 1982; Kyle et al., 1983; Bassi et al., 1988; Larsson et al., 1987), and evidence for redox sensitivity of the LHC2 kinase (Allen and Horton, 1981; Bennett, 1984). Despite its discovery and descriptive phenomenology over 30 years ago, the
The actual mechanism and physiological significance of state transitions is still controversial today. The subsequent discussion will focus on the most recently reviewed model for the state transition in green algae and higher plants (Allen, 1992) and will incorporate some additional recent developments (Anderson, 1992).

**Phosphorylation and the mobile antenna/cyt b₆f model:**

In green algae and higher plants, the mechanism proposed to explain the state transition *in vivo* is the phosphorylation mobile antenna model (see Figure 3). If the PQ pool and quinol binding sites on the cyt b₆f complex become reduced a protein kinase is activated which phosphorylates LHC2 and/or cyt b₆f (Gal et al., 1992). Although it was originally proposed that phosphorylation caused an increase in the negative charge on phospho-LHC2 and the subsequent electrostatic repulsion provided the driving force for movement of the phospho-LHC2 from the appressed to non-appressed regions of the grana (Barber, 1986), it has recently been proposed that conformational changes in docking sites on LHC2 and cyt b₆f due to phosphorylation are responsible for disassociation of these two proteins with PS2 and diffusion to the non-appressed thylakoids where the conformational changes may, in fact, favour association of phospho-LHC2 and cyt b₆f with PS1 (Allen, 1992; Anderson, 1992). This state, where the light harvesting antennae complement (and consequently fluorescence) of PS2 is decreased is termed state 2. Transition back to state 1 (PS2 has full antennae complement and high fluorescence) occurs if PQ(cyt b₆f) is oxidized which deactivates the kinase. Phosphatases cleave the phosphate groups from LHC2 and cyt b₆f and they diffuse back to the grana to reassociate with PS2. Transitions between these two conditions are known as state transitions.

**Function of the state transition:**

Although it is generally agreed that the state transition involves reversible phosphorylation and migration of LHC2 from appressed to non-appressed thylakoids, the exact function of the state transition is still disputed. On the basis of differential light excitation of the
Figure 3: The Phosphorylation Mobile Antennae Model: A putative model for the mechanism of the state transition (modified from Allen, 1992 and Anderson, 1992). In state 1, LHC2 and cyt b6f associate with PS2 in the appressed thylakoids (1). This configuration favours linear electron transport from PS2 to PS1. When PS2 is overexcited relative to PS1, the PQ(cyt b6f) pool becomes reduced and activates a kinase. This kinase phosphorylates LHC2, cyt b6f and itself (2). Phosphorylation of LHC2 and cyt b6f results in conformational changes and affects their association with PS2 leaving them free to diffuse to the non-appressed membranes where they reassociate with PS1. This association favours cyclic electron transport around PS1 and is known as state 2 (3). Over excitation of PS1 relative to PS2 oxidizes the PQ(cyt b6f) pool and results in deactivation of the kinase. Phosphatases dephosphorylate LHC2 and cyt b6f which then diffuse back to the appressed thylakoids and reassociate with PS2 (4).
two photosystems it has been proposed that the light state transition functions to maximize the quantum yield of linear electron transport by minimizing non-photochemical de-excitation (Bonaventura and Myers, 1969). However, reversible phosphorylation and migration of phospho-LHC2 has also been observed under conditions of constant light quality and/or intensity. Under these conditions, it has been proposed that the state transition may function to optimize the relative rates of ATP synthesis and NADPH reduction in response to metabolic demands (Allen et al., 1981; Allen and Bennett, 1981; Allen and Horton, 1981; Fernyhough et al., 1984; Turpin and Bruce, 1990). Thus, it is envisaged that, in state 1, LHC2 and cyt b6f are largely associated with PS2 which favours linear electron transport and maintains a high ratio of linear to cyclic electron transport (Figure 3, Anderson, 1992). However, upon phosphorylation and migration of LHC2 and cyt b6f to the non-appressed grana, association with PS1 is favoured and this, in turn, enhances cyclic electron flow around PS1. At constant non-saturating light intensity and quality, the overall effect of a state 1 to 2 transition is, therefore, to decrease the ratio of linear to cyclic flow. This would have the effect of down-regulating NADPH production and decreasing the NADPH/ATP production ratio.

Evidence for a structural basis for changes in the ratio of linear to cyclic e\textsuperscript{-} flow:

Evidence for a structural basis for changes in the ratio of linear to cyclic electron flow after a state 1 to 2 transition has come from a variety of sources including studies of a) the lateral distribution of cyt b6f complexes (Wollman and Bulte, 1990; Vallon et al., 1991), b) super-complex formation (Wollman and Bulte, 1990), c) phosphorylation of FNR (Hodges et al., 1987), and d) the functional absorption cross section of PS1 (Allen, 1984, 1992).

On the basis of highly accurate membrane fractionation techniques and immunocytochemical microscopy, Vallon et al. (1991) demonstrated a marked change in the lateral distribution of cyt b6f complex during state transitions induced both in vivo and in vitro in maize and Chlamydomonas reinhardtii. When cells were adapted to state 2, the proportion of cyt b6f located in the stromal thylakoids was significantly greater than after a transition to
state 1. This marked change in cyt b6f distribution was accompanied in all cases by the redistribution of phospho-LHC2 although the proportion of cyt b6f redistributed was significantly larger (30-40%) than that of LHC2 (10-20%). Similarly, changes in lateral distribution of cyt b6f or phospho-LHC2 in vitro required addition of exogenous ATP, suggesting that the distribution of cyt b6f depended on LHC2 kinase activity. Vallon et al. (1991) postulated that changes in lateral distribution of cyt b6f during state transitions provide a mechanism for the modulation of linear and cyclic flow. Cytochrome b6f is less abundant than either PS1 or PS2 in thylakoids isolated from plants grown at a variety of light intensities (Anderson, 1992). Thus, increased cyt b6f in the stroma during state 2 would increase the amount of cyt b6f available for interaction with PQ and PS1 for cyclic flow.

Studies on super-complex formation have also suggested a physical basis for changes in the ratio of linear to cyclic electron transport. Precedence for super-complex formation comes from kinetic studies in photosynthetic bacteria which have suggested super-complex formation between cyt bc1, the cytochrome oxidase, and the photochemical reaction center (Joliot et al., 1989). In green algae, the evidence for super-complex formation comes from several sources. First, cyt b6f co-migrated with PS1 in sucrose density gradients following centrifugation of solubulized Chlamydomonas membranes isolated from cells in state 2 (Wollman and Bulte, 1990). Second, in procedures used to purify cyt b6f, both the LHC2 kinase and FNR were shown to co-purify with cyt b6f, indicating some level of association between these three complexes (Clark et al., 1984; Gal et al., 1990a). Third, the rate of cyt f oxidation by PS1 after a single turnover flash was increased under conditions very similar to those used to induce a state 2 transition while state 1 conditions decreased the rate of cyt f oxidation (Delosme, 1991). Delosme (1991) suggested that the faster rate of cyt f oxidation is due to the closer association of cyt b6f with PS1 during state 2. Finally, the size and distribution of freeze-fracture particles suggested that some cyt b6f complexes were associated in the same intermembrane particles with PS2α in the stacked membranes while others were associated in the same inter-membrane particles with PS1β in the unstacked regions (Olive et al., 1986). Vallon et al. (1991) proposed
that, in state 1, cyt b\(_{6f}\) may be able to migrate freely between the two compartments but that state 1 to 2 transitions would favor super-complex formation between cyt b\(_{6f}\) and PS1, thus, enhancing the amount of cyt b\(_{6f}\) found in the stroma.

Studies on the effects of phosphorylation on FNR activity have suggested another mechanism whereby the state transition may affect the ratio of linear to cyclic electron transport. Hodges et al. (1987) report an increased recovery of phosphorylated FNR in the soluble fraction after incubation of pea thylakoids with ATP in the light or dark. FNR is weakly bound to the stromal side of the thylakoid where it forms a cation-sensitive complex with Fd. Its modification might be expected to alter binding to both Fd and the membrane and affect e\(^-\) transfer to NADP. The kinase which phosphorylates FNR is redox-controlled and has properties similar to those of the LHC2 kinase. Hodges et al. (1987) reported that PS1 inhibits reduction of NADP and that this inhibition is ATP-dependent but independent of effects of phosphorylation of LHC2. The inhibition of NADP reduction was reversed by antimycin A which also inhibits cyclic electron transport. This led them to suggest a role for FNR phosphorylation in the regulation of the relative rates of cyclic and non-cyclic photophosphorylation.

The final factor which could provide a structural basis for an increase in the ratio of cyclic to linear electron transport after a state 1 to state 2 transition is the complementary change in light harvesting allocation which is thought to accompany the state transition. It is generally agreed that a mobile sub-population of the phospho-LHC2 dissociates from PS2 and moves from the grana to the PS1 rich stroma. However, determination of the functional association of phospho-LHC2 with PS1 has been far more controversial (for a review see Allen, 1992; see also Wendler and Holzwarth, 1987; Allen and Melis, 1988). It has been suggested that discrepancies in functional phospho-LHC2 association with PS1 may arise from variability induced by temperature (Havaux, 1988; Timmerhaus and Weis, 1990) and zeaxanthin formation associated with the development of thylakoid \(\Delta p\text{H}\) (Horton, 1989). In a recent review, Allen (1992) proposes that mechanisms which can switch between excitation-transfer to PS1 and
dissipation as heat, depending upon light intensity and temperature, would determine whether
the state 2 transition functions to increase or decrease the quantum yield of PS1, that is, to
conserve or dissipate excitation energy.

Phosphorylation of LHC2 could affect either the efficiency of cyclic electron flow or the
relative extent of cyclic vs. linear flow dependent on whether phospho-LHC2 was functionally
associated with PS1. As was discussed previously, under normal conditions of CO2 fixation
linear electron transport would not be able to provide the ATP/NADPH ratio required for CO2
fixation suggesting that cyclic or pseudocyclic electron transport would be required to provide
the excess ATP requirements for efficient CO2 fixation (Heber and Walker, 1992). At constant
sub-saturating light intensity and quality, the quantum yield of PS1 cyclic flow would increase
only if phospho-LHC2 redirected its excitation energy to PS1. The effect of dissociation of
phospho-LHC2 from PS2 under these conditions, however, would be to decrease the quantum
yield of linear electron transport. Assuming that some cyclic electron transport occurred in both
state 1 and state 2, a decrease in the quantum yield of linear electron transport would still
function to decrease the ratio of linear to cyclic transport although it might not increase the
absolute quantum yield of cyclic electron transport.

**Can respiratory carbon flow influence the redox state of the PQ(cyt b6f) pool and poise
the ratio of linear to cyclic electron flow?:**

It is likely that respiratory carbon flow could interact directly with photosynthetic
electron transport by changing production of ATP and/or NAD(P)H. The pertinent question
then becomes: can changes in the production of ATP or NAD(P)H, as a result of respiratory
carbon flow, affect the redox state of the PQ(cyt b6f) pool and result in a state 1 to 2
transition? In the light, there are two potential mechanisms whereby increases in the
NAD(P)H/ATP production ratio by respiratory carbon flow might affect the redox state of the
PQ(cyt b6f) pool. Linear electron transport produces approximately 0.77 NADPH per ATP
(Nelson and Prezelin, 1990). Hence, if the ratio of NADPH/ATP in the chloroplast were to
increase significantly, due to an increase in the NAD(P)H/ATP production ratio from respiratory carbon flow, this could potentially affect the poising of the state 1 to 2 transition in at least two ways. The first involves direct substrate (NADP) limitation of electron transport and the second involves direct reduction of the PQ(cyt b₆f) pool by NAD(P)H.

**Substrate (NADP) limitation of the PETC:**

If respiratory carbon flow were to increase in the light, an increase in NADPH or ATP in the chloroplast could potentially affect the reduction of the PQ(cyt b₆f) pool. An increase in the ATP/ADP ratio would lead to substrate limitation of the chloroplast ATP synthase and limit dissipation of the proton gradient. Since electron flow from PQ to cyt b₆f is coupled to proton translocation, the rate of electron flow is decreased by an increase in thylakoid ΔpH (Lawlor, 1987). However, it is quite unlikely that an increase in respiratory carbon flow would be accompanied by an increase in the ATP/ADP ratio since this would tend to limit rather than enhance respiratory carbon flow (Day et al., 1987). It is much more likely that increases in respiratory carbon flow would lead to an increase in the ratio of reduced/oxidized reductant (Lambers, 1990). An increase in reduced/oxidized pyridine nucleotide ratios in the chloroplast could potentially affect the redox state of the PQ(cyt b₆f) pool by substrate-limiting linear electron transport due to a lack of NADP. Testing this hypothesis would involve examining changes in respiratory carbon flow which occur in the light during photosynthetic electron transport. Such tests would be extremely difficult to perform because of the difficulty in resolving the effect of changes in the NAD(P)H/NAD(P) ratio directly attributable to respiration.

**Direct interaction between reductant and the PQ(cyt b₆f) pool:**

Respiratory carbon flow could also affect the reduction of the PQ(cyt b₆f) pool by direct reduction of PQ with NAD(P)H. Evidence for direct interaction between pyridine nucleotides and the photosynthetic electron transport chain has come from two independent
lines of research. One line of investigation has focused on photoevolution of H₂ in green algae while a second has developed from studies of respiratory O₂ uptake in the chloroplast in the dark. The electron transport pathways involved in both of these processes and their interaction with the PETC are outlined in Figure 4.

**H₂ photoevolution:** In hydrogenase-inducible green algae under anaerobic conditions, sustained H₂ production occurs in the absence of CO₂ or when CO₂ fixation is inhibited (Gaffron and Rubin, 1942; Stuart and Gaffron, 1972; Kaltwasser et al., 1969; Graves et al., 1989). This has led Graves et al. (1989) to suggest that H₂ photoevolution competes with CO₂ fixation for reductant. Kaltwasser et al. (1969) were the first to propose that in vivo PS1-dependent H₂ photoevolution depended upon degradation of organic substrate in *Scenedesmus obliquus* (see Figure 4). H₂ photoevolution was inhibited by starvation and stimulated by exogenous substrates such as glucose, acetate and ethanol (Wiessner and Gaffron, 1964; Tanner et al., 1965; Kaltwasser et al., 1969; Bishop et al., 1977; Senger and Bishop, 1979). In addition, H₂ photoevolution was accompanied by CO₂ release and was inhibited by monofluoroacetic acid (a TCA cycle inhibitor) suggesting that oxidative carbon metabolism was required (Healey, 1970b; Stuart and Gaffron, 1971). These observations were consistent with the existence of PS1-dependent e⁻ flow from reductant, produced by oxidative carbon metabolism, to a high potential redox carrier capable of reducing H⁺ (Healey, 1970b). Although the pathway for H₂ photoevolution appears to be species-specific, in *Chlamydomonas sp.*, H₂ photoevolution was shown to be independent of PS2 on the basis of work with PS2 mutants and lack of inhibition by DCMU (Healey, 1970a). Stimulation of H₂ photoevolution by reduced pyridine nucleotides was first observed in vitro by Abeles (1964) in thylakoid preparations of *Chlamydomonas eugmetos*. NADH-dependent H₂ photoevolution in *Chlamydomonas reinhardtii* thylakoid preparations (Ben-Amotz and Gibbs, 1975) and whole
Figure 4: Thylakoid electron transport pathways which share PQ in green algae (modified from Maione and Gibbs, 1986b). Black arrows, electron transport which occurs only in the dark; grey arrows, electron transport which occurs only in the light; white arrows, electron transport which can occur in both the dark or the light. Double lines indicate sites of inhibitor action. Electron flow from NAD(P)H to O$_2$ via the NAD(P)H-PQ oxidoreductase is defined as the chloroplast respiratory electron transport chain (CRETC). Flow from H$_2$O to Fd via PS2 and PS1 is defined as the photosynthetic electron transport chain (PETC). Electrons from Fd can be used to a) reduce NADP (linear electron transport), b) reduce O$_2$ (pseudocyclic electron transport), c) produce H$_2$ (H$_2$ photoevolution), d) reduce PQ (PS1 cyclic electron transport). Depending upon illumination, PQ can accept electrons from PS2 and/or NAD(P)H and can donate electrons to PS1 or the terminal oxidase of the CRETC. See text for further discussion. Abbreviations are as in the list of abbreviations.
cells (Gfeller and Gibbs, 1985) was observed to be sensitive to DBMIB but not DCMU, consistent with e\textsuperscript{-} transfer from NADH to PQ.

A thylakoid-bound protein component responsible for the transfer of e\textsuperscript{-} from NAD(P)H to PQ was partially purified by Godde and Trebst (1980) and further characterized by Godde (1982). Photosynthetically active chloroplast particles from C. reinhardtii were observed to evolve H\textsubscript{2} in the light at the expense of intermediates of glycolysis which produced NAD(P)H (G6P, FBP, lactate). Both exogenous NADPH and NADH were observed to act as substrates for this activity although NADPH interacted with 2-fold less activity (Godde and Trebst, 1980). The H\textsubscript{2} photoevolution activity was inhibited by rotenone and thenoyl trifluoroacetate, both of which act to inhibit iron sulfur centres in mitochondrial NADH dehydrogenase (complex 1) and succinate dehydrogenase, respectively (Figure 4). The activity was also shown to utilize PQ with much higher efficiency than ubiquinone (UQ) making mitochondrial contamination unlikely (Godde, 1982). The involvement of PQ led to the conclusion that this protein component acted as an NAD(P)H-PQ oxidoreductase.

Succinate dehydrogenase (SDH) may also act as an e\textsuperscript{-} donor to PQ. 25% of the cellular SDH activity is observed in isolated Chlamydomonas chloroplasts and exogenous succinate has been observed to support PS1 activity in thylakoid preparations (Willeford et al., 1989).

**Respiratory O\textsubscript{2} consumption in the chloroplast:** It has been proposed that in the dark, electrons from NAD(P)H are transferred to O\textsubscript{2} via NAD(P)H-PQ oxidoreductase and the PQ pool (Bennoun, 1982; Diner and Mauzeral, 1973; Peltier et al., 1987) (see Figure 4). The term chlororespiration was first coined by Bennoun (1982) to describe the pathway of electron transport from NAD(P)H to O\textsubscript{2} and to distinguish it from the pathway in the mitochondria. However, the term "chlororespiration" has since become ambiguous because respiratory carbon flow (Singh et al., 1992; Gibbs et al., 1990), electron transport from NAD(P)H to O\textsubscript{2} via PQ (Bennoun, 1982), and electron transport from NAD(P)H to O\textsubscript{2} via FNR (Kow et al., 1982) are all thought to occur in the chloroplast. For the purposes of this work, chloroplastic respiratory
e^- transport chain (CRETC) activity involving PS1- and PS2- independent electron transport from NAD(P)H to O_2 via PQ will be distinguished from both chloroplastic respiratory carbon flow and PS1/FNR-associated O_2 consumption.

Goedheer (1963) was the first to propose the existence of a putative thylakoid respiratory electron transport chain which consumed O_2 and shared components with the PETC. Evidence consistent with dark e^- flow from components of the PETC to O_2 was provided by observations that the redox state of both the PQ pool (Diner and Mauzeral, 1973; Diner, 1977) and cyt b563 (Hiyama et al., 1969) was affected by anaerobiosis. On the basis of O_2 flash yield measurements and photosynthetic light intensity curves in anaerobically treated Chlorella, Diner and Mauzeral (1973) hypothesized that PQ was shared between the PETC and a chloroplastic respiratory e^- transport chain which accepted electrons from reduced pyridine nucleotides and donated them to O_2 (Figure 4). It was subsequently shown that PQ could be reduced by the products of anaerobic starch metabolism in Chlamydomonas reinhardtii (Gfeller and Gibbs, 1985). At the extinction point (the lowest pO_2 at which anaerobic fermentation is eliminated), DBMIB could be shown to increase fermentative ethanol production which, in turn, resulted in a further increase in the extinction point from 2 to 5% O_2. This led Gfeller and Gibbs (1985) to propose that oxidation of pyridine nucleotides occurs via PQ in the presence of O_2 or via ethanol formation in the absence of electron transfer to O_2 (anaerobiosis or DBMIB inhibition).

Oxidation of reductant (via the CRETC) was shown to involve the NAD(P)H-PQ oxidoreductase because a) respiratory CO_2 efflux associated with CRETC activity in isolated C. reinhardtii chloroplasts was inhibited by rotenone, an inhibitor of the NAD(P)H-PQ oxidoreductase (Singh et al., 1992) and b) exogenous NAD(P)H increased the reduction of PQ as measured by fluorescence induction in open cell preparations of a PS1 mutant of C. reinhardtii (Bennoun, 1982).

Although the NAD(P)H-PQ oxidoreductase has been isolated and partially characterized (Godde and Trebst, 1980), very little is known about the identity of the components that accept
e$^-$ from the PQ pool and transfer them to O$_2$. Using *C. reinhardtii* mutants, Bennoun (1983) demonstrated that the Rieske iron sulfur protein, cyt f, PC, PS1, and the ATP synthase were not necessary for CRETC activity, suggesting that interaction between the CRETC and PETC occurred only at the level of PQ. An amperometric signal associated with CRETC flow to O$_2$ (Peltier et al., 1987) was inhibited by myxothiazol and 5 μM antimycin A, inhibitors which specifically affect cytochrome bc$_1$ but not b$_6$f complexes. This fact led Ravenel and Peltier (1991) to suggest that a distinct cyt bc type complex was involved in the CRETC. Furthermore, two novel cytochromes, cyt h$_1$ and h$_2$ were identified (Lemaire et al., 1986; Rolfe et al., 1987) and were shown to increase under conditions of N-limitation, which enhances CRETC activity in *C. reinhardtii* (Peltier and Schmidt, 1991). Ravenel and Peltier (1991) suggested that these two cytochromes might be components of the putative myxothiazol- and antimycin A-inhibited cyt bc complex.

The existence of a terminal oxidase was suggested by inhibitor studies. In *Chlamydomonas*, dark oxidation of the PQ pool was inhibited by CO, NO, KCN, and sodium azide, indicating the involvement of a cytochrome-type oxidase in the oxidation of PQ. In *Chlorella*, however, SHAM (but not KCN) inhibited PQ oxidation suggesting that the terminal oxidase may act more like an alternative oxidase in this organism (Bennoun, 1982).

**Competition between O$_2$ and PS1 as terminal electron acceptors for the CRETC / The effects of illumination:**

**Kok effect:** Kok (1949) first observed a non-linearity, at low light intensities, of the photosynthetic light saturation curve in the green alga *Chlorella*. On the basis of DCMU insensitivity, it was proposed that the Kok effect resulted from inhibition of mitochondrial respiration, in the light, by an increase in the ATP/ADP ratio from cyclic photophosphorylation (Kok 1949; Hoch et al., 1963). However, since the Kok effect was also insensitive to CCCP and was enhanced in far red light, Healey and Myers (1971) suggested that inhibition of O$_2$ consumption at low light intensities was a result of the diversion of reductant from respiratory
electron transport to PS1. Similarities in the Kok effect observed in *Anacystis nidulans*, a cyanobacterium in which the respiratory and photosynthetic electron transport chains occur in the same membrane and share components, led Jones and Myers (1963) to suggest that a respiratory activity was directly inhibited by competition with PS1 for reductant (Jones and Myers, 1963).

In *C. reinhardtii*, mass spectrometric measurements of gross gas exchange allowed Peltier and Sarrey (1988) to distinguish between two respiratory components. The first component was inhibited by low concentrations of antimycin A and SHAM, was light-independent and was thought to result from METC activity. A second component was inhibited by light but insensitive to low concentrations of antimycin A and SHAM, suggesting that it resulted from CRETC and not METC activity. Consistent with these observations is the observation that, in *Chlorella vulgaris*, the Kok effect was enhanced by anaerobiosis (which would prevent oxidation of PQ) and abolished by benzoquinone (which rapidly oxidizes the PQ pool) (Diner and Mauzeral, 1973).

**Flash inhibition of CRETC:** Using amperometric and mass spectrometric measurements in *C. reinhardtii*, Peltier et al. (1987) observed a respiratory O$_2$ consumption component which was inhibited by short (2 μsec) saturating flashes. This component was related to $^{18}$O$_2$ uptake, was insensitive to DCMU, and was stimulated by acetate and high pO$_2$. It was suggested that the flash-inhibited component resulted from inhibition of O$_2$ consumption via the CRETC because it a) had a higher $k_M$ for O$_2$ than the METC, b) was insensitive to concentrations of antimycin A and SHAM which completely inhibited respiration and c) was inhibited by concentrations of KCN which have no effect on the METC. In addition, in mutants which were missing either PS1 or cyt b$_6$f, this respiratory component was enhanced after illumination (Ravenel and Peltier, 1992; Peltier and Thibault, 1988). De-convolution of the flash-induced amperometric signal in wild-type cells indicated that flash-induced stimulation also occurred but that this stimulation developed more slowly than flash-induced inhibition by PS1 (Ravenel and Peltier, 1992). All
these data support the idea that light inhibition of CRETC activity is due to competition, between PS1 and O₂, for e⁻ from PQ. Stimulation of the respiratory component by illumination suggests that O₂ may accept e⁻ from PS2 as well as from reductant and highlights PQ as a cross-over point between oxidizing and reducing reactions in the thylakoid. Similarities between this flash-inhibited component and that seen in photosynthetic prokaryotes, where the PETC and respiratory electron transport chain share components, lends further support to the idea that PS1 and O₂ compete for e⁻ from PQ (Vermeglio and Carrier, 1984; Lavorel et al., 1989; Baccarini et al., 1978; Vermeglio and Joliot, 1984; Richaud et al., 1986).

Evidence for the existence of a CRETC in higher plants:

Although strong evidence exists for the occurrence of a CRETC in chl b-containing (Bennoun, 1982) and chl c-containing (Wilhelm and Duval, 1990; Buchel and Wilhelm, 1990; Ting and Owens, 1993) unicellular algae, the existence of a CRETC in higher plant cells is still debated. Chloroplast genome analysis in liverwort, tobacco, sugar beet, rice, and broad bean has revealed the presence of open reading frames which have a high homology with the mitochondrial NADH dehydrogenase (complex 1) (Umesono and Ozeki, 1987; Oyhama et al., 1988; Meng et al., 1986; Shinozaki et al., 1986). A recent study has also shown that this complex is expressed in mono- and dicotyledonous plants and is localized in the stromal lamellae of the thylakoid membrane (Berger et al., 1993). H₂ photoevolution, correlated with NAD(P)H-PQ oxidoreductase activity, was not detected in spinach thylakoids provided with exogenous hydrogenase (Ben-Amotz and Gibbs, 1975; Godde and Trebst, 1980). However, NADPH reduced cyt b₅₆₀ (Cramer and Butler, 1967) and PQ (Mills et al., 1979) in spinach chloroplasts. Flash-induced inhibition of respiratory O₂ uptake was not observed in photoautotrophic cell cultures or protoplasts of Euphorbia (Avelange and Rebeille, 1991; Ravenel and Peltier, 1992). However, Garab et al. (1989) demonstrated in tobacco and sugar beet that both KCN and SHAM inhibited the oxidation kinetics of PQ in vitro and in vivo. In addition, oxidation of exogenous glucose in isolated spinach chloroplasts was inhibited by
rotenone, amytal, antimycin A, propyl galate, sodium azide (Singh et al., 1992), and anaerobiosis (Ahluwalia et al., 1989), all of which have themselves been demonstrated to inhibit CRETC activity in isolated Chlamydomonas reinhardtii chloroplasts (Singh et al., 1992). In spinach leaf discs, reduction of the PQ pool resulted from anaerobiosis and the PQ pool was re-oxidized by either O₂ or far red light, suggesting that electron transport from PQ to a terminal oxidase occurs in higher plants (Harris and Heber, 1993).

Evidence to suggest that changes in the cellular NAD(P)H/ATP ratio can regulate the state 1 to 2 transition:

In the light: Treatments thought to cause an increase in the NADPH/ATP ratio have been shown to induce a state 1 to 2 transition in maize (Horton and Lee, 1986; Horton, 1987, 1989; Horton et al., 1989; Turpin and Bruce, 1989). In maize mesophyll chloroplasts, the NADPH/ATP ratio can be manipulated by providing endogenous carbon with differing NAD(P)H/ATP requirement ratios (Fernyhough et al., 1984; Horton et al., 1989). OAA (1 NADPH required per OAA assimilated to malate via NADP-malate dehydrogenase), which would decrease the cellular NADPH/ATP ratio, was observed to strongly inhibit LHC2 phosphorylation. Pyruvate (2 ATP required per pyruvate reacted to PEP via pyruvate PPi dikinase), which would increase the cellular NADPH/ATP ratio, increased LHC2 phosphorylation (Fernyhough et al., 1984; Fernyhough et al., 1989). Similarly, in the light in N-limited green algae, it was shown that assimilation of NH₄⁺, which requires 0.2 NADPH per ATP, resulted in a state 1 to 2 transition (as measured by absolute 77K fluorescence), while NO₃⁻ and CO₂ assimilation which require 0.83 and 0.67 NADPH per ATP, respectively, did not cause a state 1 to 2 transition (Turpin and Bruce, 1990). A transition from photoautotrophic to photoheterotrophic acetate metabolism, which has a low NAD(P)H/ATP requirement ratio, has been observed to increase the absorption cross section of PS1 as measured by the quantum yield of H₂ photoevolution in Chlamydomonas stellata (Boichenko et al., 1992). An extreme state 1 to 2 transition was also observed in high CO₂-grown Chlorella
vulgaris upon transfer to low CO$_2$ conditions leading Demidov and Elfimov (1992) to suggest that inhibition of the Calvin cycle (due to CO$_2$-limitation) and increased ATP consumption, necessary to induce the CO$_2$-concentrating mechanism, decreased the cellular NADPH/ATP requirement ratio. In all of the above cases, it is likely that a NADPH/ATP utilization ratio lower than the linear electron transport NADPH/ATP production ratio of 0.77 would tend to increase the cellular NADPH/NADP ratio. This, in turn, could influence the redox state of the PQ(cyt b$_6$f) pool either by substrate-limitation or by reduction of PQ via the NAD(P)H-PQ oxidoreductase. It is, however, very difficult to differentiate between these two possibilities in the light.

**In the dark:** State 1 to 2 transitions have also be shown to occur in the dark, leading several authors to suggest that an increase in the dark NAD(P)H/NAD(P) ratio might increase the redox state of the PQ(cyt b$_6$f) pool and result in a state 1 to 2 transition (Rebeille and Gans, 1988; Gans and Rebeille, 1990; Bulte et al., 1990; Mohanty et al., 1990). In *Chlamydomonas reinhardtii*, treatment of darkened cells with uncouplers or antimycin A and SHAM increased cellular NADPH/NADP ratios and resulted in a state 1 to state 2 transition (Bulte et al., 1990; Gans and Rebeille, 1990). Similarly, assimilation of NH$_4^+$ by N-limited *Selenastrum minutum* resulted in an increase in the cellular NADPH/NADP ratio (Vanlerberghe et al., 1992) and a state 1 to 2 transition (Mohanty et al., 1990). In the absence of illumination, substrate-limitation of the PETC would not occur and it is likely that the above treatments increased the reduction of the PQ(cyt b$_6$f) pool as a result of NAD(P)H-PQ oxidoreductase activity.

**The rationale for working in the dark:**

In most studies to date, the direct measurement of either pyridine or adenine nucleotide levels has been limited and a correlation between a decreased NADPH/ATP requirement ratio and a state 1 to 2 transition has been implied but not observed. It is extremely difficult to determine the mechanism responsible for the reduction of PQ in the light because of the variety
of processes which may contribute to regulation of the PETC. In the light, NAD(P)H can be consumed by carbon fixation, \(\text{NO}_3^-\) assimilation, and amino acid biosynthesis. At the same time, NAD(P)H is produced by the PETC and respiratory carbon flow. In the presence of multiple sources and sinks for NAD(P)H, it is extremely difficult to isolate the effects of reductant production from respiratory carbon flow on the PETC \textit{in vivo} during illumination. In addition, the redox status of the PQ(cyt b₆f) pool can be affected by its rate of reduction by PS2 or reductant (Figure 4, see also Ravenel and Peltier, 1992) and by its rate of oxidation by PS1, which, in turn, can be affected by light-induced trans-thylakoid \(\Delta pH\). Measurement of a state transition using fluorescence quenching techniques would be complicated because at least 2 other processes are thought to quench fluorescence in the light; these are energized quenching (due to thylakoid \(\Delta pH\)) and photoinhibitory quenching (Krause and Weis, 1991). All of these factors make it extremely difficult to clearly demonstrate the regulation of the photosynthetic NADPH/ATP production ratio by respiratory carbon flow in the light.

It is equally possible, however, that respiratory activity in the dark might affect the redox state of the PQ(cyt b₆f) pool and poise the PETC for a decrease in the NADPH/ATP production ratio upon illumination. Testing this hypothesis would be considerably easier. The multiple sources and sinks for NAD(P)H in the dark would be absent largely because production and consumption of NAD(P)H, via photosynthetic processes, would not occur. In addition, the redox state of PQ would not be affected by PS1 and PS2 activity making it possible to isolate the effects of NAD(P)H-PQ oxidoreductase activity on the PQ redox state. Under these conditions, it is possible to isolate and test the effects of increases in the NADPH/NADP ratio on the redox state of the PQ(cyt b₆f) pool by direct electron transfer from the NAD(P)H-PQ oxidoreductase (Godde and Trebst, 1980).

\textbf{The hypothesis to be tested:}

The theoretical relationship between respiratory carbon flow and the PETC via NAD(P)H-PQ oxidoreductase activity in the dark can be formalized as a testable hypothesis.
Essentially, I propose that increases in respiratory carbon flow which cause an overall increase in the NAD(P)H/NAD(P) ratio will increase the reduction of the PQ(cyt b6f) pool and consequently poise the PETC to decrease the ratio of linear/cyclic electron transport via a state 1 to 2 transition. The corollary to this hypothesis is that increases in respiratory carbon flow, which do not result in an overall increase in the NAD(P)H/NAD(P) ratio, will not increase the reduction of the PQ pool and will not affect the poising of the PETC for cyclic vs. linear electron transport. The purpose of this thesis will be to test this hypothesis and its corollary in vivo, in the green alga Selenastrum minutum, as stringently as possible, to develop a model for the interaction between respiratory carbon flow and photosynthetic light harvesting.

It has been suggested that the role of the CRETC is to recycle reductant for respiratory carbon flow in the chloroplast in much the same manner that the METC functions in mitochondria (Peltier et al., 1987; Peltier and Schmidt, 1991; Singh et al., 1992). If the above hypothesis is supported, however, it will have major implications for our understanding of the role of the CRETC in metabolism. Essentially, the CRETC would allow respiration to directly modulate photosynthesis. First, the CRETC could allow respiration to communicate directly with the PETC and decrease NADPH production via the state 1 to 2 transition in the light. A second implication is that respiratory activity in the dark could affect the poising of the photosynthetic NADPH/ATP production ratio upon illumination. Increases in NAD(P)H in the chloroplast due to respiratory carbon flow in either the dark or light could, therefore, be communicated to the PETC and cause a modulation of the NADPH/ATP production ratio to compensate.
CHAPTER 2: EXPERIMENTAL RATIONALE: A FRAMEWORK TO TEST THE HYPOTHESIS

INTRODUCTION

Rationale for the hypothesis to be tested:

Considerable evidence exists to suggest that the activity of both the PETC and photosynthetic carbon fixation can affect respiration through changes in the ratio of pyridine and/or adenine nucleotides (Graham, 1980; Dry and Wiskich, 1987; Turpin and Weger, 1990). Considerably less information is known, however, about whether respiration regulates photosynthesis. Since both the PETC and respiratory carbon flow produce NAD(P)H and ATP but with potentially different stoichiometries, it is possible that changes in respiratory carbon flow could affect NAD(P)H/ATP ratios in the cell, making it necessary for the PETC to down-regulate the NADPH/ATP production ratio to prevent over-reduction of the cell.

The previous chapter examined the theoretical possibilities of an interaction between respiratory carbon flow in some detail. It was proposed that the state 1 to 2 transition might function to down-regulate the ratio of NADPH/ATP production by decreasing the ratio of linear to cyclic e\textsuperscript{-} transport (Allen and Horton, 1981; Horton et al., 1989). To support this contention, state 1 to 2 transitions were observed during treatments which are thought to increase the cellular NADPH/ATP ratio in the light (Fernyhough et al., 1984; Horton and Lee, 1986; Horton et al., 1989; Turpin and Bruce, 1989; Boichenko et al., 1992; Demidov et al., 1992). During these treatments, it was proposed that the increase in the cellular NADPH/ATP ratio resulted in reduction of the PQ pool, either by NADP\textsuperscript{+}-limitation of linear e\textsuperscript{-} transport or by NAD(P)H-PQ oxidoreductase activity, and initiated a state 1 to 2 transition (Turpin and Bruce, 1989; Horton et al., 1989). It is, however, extremely difficult to distinguish between the two mechanisms which might contribute to PQ reduction in the light.

It is particularly difficult to isolate the interaction between respiratory carbon flow and the PETC in the light. Physiological measurements of gas exchange are hampered by the superimposition of a variety of processes (Turpin and Weger, 1990) and biochemical
measurements are difficult because cofactors (ATP and NAD(P)H) potentially responsible for
the interaction between respiratory carbon flow and the PETC can be affected by several source
and sink reactions at once. In the dark, however, it is considerably easier to isolate the effects
of changes in the NADPH/ATP ratio on the redox state of the PQ(cyt b6f) because neither
photosynthetic carbon fixation or electron transport occur. Under these conditions, it should be
possible to isolate and test the effects of increases in NAD(P)H/NAD(P) ratios on the state 1 to
2 transition resulting from increased reduction of the PQ(cyt b6f) pool by direct electron
transfer from the NAD(P)H-PQ oxidoreductase.

**Methods which can be used to measure state transitions:**

In order to examine the interaction between respiratory carbon flow and the state
transition in the dark, accurate measurements of the state transition were required. The state
transition has traditionally been measured by changes in fluorescence emission or LHC2
phosphorylation. LHC2 phosphorylation measurements have proven extremely useful for the
measurement of state transitions *in vitro* but are intrusive and do not provide any information
about the functional association of phospho-LHC2. On the other hand, chlorophyll *a*
fluorescence provides a sensitive, non-intrusive probe of the photosynthetic apparatus and,
when several methods are used, allows for resolution and rapid measurement of state transition
kinetics.

Light emission (or fluorescence) arises from chl *a* if excitation energy is not dissipated
by photochemistry or radiationless dissipation. At room temperature, most fluorescence is
emitted by chl *a* molecules in the light harvesting antennae of PS2 (Krause and Weis, 1991).
Fluorescence yield is rarely maximal due to the existence of photochemical and non-
photochemical processes which can "quench" fluorescence. Photochemical quenching (qp) is
related to the redox state of QA, and occurs when excitation energy of the reaction centre
chlorophyll is used for photochemistry. If the primary quinone acceptor associated with the PS2
reaction centre (QA) is reduced, the reaction centre is "closed" and cannot contribute to
photochemical quenching of fluorescence. On the other hand, an "open" or oxidized reaction centre is capable of quenching fluorescence photochemically (Bradbury and Baker, 1981; Krause and Weis, 1984; Sivak and Walker, 1985).

Quenching of fluorescence may also result from non-photochemical processes which include energization of the thylakoid membrane, the state transition, and photoinhibition (Krause and Weis, 1991; Horton and Bowyer, 1991). A major component of non-photochemical quenching ($q_{NP}$) is energized quenching, or $q_E$, which is associated with the buildup of the thylakoid proton gradient. The molecular mechanism of $q_E$ is still not well understood (Ruban and Horton, 1992) but it is thought that $q_E$ results in energy release in the form of heat rather than fluorescence (Krause et al., 1982; Horton, 1982). A xanthophyll cycle is one mechanism that has been proposed to account for $q_E$. The xanthophyll cycle would involve dissipation of excess excitation energy by zeaxanthin, which is formed by de-epoxidation of violoxanthin when the thylakoid lumen pH is low (Demmig-Adams, 1990). Horton and coworkers have recently proposed that $q_E$ involves a protonation-induced aggregation of LHC2, which serves to decrease fluorescence yields and may be enhanced by zeaxanthin (Ruban and Horton, 1992; Ruban et al., 1992; Horton et al., 1991). It is important to note, though, that $q_E$ cannot be attributed solely to the formation of thylakoid $\Delta pH$. In higher plants, antimycin A has been demonstrated to inhibit $q_E$ without affecting thylakoid $\Delta pH$, suggesting that $q_E$ may be regulated by both the thylakoid $\Delta pH$ and the redox state of an electron transport chain component (Oxborough and Horton, 1987).

The D1 protein of PS2 appears to be highly susceptible to photodamage and this can be accelerated by prolonged illumination at high light intensities or at extreme temperatures. Photodamage of PS2 can convert PS2 from high fluorescent, photochemically active centres to low fluorescent, photochemically inactive centres. The quenching of fluorescence resulting from this process is referred to as photoinhibitory quenching, or $q_I$ (Krause and Weis, 1991).

Room temperature fluorescence can also be affected by the occurrence of state transitions. In state 1, the amount of LHC2 functionally associated with PS2 is greater than in
state 2 (Allen, 1992). In the absence of other non-photochemical quenching mechanisms, the intensity of fluorescence emitted from antennae pigments is dependent upon the absorption cross section or amount of antennae pigments associated with the reaction centre (Krause and Weis, 1991). Thus, a decrease in the absorption cross section of PS2 due to a state 1 to state 2 transition can be measured as a decrease in fluorescence emitted from PS2 at room temperature. This quenching of fluorescence is referred to as state transition quenching, or qT.

It is possible to distinguish between photochemical and non-photochemical quenching with room temperature fluorescence measurements using the light doubling technique of Bradbury and Baker (1981). A non-actinic measuring beam (does not cause photochemistry) is used to measure the minimal level of fluorescence of the antennae pigments, $F_0$, where $qp = 1$ and $qNP = 0$. Application of a short, saturating flash of light results in full reduction of QA and provides a measure of the maximal fluorescence possible when $qp$ is 0. The level of fluorescence excited by a saturated flash, under conditions where $qNP$ is negligible, is defined as $F_M$. Differences between $F_M$ and $F_M'$ (the saturated flash level of fluorescence when $qNP > 0$) allow a determination of the degree to which $qNP$ contributes to fluorescence quenching. Resolution of the separate components of $qNP$ has been made possible by examining the kinetics of fluorescence relaxation in the dark in the presence of DCMU, sodium fluoride (a phosphatase inhibitor which inhibits a state 2 to 1 transition), and chloramphenicol. A fast phase of relaxation ($t_{1/2}=1$ min) is attributed to $qE$, the second phase ($t_{1/2}=8$ min) is attributed to $qT$, and a slow phase ($t_{1/2}=40$ min) is attributed to $qi$ (Horton and Hague, 1988). Resolution of these components, however, can be complicated in green algae and should be performed with caution (Lee et al., 1990). Quenching of PS2 fluorescence at room temperature which persists after uncoupling of the proton gradient is thought to be due to $qT$ (if the light intensity is non-photoinhibitory). This, however, should be confirmed by alternative measurements (Lee et al., 1990).

Absolute 77K fluorescence measurements have traditionally been used to confirm the occurrence of a state transition (Krause and Behrend, 1983; Saito et al, 1983; Catt et al, 1984;
Rebeille and Gans, 1988). At room temperature, most fluorescence emission arises from PS2. However, upon cooling to liquid nitrogen temperatures (77K), fluorescence emission peaks associated with both PS2 and PS1 are observed. Peaks associated with the CP43 and CP47 proteins of PS2 are observed at 685 and 695 nm, respectively (Krause and Weis, 1991). Longer wavelength peaks with maxima ranging from 715 to 735 nm are thought to arise from the PS1 core ("PS1-65") and LHC1 (Krause and Weis, 1991). When fluorescence emission spectra are normalized to an external fluorescence standard (e.g. fluorescein), it is possible to determine the "absolute" fluorescence emission associated with PS2 and PS1. A complementary decrease in the absolute fluorescence emission arising from PS2 (F686, F695) and an increase in that arising from PS1 (F715- F735) have been strongly correlated with the occurrence of LHC2 phosphorylation and a state 1 to 2 transition both in vitro (Krause and Behrend, 1983) and in vivo (Saito et al, 1983) in higher plants and green algae. Furthermore, Krause et al (1983) have shown that the absolute fluorescence arising from PS2 is greatly affected by ΔpH-dependent quenching (qE) while the absolute fluorescence arising from PS1 is not. Since both qE and qI are thought to have negligible effect on PS1 fluorescence, absolute 77K fluorescence provides a useful tool to resolve quenching mechanisms and determine the contribution of the state transition to qNP at room temperature (Krause and Weis, 1991).

Rationale for working with Selenastrum minutum:  

In this study, the green alga Selenastrum minutum was chosen to test the interaction between respiratory carbon flow and the state transition. Regulation of respiratory carbon flow has been well characterized physiologically and biochemically in this alga (for a review see Turpin, 1991). Furthermore, S. minutum is easily grown at steady-state under nutrient limitation in chemostats. Nutrient limitation of this alga results in large accumulation of starch which can support high rates of respiratory carbon flow in the dark (Elrifi and Turpin, 1985). In addition, it has been shown that activity of the CRETC, in particular the NAD(P)H-PQ oxidoreductase, is enhanced under N-limitation in green algae (Peltier and Schmidt, 1991).
This, in turn, suggested that the effects of respiratory carbon flow on PETC poising might be more significant and better observed in nutrient-limited cells.

This chapter will focus on the question of whether respiratory carbon flow in the dark can affect the poise of the PETC for the ratio of NADPH/ATP production via a state transition. In order to answer this question, respiratory carbon flow was enhanced by a variety of treatments and fluorescence characteristics were examined to determine whether a state 1 to 2 transition had occurred. Two classes of treatments were distinguished. Both classes resulted in increases in respiratory carbon flow but the first class resulted in complementary changes in PS2 and PS1 fluorescence emission indicative of a state 1 to 2 transition. The second class of treatments did not.

**MATERIALS AND METHODS**

**Cell culture (chemostats)**

The green alga *Selenastrum minutum* (Naeg.) Collins (UTEX 2459) was cultured axenically under conditions of NO$_3^-$ or Pi-limitation in temperature-regulated (20 °C) chemostats as described previously (Elrifi and Turpin, 1985). Cells were aerated with 5% CO$_2$ and magnetically stirred. Continuous illumination was provided by Sylvania cool white fluorescence VHO tubes with an average PFD of 150 μEinstein·m$^{-2}$·s$^{-1}$. Cells were grown on a substantially modified Hughes medium buffered with 25 mM Hepes/KOH at pH 8.0. NO$_3^-$ limited medium contained 1 mM NaNO$_3$ and 200 uM K$_2$PO$_4$ while Pi limited medium contained 12 mM NaNO$_3$ and 30 uM K$_2$PO$_4$. Because the chemostat has a fixed volume, each drop of fresh medium forces an equal volume out of the chemostat. Consequently, when cells are provided with medium which is limited by a particular nutrient, the steady state growth rate of cells is equal to the dilution rate of the culture. NO$_3^-$ and Pi-limited cells were grown under
steady-state conditions at 0.3 and 0.6 d⁻¹, respectively. This represents 18 and 36% of their maximal growth rate. For an overview of chemostat theory see Turpin et al. (1985).

**Treatments:**

1) **NH₄⁺:** Re-supply of NH₄⁺ to dark, aerobic N-limited *S. minutum* results in rates of N assimilation into amino acids of up to 180 μmol N·mg⁻¹ Chl·h⁻¹ (Weger and Turpin, 1989). To ensure that cells acclimated during N-assimilation, cells for these experiments were provided with 180 μmol NH₄Chl·mg⁻¹ Chl which required approximately 1 hour of assimilation.

2) **Anaerobiosis:** Initiation of strict anaerobiosis was achieved by incubation of cells with a glucose/glucose oxidase O₂ scavenging system (Vanlerberghe et al., 1990), consisting of 5 mM glucose, 40 μg mL⁻¹ catalase, 400 μg mL⁻¹ glucose oxidase, and replacement of air bubbling with N₂. It has been shown previously that these cells are unable to utilize exogenous glucose (Vanlerberghe et al., 1989).

3) **CCCP:** CCCP was provided to a final concentration of 3.3 μmol CCCP mg⁻¹ Chl. This concentration was shown to uncouple both mitochondrial and chloroplastic electron transport chains.

4) **NO₃⁻:** NO₃⁻ was provided at a concentration of 55 μmol NaNO₃ mg⁻¹ Chl which allowed cells to fully acclimate to N-assimilation and required approximately 1 hour for assimilation (Weger and Turpin, 1989).

5) **Phosphate:** KPi (K₂HPO₄/KH₂PO₄, pH 8.0) was re-supplied to Pi-limited cells at a concentration of ≈ 80 μmol mg⁻¹ Chl which was shown to require approximately 1 hour for assimilation (Gauthier and Turpin, 1993).
Experimental:

Cells were concentrated by centrifugation (5000 rpm, 5 min) and resuspension in supernatant at the concentrations noted in each technique. All experiments were performed in a darkened, temperature-regulated (20 °C) cuvette bubbled with 5% CO₂ in air and magnetically stirred unless otherwise stated. Cells were dark-adapted for 20 minutes before treatment. Treatments 1 to 4 were applied to N-limited cells while treatment 5 was applied to Pi-limited cells. In all cases, cells had fully acclimated to treatment within 20 minutes.

Fluorescence measurements:

Room temperature steady state fluorescence:

All room temperature fluorescence was measured using a PAM fluorometer (Heinz Walz, Effeltrich, FRG) as previously described (Schreiber et al., 1986). Dilute cells (3 -5 μg Chl mL⁻¹) were dark adapted for 20 minutes in a temperature-regulated, magnetically-stirred, aerated, 3.0 mL disposable acrylic cuvette. The fibre optic cable was placed at the surface of the cuvette. Fluorescence parameters were essentially the same as those designated by van Kooten and Snel (1990) although some measurements were approximations rather than true measurements (see Appendix 1). For the purposes of these measurements, F₀ was designated as the level of fluorescence induced in dark aerobic control cells by the 1.6 kHz measuring beam (0.3 μEi·m⁻²·s⁻¹, < 680 nm). Increases in the fluorescence induced by the measuring beam after treatments were designated as F. Maximal fluorescence (Fₘ) was induced by a saturating (10,000 μEi·m⁻²·s⁻¹) 50 msec multiple turnover xenon flash provided by the PAM XMT 103 unit. (The 50 msec pulse results in induction to the I2 level which reflects full suppression of photochemical quenching at PS2 (Schreiber et al., 1989)). The 50 msec pulses were used to minimize the actinic effects of the saturating pulses in dark-adapted cells. Changes in Fₘ resulting from treatments were designated as Fₘ'. Because changes in photochemical and non-photochemical quenching (qP and qNP, respectively) and not absolute amounts of quenching
coefficients were of interest, \( q_p \) and \( q_{NP} \) were estimated relative to the control with control values arbitrarily set to \( q_p = 1 \) and \( q_{NP} = 0 \) (see Appendix 1). Calculation of the potential corrected quantum yield of linear electron transport for each treatment was performed as described in Holmes et al. (1989): \( \Phi_p = J/I = q_p(0.4777 - 0.3282 q_{NP}) \), where \( \Phi_p \) is the corrected quantum yield, \( J \) is gross linear photosynthetic electron flow and \( I \) is the incident light intensity.

**Absolute 77K Fluorescence:**

For measurement of absolute 77K fluorescence, cells (200 \( \mu \)L, 3-5 \( \mu \)g Chl \( mL^{-1} \)) were injected into uniform NMR tubes and rapidly frozen in liquid \( N_2 \). Absolute emission spectra were measured on a custom-built spectrofluorometer fitted with a low temperature quartz dewar filled with liquid \( N_2 \) (Bruce et al., 1989). Fluorescence was induced by chlorophyll \( a \) excitation (435 nm, 10 nm bandwidth). NMR tubes were mounted in a spinning sample holder driven by compressed \( N_2 \). Fluorescence from the whole tube surface was averaged by spinning the sample at approximately 1000 rpm, during the 10 second measuring period. Repeated fluorescence yield determinations from identical samples were within 4%. Higher accuracy was achieved via the spinning method than with the internal fluorescence standard fluorescein.

**Other measurements:**

All measurements of metabolites and gas exchange were standardized to chlorophyll. Chlorophyll was extracted for 1 to 4 hours in 100% methanol at -20°C and samples were centrifuged to remove particulate matter before reading absorbance (Elrifi and Turpin, 1985). Chlorophyll concentration (\( \mu \)g \( mL^{-1} \)) was calculated as 25.5*(\( A_{650} \)) + 4.0*(\( A_{665} \)) (Holden, 1965).
RESULTS

Effect of treatments on CO₂ efflux and starch degradation:

1) NH₄⁺: Assimilation of NH₄⁺ resulted in an increase in respiratory carbon flow which was observed as a 2.5-fold increase in respiratory CO₂ efflux or a 6-fold increase in the rate of starch breakdown (Table 1).

2) Anaerobiosis: Anaerobiosis in N-limited S. minutum resulted in a "Pasteur effect", which was observed as a 2-fold increase in the rate of starch breakdown (Table 1). The rate of respiratory CO₂ efflux declined 4.7-fold during anaerobiosis (Table 1).

3) CCCP: Uncoupling of N-limited cells with CCCP resulted in a 2.1-fold increase in starch degradation and a 1.8-fold increase in respiratory CO₂ efflux (Table 1; see also Figures 16 and 21, Chapter 5).

4) NO₃⁻: Treatment of N-limited cells with NO₃⁻ resulted in a 3.5-fold increase in the rate of CO₂ efflux and an 8-fold increase in the rate of starch breakdown (Table 1).

5) Pi: In cells grown under phosphate limitation, re-supply of Pi was correlated with a 2.5-fold increase in both respiratory CO₂ efflux and the rate of starch breakdown (Table 1).

Effect of treatments on fluorescence emission:

Figure 5 shows the effects of the 5 treatments which caused increased respiratory carbon flow on steady state room temperature fluorescence emission from S. minutum. These effects were tentatively grouped into two classes on the basis of their effects on room temperature and 77K fluorescence emission (Table 2).

Class 1 treatments (NH₄⁺, anaerobiosis and CCCP):

Perturbation in both Fₘ and F₀ occurred after treatment of N-limited cells with NH₄⁺, anaerobiosis or CCCP. F, the level of fluorescence induced by the measuring beam, increased
Table 1: The effect of a variety of treatments on the rate of respiratory carbon flow in *S. minutum* measured either as the rate of CO$_2$ efflux or starch degradation. Values are normalized to the respective dark, aerobic control. Pi treatment was made to Pi-limited cells, all other treatments were made to N-limited cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>rate of CO$_2$ efflux</th>
<th>rate of starch breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>dark, aerobic control (N or Pi-limited)</td>
<td>1.0 *</td>
<td>1.0 **</td>
</tr>
<tr>
<td>+ NH$_4^+$ †</td>
<td>2.5</td>
<td>6.0</td>
</tr>
<tr>
<td>+ anaerobiosis ‡</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>+ CCCP ‡‡‡</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>+ NO$_3^-$ ‡</td>
<td>3.5</td>
<td>8.0</td>
</tr>
<tr>
<td>+ Pi ‡‡‡</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Actual values were 88.5 (± 32.6) μmol CO$_2$ mg$^{-1}$ Chl h$^{-1}$ and 724 nmol CO$_2$ mg$^{-1}$ Chl h$^{-1}$ for N- and Pi-limited cells, respectively.

** Actual values were 12.7 (± 0.7) μmol gluc equiv mg$^{-1}$ Chl h$^{-1}$ and 10.7 μmol gluc equiv mg$^{-1}$ Chl h$^{-1}$ for N- and Pi- limited cells, respectively.

† from Weger and Turpin, 1989.
††† see Figures 14 and 19, Chapter 5.
‡ from Weger and Turpin, 1989; Turpin, 1992.
‡‡ from Gauthier and Turpin, 1993.
Figure 5: The effect of treatments which increase respiratory carbon flow on steady-state saturation pulse analysis as measured with a pulse amplitude modulated (PAM) fluorometer. Control cells (3-5 μg Chl mL⁻¹) were dark, aerobically adapted for 20 minutes before treatment with A. 750 μM NH₄Cl; B. anaerobiosis; C. 10 μM CCCP; D. 10 μM DCMU; E. 200 μM NaNO₃; F. 400 μM KPi. Treatments A-E and F were made to N-limited and Pi-limited cells, respectively.
approximately 24% within 1 minute of NH$_4^+$ treatment and then recovered gradually to control levels within 20 minutes (Fig 5A). Similarly, F increased a maximum of 38% within 4 minutes of the onset of anaerobiosis (Fig 5B). Maximal change in F did not occur until approximately 10 minutes after uncoupling with CCCP by which time it had increased by 50% (Fig 5C). The maximal level of fluorescence, F$_M$, decreased 29 and 20%, respectively, after 20 minutes of NH$_4^+$ assimilation or anaerobiosis in N-limited cells. Uncoupling with CCCP initially caused a 6% increase in F$_M$ and then resulted in a 14% decrease in F$_M$ within 20 minutes (Figure 5C). This resulted in a 20% overall decrease in fluorescence after treatment with CCCP.

F$_0$ is defined as the minimal level of fluorescence when all the reaction centers are open (QA is oxidized). The fluorescence resulting from the low intensity measuring beam in the "dark" was not the true F$_0$ because it increased upon the addition of 10 μM DCMU (Figure 5D). This problem is inherent in the fluorometer because, at the lowest possible measuring beam light intensity, 10 μM DCMU still resulted in an increase in fluorescence induced by the measuring beam (data not shown). This increase was presumably due to photochemistry caused by the measuring beam (i.e. the measuring beam has an actinic effect) in the presence of DCMU. Thus the fluorescence level measured under dark aerobic control conditions would more accurately be defined as variable fluorescence, F$_V$, but, following convention, has been termed F$_0$.

The assumptions used to approximate the quenching parameters (qp and q$_{NP}$) from the fluorescence transients shown in Fig 5 are provided in Appendix 1. In all cases, the amount of non-photochemical quenching (q$_{NP}$) induced by treatment with NH$_4^+$, anaerobiosis or CCCP increased greater than 0.23 in comparison to dark aerobic control cells within 20 minutes of treatment (Table 2). Treatment with NH$_4^+$, CCCP, and anaerobiosis decreased the amount of photochemical quenching (qp) by 6, 27, and 31%, respectively, relative to dark control cells (Table 2). Approximation of Φp, the potential quantum yield of linear electron transport (quantum yield which would be observed immediately upon illumination or that observed under steady-state illumination assuming q$_{NP}$ and qp values were the same as those calculated in the
Table 2: A summary of the effect of treatments which increased respiratory carbon flow on room temperature and 77K fluorescence parameters in *S. minutum*. $q_{NP}$ and $q_P$ were approximated relative to the dark aerobic control as described in Appendix 1. The potential quantum yield of linear electron transport, $\Phi_p$, was calculated as in Holmes et al. (1989). $F_{686}/F_{717}$ were calculated from absolute fluorescence spectra. All values indicate measurements taken after 20 minutes adaptation to a treatment. Pi treatment was made to Pi-limited cells, all other treatments were made to N-limited cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$q_{NP}$</th>
<th>$q_P$</th>
<th>$\Phi_p$</th>
<th>$F_{686}/F_{717}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dark aerobic controls:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-limited cells</td>
<td>0.0</td>
<td>1.0</td>
<td>0.478</td>
<td>2.33</td>
</tr>
<tr>
<td>Pi-limited cells</td>
<td>0.0</td>
<td>1.0</td>
<td>0.478</td>
<td>2.82</td>
</tr>
<tr>
<td><strong>Class 1 treatments:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NH$_4^+$</td>
<td>0.32</td>
<td>0.94</td>
<td>0.344</td>
<td>1.95</td>
</tr>
<tr>
<td>+ anaerobiosis</td>
<td>0.27</td>
<td>0.73</td>
<td>0.260</td>
<td>1.90</td>
</tr>
<tr>
<td>+ CCCP</td>
<td>0.23</td>
<td>0.69</td>
<td>0.254</td>
<td>1.73</td>
</tr>
<tr>
<td><strong>Class 2 treatments:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NO$_3^-$</td>
<td>0.08</td>
<td>0.92</td>
<td>0.439</td>
<td>2.25</td>
</tr>
<tr>
<td>+ Pi</td>
<td>0.03</td>
<td>0.98</td>
<td>0.458</td>
<td>2.83</td>
</tr>
</tbody>
</table>
dark) was made for each treatment (Table 2). Relative to the dark control, $\Phi_p$ decreased 85% after 20 minutes of anaerobiosis or CCCP uncoupling and 40% during NH$_4^+$ assimilation.

Absolute 77K fluorescence emission spectra from cells which were treated with NH$_4^+$, CCCP, or anaerobiosis are shown in figure 6. The fluorescence at 686 nm (F686) arises from PS2 (CP43) while the fluorescence at 717 nm arises from the peripheral antennae of PS1 (Krause and Weis, 1991). The effect of NH$_4^+$, anaerobiosis, or CCCP was to decrease F686 by 10, 9 and 14% respectively and increase F717 by 21, 13, or 27%, respectively. The absolute decrease in F686 after treatment with NH$_4^+$, anaerobiosis or CCCP was 1.3-, 1.6- and 1.2-fold greater, respectively, than the absolute increase in F717 (Figure 6). NH$_4^+$, anaerobiosis and CCCP treatment decrease the ratio of F686/F717 fluorescence by $\geq$ 20% (Table 2).

**Class 2 treatments (NO$_3^-$ and Pi):**

In contrast to class 1 treatments, NO$_3^-$ assimilation by N-limited cells or Pi assimilation by Pi-limited cells resulted in minimal perturbation in room temperature fluorescence (Figure 5) and caused much smaller changes in $q_{NP}$ or $q_p$ relative to dark control cells (Table 2). NO$_3^-$ treatment resulted in a 0.08 increase in $q_{NP}$ and a 8% decrease in $q_p$. Pi treatment increased $q_{NP}$ by 0.03 and decreased $q_p$ by 2%. In addition, NO$_3^-$ and Pi treatments decreased the potential quantum yield of linear electron transport by only 9 and 4% respectively (Table 2). The effect of NO$_3^-$ or Pi assimilation on 77K emission spectra was also minimal (data not shown) and the F686/F717 ratio decreased by $< 5\%$ after treatment with NO$_3^-$ or Pi (Table 2).

**DISCUSSION**

**Effect of treatments on respiratory carbon flow:**

In green algae, starch is degraded to hexose phosphates in the chloroplast (Levi and Gibbs, 1984). Hexose phosphate can then be oxidized via the oxidative pentose phosphate pathway or the "upper half" of glycolysis to the level of phosphoglyceric acid (PGA) in the
Figure 6: The effect of class 1 treatments (NH$_4^+$, anaerobiosis or CCCP) on absolute fluorescence emission at 77K. Control cells (3-5 µg Chl mL$^{-1}$) were dark, aerobically adapted for 20 minutes (—) before treatment with 750 µM NH$_4$Cl (----), anaerobiosis (-----) or 10 µM CCCP (----). All treatments were made to N-limited cells.
chloroplast before being exported to the cytosol (Klein, 1986). PGA is converted to pyruvate (PYR) in the cytosol and imported into the mitochondria where it is converted to acetyl CoA and enters the TCA cycle (Lambers, 1990). CO₂ efflux arises from decarboxylation steps in the OPP pathway, the pyruvate dehydrogenase complex, and the tricarboxylic acid cycle (TCA cycle). Increases in respiratory carbon flow can, therefore, be measured directly as increases in the rate of starch degradation or respiratory CO₂ efflux. On the basis of these measurements, all 5 treatments increased dark respiratory carbon flow in the green alga *S. minutum* (Table 1).

1) **The effects of NH₄⁺ assimilation:** Treatment of darkened, N-limited cells with NH₄⁺ resulted in a 3-fold enhancement of respiratory CO₂ efflux and a 2-fold increase in the rate of starch degradation (Table 1). In N-limited cells, re-supply of NH₄⁺ results in rapid assimilation into amino acids (Turpin et al., 1990; Weger and Turpin, 1989). In contrast to assimilation of N in N-sufficient cells, assimilation of N by N-limited cells is independent of recent photosynthate and can occur in both the dark and the light (Amory et al., 1991). An increase in TCA cycle activity is necessary to supply carbon skeletons required for amino acid biosynthesis. This has the further effect of "drawing down" carbon from starch via glycolysis to replenish carbon in the TCA cycle.

2) **The effects of anaerobiosis:** Anaerobiosis resulted in a 2-fold increase in the rate of starch breakdown in darkened N-limited cells (Table 1). Anaerobiosis prevents oxidation of reduced pyridine nucleotide via the mitochondrial electron transport chain. This treatment results in a "Pasteur effect" in green algae (Gfeller and Gibbs, 1984; Peavey et al., 1983; Vanlerberghe et al., 1990) and higher plants (Barker et al., 1967; Faiz-ur-Rahman et al., 1974; Givan, 1968; Kobr and Beevers, 1971). In *S. minutum*, the Pasteur effect involves a stimulation of glycolytic starch degradation (Table 1) concurrent with an increase in the production of fermentative end products such as lactate, ethanol, and succinate (Vanlerberghe et al., 1990). The 5-fold decrease in CO₂ efflux, measured during anaerobiosis (Table 1), is consistent with
the observation that only partial oxidative TCA cycle activity occurs during anaerobiosis. Although CO$_2$ is released from ethanol formation (Vanlerberghe et al., 1989), only 1 CO$_2$ is released per pyruvate for ethanol formation compared to 3 CO$_2$ released per pyruvate for full TCA cycle decarboxylation. The occurrence of other fermentative pathways which do not involve CO$_2$ efflux would further decrease the rate of respiratory CO$_2$ efflux (Vanlerberghe et al., 1989).

3) The effect of uncoupling with CCCP: Uncoupling of cells with CCCP resulted in an approximately 2-fold increase in respiratory carbon flow as measured by CO$_2$ efflux or starch degradation (Table 1). This increase is thought to be a response to decreased levels of chemiosmotically-generated ATP which serves to activate glycolysis. Specifically, pyruvate kinase (which catalyzes the conversion of PYR to PEP) is thought to be ADP-limited under physiological conditions and PGA kinase has been proposed to be controlled by adenylate energy charge (Turner and Turner, 1980; Lambers, 1990). Increases in ADP during uncoupling are thought to increase the activation of PK and increase the rate of respiratory carbon flow via glycolysis and the TCA cycle (Turpin et al., 1990; Vanlerberghe et al., 1990a; Bulte et al., 1990).

4) The effects of NO$_3^-$ assimilation: Treatment of N-limited cells with NO$_3^-$ resulted in a 3.5-fold increase in the rate of CO$_2$ efflux and an 8-fold enhancement of starch degradation in the dark (Table 1). Although assimilation of NO$_3^-$ into amino acids occurs at one third the rate of NH$_4^+$ assimilation in the dark, (Weger and Turpin, 1989) it requires $\approx$ 1.5-fold more respiratory carbon flow due to the reductant requirements for NO$_3^-$ reduction to NH$_4^+$ (Turpin, 1991).

5) The effects of Pi assimilation: Treatment of Pi-limited cells with Pi resulted in a 2.5-fold increase in both the rate of CO$_2$ efflux and the rate of starch degradation (Table 1). Re-supply
of Pi to Pi-limited cells was observed to result in rapid rates of uptake and assimilation and it is thought that assimilation of Pi results in a rapid increase in respiratory carbon flow (Table 1) due to the high ATP requirements associated with Pi uptake (Gauthier and Turpin, 1993).

**Effect of treatments on fluorescence emission:**

Although all 5 treatments resulted in an increase in respiratory carbon flow, these treatments were grouped into two classes on the basis of differences in the effects of treatments on fluorescence emission at both room temperature and 77K (Table 2):

**Class 1 treatments (NH₄⁺, anaerobiosis or CCCP):**

**Effects on **$F'_M$, $F_M'$ and $q_{NP}$**:

Anaerobiosis, NH₄⁺ assimilation and CCCP uncoupling all caused large perturbations in room temperature fluorescence and were designated class 1 treatments (Figure 5 A,B,C). All of these treatments resulted in an increase in F (the fluorescence induced by the measuring beam) and a decrease in $F_M$ relative to dark control levels. The height of $F_M'$ relative to $F_M$ can give a direct approximation of the amount of $q_{NP}$ occurring (see Appendix 1). After treatment with CCCP, $F_M'$ actually increased briefly relative to $F_M$ before decreasing to 73% of the dark control $F_M$ (Figure 5C). This suggests that a thylakoid proton gradient occurred even in dark control cells and that this may quench the true $F_M$ level. Non-photochemical quenching of dark fluorescence has also been observed in the diatom *Phaeodactyllum tricornutum* and was attributed to the occurrence of a thylakoid proton gradient in the dark in this alga (Ting and Owens, 1993). Indeed, measurements of $F_O$ and $F_M$ in the dark and over a wide range of light intensities in *S. minutum*, indicated that the true $F_O$ ($q_P=1$, $q_{NP}=0$) and $F_M$ ($q_P=0$, $q_{NP}=0$) occurred at very low light intensities rather than in the dark (see Appendix 1). Quenching of PS2 fluorescence is probably due to a combination of a proton gradient in the dark and the fact that the dark state in green algae is intermediate between state 1 and state 2 (Williams and Allen, 1987). In the interest of simplicity,
approximations of the level of \( q_{NP} \) and \( q_p \) were made relative to the dark control, assuming that these values were 0 and 1 respectively (Appendix 1).

The level of \( q_{NP} \) after 20 minutes of adaptation to class 1 treatments indicated an increase of \( \geq 0.2 \) relative to the dark control. \( q_{NP} \) is thought to result from a variety of non-photochemical processes which have been resolved by relaxation of quenching in the dark or in the presence of DCMU (Horton and Hague, 1988). These include quenching related to a buildup of trans-thylakoid \( \Delta pH \) or energized quenching \( (q_E) \), quenching resulting from photoinhibition of PS2 \( (q_I) \), and quenching related to decreases in PS2 absorbance cross-section due to a state 1 to state 2 transition \( (q_T) \) (Krause and Weis, 1991). In the present study, the amount of \( q_{NP} \) was calculated relative to the dark control and only \( q_E \) and \( q_T \) were possible contributors to the total \( q_{NP} \) observed after treatment.

The fact that much of the \( q_{NP} \) measured by room temperature fluorescence arose from \( q_T \) was confirmed by measurements of absolute 77K fluorescence. Complementary changes in PS2 and PS1 fluorescence peaks have been correlated with the occurrence of a state transition \( (q_T) \) (Krause and Behrend, 1983; Saito et al, 1983) while \( q_E \) appears to affect only PS2 fluorescence emission at 77K (Krause et al, 1983). In all three treatments, a complementary change in the amplitude of F686 and F717 peaks was consistent with a state 1 to state 2 transition (Figure 6). As a result, the ratio of PS2/PS1 (F686/F717) fluorescence decreased by \( \geq 20\% \) during all class 1 treatments (Table 2). The implication that these treatments resulted in a state 1 to 2 transition in \textit{S. minutum} is consistent with the fact that LHC2 phosphorylation increased in \textit{Chlamydomonas reinhardtii} after treatment with anaerobiosis (Wollman and Delepelaire, 1984) or CCCP (Bulte et al., 1990; Gans and Rebeille, 1990). It is also possible that \( q_E \) may have contributed somewhat to quenching at room temperature although it is difficult to determine the magnitude of \( q_E \) contribution to PS2 quenching.

It has been proposed that the state transition may function to regulate the relative ratios of linear and cyclic electron transport (Allen, 1984; 1992; Turpin and Bruce, 1989). One way to examine this possibility is to examine the effects of observed changes in \( q_{NP} \) on the potential
quantum yield of linear electron transport should cells be illuminated. In illuminated cells, the corrected quantum yield of linear electron transport, $\Phi_p$, was negatively correlated with $q_{NP}$ (Weis and Berry, 1987; Holmes et al., 1989). In class 1 treated cells, the effect of the increase in $q_{NP}$ (arising from both $q_T$ and/or $q_E$) in the dark would be to decrease the quantum yield of PS2-mediated linear electron transport immediately upon illumination as compared to the dark aerobic control (Table 2). Furthermore, assuming that the $q_{NP}$ occurring in the dark state would affect the time required to reach a steady-state light state and would also be likely to affect the light state reached, it is quite possible that the changes in dark $q_{NP}$ could affect $\Phi_p$ in the long term during illumination. A decrease in the quantum yield of PS2-mediated linear electron transport would decrease the amount of NADPH produced by the PETC at a constant intensity of illumination. In addition, if cyclic electron transport were occurring, decreasing the quantum yield of linear electron transport would increase the contribution of cyclic electron flow to total electron transport which, in turn, would decrease the NADPH/ATP production ratio. The state 1 to 2 transition might also have the additional effect of enhancing the quantum yield of cyclic electron transport via increased PS1 absorption cross section (Table 2) and association with cyt b$_6$f. This, in turn, would further down-regulate NADPH production and decrease the ratio of NADPH/ATP production.

It should be noted that chloroplast respiratory e$^-$ transport may down-regulate the potential production of NADPH via the PETC in two distinct ways. First, by increasing the thylakoid $\Delta$pH via e$^-$ transport to O$_2$, the CRETC could increase $q_E$ and result in a decrease in the potential quantum yield of PS2. A decrease in the quantum yield of linear e$^-$ transport would decrease NADPH production and would subsequently decrease the ratio of NADPH/ATP produced in the light. Second, by reducing the PQ(cyt b$_6$f) pool, the CRETC would have the effect of a) decreasing the absorbance cross section of PS2 relative to PS1 and b) increasing the association of cyt b$_6$f and LHC2 with PS1. Both of these effects would decrease the quantum yield of linear electron transport and increase the quantum yield of PS1-
mediated cyclic electron transport resulting in a further decrease in the NADPH/ATP production ratio.

**Effects on F₀, F and qp:** Increases in the level of fluorescence induced by the measuring beam have been interpreted as resulting either from a decrease in connectivity between LHC2 and PS2 or changes in the redox state of QA (Krause and Weis, 1991; Buchel and Wilhelm, 1990; Govindjee and Satoh, 1986). Under the conditions used in these experiments, it is unlikely that a decrease in connectivity between LHC2 and PS2 resulted suggesting that the increase was due to changes in the redox state of QA. The increases in fluorescence observed after treatment with DCMU (Figure 5D) are most likely due to actinic effects of the measuring beam due to direct inhibition of QA⁻ oxidation. On the other hand, the increases in F observed after class 1 treatments are not likely to be due to direct inhibition of QA⁻ oxidation but are more likely to arise because these treatments resulted in reduction of the QA pool which in turn increased fluorescence (Figure 5 A,B,C). Calculation of changes in qp as a result of class 1 treatments indicated that a significant decrease in qp occurred consistent with reduction of the QA pool (Table 2). One potential mechanism whereby the QA pool could become reduced is via redox equilibration with a more reduced PQ pool. If, as hypothesized, the rate of PQ reduction was enhanced by an increase in respiratory carbon flow and reductant, this should result in an overall reduction of the PQ pool. This hypothesis will be examined in more detail in Chapter 3. An enhancement in the reduction of the PQ pool is also be consistent with the observation that a state 1 to 2 transition had occurred during class 1 treatments.

It should be noted that it is possible that CCCP may have had some independent effects on QA. In addition to being an uncoupler, CCCP is an ADRY reagent (Accelerates the Deactivation Reactions of Y, the water oxidizing complex of PS2) (Buhkov et al., 1990; Renger, 1972). It has been proposed that CCCP results in inhibition of the back recombination between oxidized Z⁺ and QA⁻; this would also result in accumulation of QA⁻ and an increase in fluorescence (Mohanty and Govindjee, 1973).
Class 2 treatments (NO$_3^-$ and Pi):

In contrast to class 1 treatments, NO$_3^-$ and Pi assimilation resulted in very little perturbation of room temperature fluorescence and were designated as class 2 treatments (Figure 5 E,F). At the same time, only minor (< 5%) changes in $q_p$ and $q_{NP}$ were observed which resulted in very little effect on the potential quantum yield of PS2 linear electron transport in comparison with dark control cells (Table 2). Comparatively small changes in the ratio of PS2/PS1 fluorescence indicated that no state 1 to 2 transition (which would change LHC2 allocation between PS2 and PS1) occurred after either of these treatments (Table 2).

SUMMARY

*Selenastrum minutum* provided an ideal system to comprehensively test the hypothesis that increases in respiratory carbon flow which result in increases in the NAD(P)H/NAD(P) ratio will increase the reduction of PQ (via the NAD(P)H-PQ oxidoreductase) and result in a state 1 to 2 transition. Five metabolically dissimilar treatments including NH$_4^+$, NO$_3^-$ and Pi assimilation, uncoupling with CCCP, and anaerobiosis resulted in large increases in the rate of respiratory carbon flow (Table 1). Furthermore, with the possible exception of uncoupling with CCCP (a naturally occurring antibiotic), all of these treatments simulate physiological conditions which can occur in nature.

This set of five treatments was further sub-divided into 2 classes on the basis of the magnitude of their effects on room temperature and 77K fluorescence. Class 1 treatments (NH$_4^+$, anaerobiosis or CCCP) resulted in large changes in fluorescence emission characteristic of a state 1 to state 2 transition. The state 1 to 2 transition has been suggested as a mechanism to decrease the efficiency of PS2-mediated linear electron transport relative to PS1-mediated cyclic electron transport and would poise the PETC to decrease the production of NADPH relative to ATP (Allen, 1981; Anderson, 1992). In contrast, class 2 treatments (NO$_3^-$ and Pi) resulted in only small changes in fluorescence emission characteristics, implying that a state 1 to
2 transition had not occurred, despite the fact that both treatments increased the rate of respiratory carbon flow. Since a basic tenet of the hypothesis is that the state 1 to 2 transition occurs in response to an over-production of NAD(P)H, the class 1 and class 2 treatments provide an excellent framework within which to begin testing other aspects of the model. With class 1 and 2 treatments, it should be possible to a) correlate the occurrence of a state 1 to 2 transition with a reduction of the PQ pool (Chapter 3) and b) determine whether reduction of the pyridine nucleotide pool is a necessary condition for reduction of the PQ pool and activation of the LHC2 kinase (Chapter 4).
CHAPTER 3: TESTING THE MODEL: THE EFFECTS OF CLASS 1 AND CLASS 2 TREATMENTS ON REDUCTION OF THE PQ(cyt b₆f) POOL

INTRODUCTION

The PQ pool has been proposed as the intersection point between a chloroplastic respiratory electron transport chain (CRETC) and the PETC (Goedheer, 1963; Diner and Mauzeral, 1973; Bennoun, 1982). Since the redox state of the PQ(cyt b₆f) pool can regulate the activation of the kinase responsible for the state 1 to 2 transition (Allen, 1992) and since the redox state of the PQ pool can be affected by electron flow from NAD(P)H to PQ via the CRETC (Bennoun, 1982), it is critical to determine the effects of increases in respiratory carbon flow on the redox state of the PQ pool. In particular, it is necessary to demonstrate a correlation between an increase in respiratory carbon flow and PQ reduction both to provide direct evidence consistent with the operation of the CRETC in vivo and to confirm measurements of the occurrence of a state 1 to 2 transition.

It was demonstrated in Chapter 2 that respiratory carbon flow affected the poise of the PETC for the NADPH/ATP production ratio via a state 1 to 2 transition. Two classes of treatments which increased respiratory carbon flow were distinguished. Class 1 treatments resulted in a state 1 to 2 transition while Class 2 treatments did not. Class 1 treatments also increased F, the fluorescence induced by the measuring beam after the treatment suggesting that the QA pool had become more reduced. It was proposed that reduction of the QA pool might be a result of redox equilibration with a more reduced PQ pool resulting from class 1 treatments. The purpose of this chapter was to determine the effects of increases in respiratory carbon flow on the redox state of the PQ(cyt b₆f) pool in more detail. The sub-hypothesis to be tested was that class 1 treatments would result in an increase in PQ(cyt b₆f) pool reduction while class 2 treatments would not.
Methods which can be used to infer the redox state of the PQ pool:

The physical reactions within the pigment beds of PS2 and PS1 are much faster than electron transport processes. Photon capture and exciton migration to the reaction centres occurs within $10^{-15}$ sec and $5 \times 10^{-12}$ seconds, respectively (Lawlor, 1987). Chlorophyll fluorescence occurs within $10^{-9}$ seconds if the reaction centre is closed (primary electron transfer acceptor is reduced), but is faster if the reaction centre is oxidized. At room temperature, fluorescence originates mainly from PS2 while fluorescence from PS1 is minimal. This is due to the fact that, although both P680$^+$ and P700$^+$ can act as traps for excitation energy (dissipation as heat) and represent "quenchers" of fluorescence, reduction of P700$^+$ is slow (ms to µs) compared to that of P680$^+$ (nsec) and, therefore, P700$^+$ acts as a much more efficient quencher of fluorescence (Krause and Weis, 1991; Horton and Bowyer, 1991). The rate-limiting step in photosynthetic electron transport is electron transfer from PQH$_2$ to the cyt b$_6$f complex. A six-fold increase in this time constant (16 - 90 msec) is observed as the thylakoid proton gradient increases from low to high levels (Lawlor, 1987). On average, an electron is transferred from water to NADP$^+$ within 20 msec (Wong, 1982; Govindjee and Waseilewski, 1990; Lawlor, 1987).

Both time-resolved fluorescence induction and decay can be used to deduce the redox state of the PQ pool. In the absence of q$_{NP}$, the fluorescence yield at any one time is proportional to the redox state of QA; that is, there is a hyperbolic relationship between the fluorescence yield at any one time and the fraction of the QA pool that is reduced (Joliot and Joliot, 1964; Cao and Govindjee, 1990; Gleiter et al., 1993). Fluorescence yield is maximal ($F_M$) when QA is reduced and minimal ($F_0$) when QA is oxidized. The kinetics of fluorescence induction from $F_0$ to $F_M$ or fluorescence decay from $F_M$ to $F_0$ are related to competing processes which govern the rate of oxidation and reduction of the QA pool (Krause and Weis, 1991). The kinetics of these individual processes can be resolved by judicious choice of the time scale on which measurements are based.
Measurements of fluorescence decay from $F_M$ to $F_O$ in the μsecond time scale can be used to resolve the rate of $Q_A$ oxidation by $Q_B$ (PQ). The dark decay of variable fluorescence back to the $F_O$ state has typically been used to examine the effects of mutations, inhibitors and photoinhibition on the re-oxidation kinetics of $Q_A$ (Cao and Govindjee, 1990; Gleiter et al., 1993; Erickson et al., 1989; Govindjee et al., 1992; Robinson and Crofts, 1983). In this method, by means of a high intensity single turnover flash, approximately all $Q_A$ (but not $Q_B$ or PQ) is reduced, resulting in a maximal level of fluorescence, $F_M$. The decline of fluorescence (induced by a weak, modulated, "non-actinic" measuring light) to the minimal level of fluorescence, $F_O$, in the ensuing dark period reflects the re-oxidation kinetics of $Q_A^-$ (Krause and Weis, 1991). When this decay is curve-fitted, it can be shown to proceed in three exponential phases with lifetimes of 200 - 900 μsec, 2-10 msec, and 1-2 seconds (Cao and Govindjee, 1990; Robinson and Crofts, 1983). The fastest phase has been attributed to electron transport from $Q_A^-$ to $Q_B$ in centres containing bound $Q_B$. The middle phase is thought to represent the binding kinetics of PQ to the $Q_B$ site in centres with no $Q_B$ bound before the flash. The slowest component of the decay has been attributed to PS2 centres that are unable to transmit electrons to the $Q_B$ pool (PS2β centres). $Q_A^-$ re-oxidation apparent from this phase is thought to result from recombination between $Q_A^-$ and the S2 state of the water oxidizing complex of PS2 (Cao and Govindjee, 1990; Etienne et al., 1990). It should noted, however, that decay models merely quantify results by describing the decay of $Q_A^-$. In reality, these processes may not necessarily be independent of each other and may not follow the first order reaction kinetics that have been assigned to them (Cao and Govindjee, 1990).

Because the rate of re-oxidation can be affected by the redox state of the PQ pool, it is also possible to infer the reduction state of the PQ pool from fluorescence decay kinetics. However, under conditions where the PQ pool is partially reduced, the $Q_A$ pool is also likely to be slightly reduced due to redox equilibration between these two pools (Robinson and Crofts, 1983). Thus, before and after the single turnover flash the $Q_A$ pool will not be fully oxidized and fluorescence will decay from a state where all $Q_A$ is reduced to a state where only part of
the QA pool is reduced. In addition, PS2 will exist in a large variety of redox conformations before and during the flash including QAQB, QAQ2B, QAQB2, QAQBH2, QA, and QA- leading to considerable complexity as compared to conditions where all QA is oxidized before the single turnover flash. However, despite this added complexity, reduction of the PQ pool will, in addition to slightly reducing the QA pool before the single turnover flash, also decrease the rate of QA- oxidation because of the decrease in oxidized PQ available to oxidize QA- (Robinson and Crofts, 1983). Thus, one would expect a decrease in the rate of fluorescence decay when the PQ pool becomes reduced.

Measurement, on a msec time scale, of the kinetics of fluorescence induction from F0 to FM allows resolution of the kinetics of QA reduction (Krause and Weis, 1991). Since PQ to cyt b6f is the rate-limiting electron transfer step in the msec time frame, the availability of oxidized PQ will determine the kinetics of QA reduction and hence fluorescence induction to FM. Increasing the reduction of the PQ pool should increase the rate of fluorescence induction. In this chapter, both fluorescence decay and induction analysis are employed to examine the effect of increased rates of respiration on PQ redox status.

MATERIALS AND METHODS

Experimental conditions:

All experimental conditions were as previously described for steady-state fluorescence saturation pulse analysis (Chapter 2) unless otherwise specified. Cells were adapted for 20 minutes under control or treatment conditions before measurement of fluorescence parameters.

Fluorescence:

Time-resolved decays:

Time-resolved fluorescence decays were measured essentially as described by Schreiber (1986) using the PAM fluorometer (Heinz Walz, Effeltrich, FRG). A single turnover flash (t1/2
= 8 μsec, 10,000 μEi·m⁻²·s⁻¹) was provided by the XST 103 (Walz) unit. Three msec before triggering of the single turnover flash, a low intensity modulated measuring beam (λ = 660 nm, 0.5 μEi·m⁻²·s⁻¹) was electronically switched from 1.6 kHz to 100 kHz. The 100 kHz measuring beam was used for a duration of 40 msec to allow resolution of the kinetics of slower fluorescence decays which resulted from class 1 treatments (in particular NH₄⁺ or anaerobiosis treatment, see Appendix 2). Due to special gating circuitry on the fluorometer detector, the first signal point was recorded 120 μsec after triggering of the single turnover flash. The signal output was measured at 25 μsec intervals and stored by the DA100 IBM-compatible interface (Kolbowski and Schreiber, Walz, Effeltrich, 1991). In order to optimize signal to noise ratios for curve fitting analysis, 8 consecutive decays (dark time interval between flashes, 15 sec) were averaged. The actinic effect of the measuring beam was measured by disconnecting the XST 103 flash lamp and electronically triggering the measuring beam to switch from 1.6 to 100 kHz.

ASCII files were transferred to Sigma Plot for further analysis. Time zero for the fluorescence decay measurements was fixed at the time the actinic flash reached its maximum intensity (Gleiter et al., 1993). Due to gating circuitry, the first data point used in decay analysis was at 120 μsec. For curve fitting, data sets were reduced to 150 data points by averaging points in the asymptotic part of the decay. Fluorescence decay curves were corrected for the actinic effects of the measuring beam by subtracting the fluorescence induced by the measuring beam from the observed fluorescence decay (see Appendix 2 for details). [QA⁻] was calculated from variable chl a fluorescence according to Joliot and Joliot (1964) assuming that the intersystem exciton transfer probability (p) was 0.5 (Cao and Govindjee, 1990). This yields a hyperbolic relationship between fluorescence yield, F(t), and the fraction q(t) of closed reaction centres at time t such that:

\[
q(t) = \frac{2(F(t) - F_0)}{[F_M - F_0 + (F(t) - F_0)]}
\]

In curves corrected for the actinic effects of the measuring beam (see Appendix 2), this relationship assumed the form:
\[ q(t) = \frac{2(F(t)_{MB+ST} - F(t)_{MB})}{[(F_M - F(t)_{MB}) + (F(t)_{MB+ST} - F(t)_{MB})]} \]

where \( F(t)_{MB+ST} \) was the fluorescence measured at time, \( t \), by the 100 kHz measuring beam in conjunction with a single turnover flash and \( F(t)_{MB} \) was the fluorescence measured at time, \( t \), by the 100 kHz measuring beam in the absence of a single turnover flash. In the case of treated cells, \( F_M' \) was substituted for \( F_M \). The fraction of closed reaction centres at time \( t \), \( q(t) \), was then be fitted by an iterative least squares method to a sum of exponentials of the form:

\[
q(t) = q(0) \sum_{i=1}^{N} \alpha_i \exp\left(-t/\tau_i\right)
\]

where \( \alpha_i \) is the amplitude and \( \tau_i \) is the lifetime of the \( i^{th} \) component of the decay. \( \tau_i \), the \( t_{1/2} \) of each decay was calculated as \( 0.69/\tau_i \).

**Time-resolved inductions:**

Fluorescence inductions were recorded in the same manner as decays with the following exceptions. A 50 msec, multiple turnover, 10,000 \( \mu \)Ei-m\(^{-2}\cdot s\(^{-1}\) flash was provided by the XMT 103 unit (Walz). This multiple turnover flash has essentially square on/off characteristics and should not affect the shape of the fluorescence induction curve (Heinz Walz, 1987). A lower intensity measuring beam (0.05 \( \mu \)Ei-m\(^{-2}\cdot s\(^{-1}\)) was switched from 1.6 to 100 kHz for 50 msec and signal output was recorded at 300 \( \mu \)sec intervals. Induction curves were signal-averaged 8 times with a frequency of 0.5 min\(^{-1}\). \( F_M \) was calculated as the average of the final 3 msec of the induction curve, or the highest point achieved in the induction curve (anaerobiosis, DCMU). The area above the fluorescence induction curve, \( A_{\text{max}} \), was calculated as the area between the fluorescence curve and its asymptote (\( F_M \) or \( F_M' \)).
RESULTS

Time-resolved fluorescence decays:

DCMU and Class 1 treatments (NH$_4^+$, anaerobiosis, CCCP):

The effect of class 1 type treatments on fluorescence decays (corrected for the actinic effect of the measuring beam) is shown in figure 7 (A, B, and C). NH$_4^+$ and anaerobic treatment resulted in a large decrease in the rate of decay which was observed as an increase in the area between the treated decay curve and the dark control decay curve (Figure 7 A, B,). Inhibition, with DCMU, of electron transfer from QA$^-$ to QB also resulted in a decrease in the rate of decay seen as an increase in the area between the treated decay and the dark control curve (Figure 7 D). Uncoupling of cells with CCCP did not result in an increase in the area between the treated and control decay curves but affected the shape of the fluorescence decay (Figure 7 C). Fluorescence decays in Figure 7 were transformed to q(t) decays as described in Materials and Methods. The dark control q(t) decay was a sum of two exponential decay components including a fast component with a half-time, T$_1$, of 185 $\mu$s and a medium component with T$_2$ of approximately 5 msec (Table 3). The fast component of the dark control q(t) decay contributed 64% (as measured by the amplitude of the fast component, $\alpha_1$=0.64) while the slow component contributed only 36% ( $\alpha_2$=0.36) to the total q(t) decay. All Class 1 treatments resulted in a loss of the fast ($\mu$s) decay component ($\alpha_1$=0) and an increase in the total contribution of the medium (msec) decay component ($\alpha_2$=1.0) (Table 3). Although the half-time of the msec decay component appeared to increase after treatment with DCMU and NH$_4^+$, this increase was not statistically significant as determined by a student t test.

Class 2 treatments (NO$_3^-$ or Pi):

Class 2 type treatments had a much smaller effect compared to class 1 treatments on the observed rate of fluorescence decay (Figure 7 E, F). NO$_3^-$ treatment resulted in a small difference between the NO$_3^-$ decay and the dark control decay between 0 and 10 msec (Figure
Figure 7: The effect of class 1 and class 2 treatments on corrected time-resolved fluorescence decay kinetics. Decays were measured using a 40 msec duration, 100 kHz measuring beam and were corrected for the actinic effect of the measuring beam as shown in Appendix 2. Open circles: dark, aerobic control cells; closed circles: cells treated with A. NH$_4^+$; B. anaerobiosis; C. CCCP; D. DCMU; E. NO$_3^-$; F. Pi. Treatments A-E and F were made to N-limited and Pi-limited cells (3-5 μg Chl mL$^{-1}$), respectively. The single turnover flash ($t_{1/2}=8$ μsec) was initiated 3 msec after the measuring beam was switched from 1.6 to 100 kHz. Decays were normalized to $F_M-F_O$ (control) or $F_{M'}-F$ (treated) where $F_M$ and $F_{M'}$ were the maximal fluorescence reached during the flash and $F_O$ or $F$ were the fluorescence measured over a 10 msec period before flash initiation. All curves were an average of 8 measurements.
7 E) while Pi treatment resulted in a difference between the treated and control curves after 10 msec of treatment (Figure 7 F). NO$_3^-$ assimilation decreased the contribution of the μsec $q(t)$ decay component from 64 to 48% and increased the contribution of the msec decay component from 36 to 52% (Table 3). $T_1$, the half time of the μsec decay component, was also slightly increased after treatment with NO$_3^-$ (Table 3). The increase in $T_2$, the half-time of the msec decay component, after treatment with NO$_3^-$ was not statistically significant. In Pi-limited control cells, the 44% contribution of the μsec $q(t)$ decay component ($\alpha_1$) was significantly smaller than the 64% contribution of this component in N-limited control cells (Table 3). Treatment with Pi resulted in no significant effect on the amplitudes of either the μsec or msec components. The half-time of the msec component ($T_2$), however, was significantly increased by treatment with Pi.

**Time-resolved fluorescence inductions (msec time scale):**

**DCMU and Class 1 treatments:**

Treatment with NH$_4^+$, anaerobiosis, or CCCP resulted in an increase in the rate of fluorescence induction compared to the dark aerobic control (Figure 8 A, B, C). The area above the fluorescence curve is proportional to the rate at which a curve reaches $F_M$. Treatment with NH$_4^+$ resulted in a rapid increase in fluorescence to $F_M$ within 900 μsec (Figure 8A) and a 95% decrease in the $A_{\text{max}}$, the area above the induction curve (Table 4). Anaerobiosis resulted in an increase to $F_M$ within 900 μsec but, after reaching a plateau, the fluorescence level declined to a level 10% less than $F_M$ (Figure 8B). $A_{\text{max}}$ was decreased by 97% compared to the control after treatment with anaerobiosis (Table 4). CCCP treatment resulted in a rapid increase to a level which was 10% below $F_M$ and was followed by a slow increase to $F_M$ (Figure 8C). This corresponded to a 64% decrease in the area above the induction curve as compared to the dark aerobic control (Table 4). Similarly to treatments with NH$_4^+$, treatment with DCMU resulted in induction of fluorescence $F_M$ within 900 μsec; subsequently fluorescence slowly decayed to a level 10% below $F_M$ within 50 msec (Figure 8D). In cells treated with
Table 3: The effect of DCMU and class 1 and class 2 treatments on the amplitude ($\alpha_i$) and half times ($T_i = T_f/0.69$) of the fast (\mu sec; $\alpha_1$ and $T_1$) and medium (msec; $\alpha_2$ and $T_2$) components of time-resolved q(t) decays which were corrected for the actinic effect of the 100 kHz measuring beam (see Appendix 2). Cells were adapted to treatments for 20 minutes before measurements of decays. Fluorescence decays (Figure 7) were transformed to q(t) decays and then curve-fitted for either a single exponential or a sum of two exponentials (see Materials and Methods).\footnote{In all cases where curves fitted a single exponential, the $r^2$ for the single exponential fit was greater than 0.98 and/or greater than the $r^2$ for the sum of two exponentials.}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\alpha_1$ (SE)</th>
<th>$\alpha_2$ (SE)</th>
<th>$T_1$ (\mu sec) (SE)</th>
<th>$T_2$ (msec) (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark aerobic controls:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-limited cells</td>
<td>0.64 (.07)</td>
<td>0.36 (.07)</td>
<td>185.3 (24.8)</td>
<td>4.89 (1.59)</td>
</tr>
<tr>
<td>Pi-limited cells</td>
<td>0.44 (.02)*</td>
<td>0.56 (.02)*</td>
<td>192.4 (14.6)</td>
<td>6.67 (.34)</td>
</tr>
<tr>
<td>$+$ DCMU</td>
<td>0.0*</td>
<td>1.0*</td>
<td>0.0*</td>
<td>10.8 (1.21)</td>
</tr>
<tr>
<td>Class 1 treatments:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$+$ NH$_4^+$</td>
<td>0.0*</td>
<td>1.0*</td>
<td>0.0*</td>
<td>9.91 (1.66)</td>
</tr>
<tr>
<td>$+$ anaerobiosis</td>
<td>0.0*</td>
<td>1.0*</td>
<td>0.0*</td>
<td>4.34 (0.34)</td>
</tr>
<tr>
<td>$+$ CCCP</td>
<td>0.0*</td>
<td>1.0*</td>
<td>0.0*</td>
<td>3.35 (0.04)</td>
</tr>
<tr>
<td>Class 2 treatments:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$+$ NO$_3^-$</td>
<td>0.48 (.01)*</td>
<td>0.52 (.01)*</td>
<td>291.4 (41.9)*</td>
<td>9.09 (1.31)</td>
</tr>
<tr>
<td>$+$ Pi \footnote{Pi treatment was made to Pi-limited cells, all other treatments were made to N-limited cells.}</td>
<td>0.51 (.04)</td>
<td>0.49 (.04)</td>
<td>201 (13.6)</td>
<td>10.5 (1.0)**</td>
</tr>
</tbody>
</table>

\footnote{Indicates that this value was significantly different from the N-limited dark aerobic control, i.e. the value was outside the 95\% confidence interval as determined by a student t test.}
\footnote{** Indicates that this value was significantly different from the Pi-limited dark, aerobic control.}
Figure 8: The effect of class 1 and class 2 treatments on time-resolved fluorescence induction kinetics. Inductions were initiated at t=0 with a multiple turnover, 50 msec duration, saturating flash (10,000 μEi m⁻² s⁻¹). Inductions were normalized to $F_M - F_O$ (control) or $F_M' - F$ (treated), where $F_M$ and $F_M'$ were the maximal fluorescence reached within 50 msec. Open circles: dark, aerobic control cells; closed circles: cells treated with A. NH₄⁺; B. anaerobiosis; C. CCCP; D. DCMU; E. NO₃⁻; F. Pi. Treatments A-E and F were made to N-limited and Pi-limited cells (3-5 μg Chl mL⁻¹), respectively. All treatments were an average of 8 measurements.
Table 4: The effect of DCMU and treatments which increase respiratory carbon flow on $A_{\text{max}}$, the area above time-resolved fluorescence induction curves. $A_{\text{max}}$ was calculated as

$$A_{\text{max}} = \sum_{t=0}^{F_M} [t \times (F_M - F(t))],$$

where $F(t)$ is the fluorescence at time $t$ and $F_M$ was substituted for $F_M$ in treated cells. Samples were adapted for 20 minutes of treatment before measurement. Pi treatment was made to Pi-limited cells. All other treatments were made to N-limited cells.

<table>
<thead>
<tr>
<th>Treatment (n=)</th>
<th>Area above induction curve, $A_{\text{max}}$ (mV·μsec) (SE)</th>
<th>% of control $A_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dark aerobic controls:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-limited cells (19)</td>
<td>3311.5 (208)</td>
<td>100.0</td>
</tr>
<tr>
<td>Pi-limited cells (3)</td>
<td>637.5 (35)</td>
<td>100.0</td>
</tr>
<tr>
<td>+ DCMU (2)</td>
<td>47.3 (14.6)</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Class 1 Treatments:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NH$_4^+$ (3)</td>
<td>125.2 (48.3)</td>
<td>3.8</td>
</tr>
<tr>
<td>+ anaerobiosis (3)</td>
<td>99.4 (5.8)</td>
<td>3.0</td>
</tr>
<tr>
<td>+ CCCP (3)</td>
<td>1182.8 (172.1)</td>
<td>35.7</td>
</tr>
<tr>
<td><strong>Class 2 Treatments:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NO$_3^-$ (5)</td>
<td>610.4 (116)</td>
<td>18.4</td>
</tr>
<tr>
<td>+ Pi (3)</td>
<td>844.3 (41)</td>
<td>132.4</td>
</tr>
</tbody>
</table>

* Significantly different from the dark control as determined by a student t test.
DCMU, the area above the fluorescence induction curve was decreased by 98% compared to the $A_{\text{max}}$ of dark control cells (Table 4).

**Class 2 treatments:**

Treatment with NO$_3^-$ resulted in a rapid increase to a level which was 90% of $F_M$ and then a slow increase to $F_M$ within 50 msec (Figure 8E). This resulted in an 82% decrease in $A_{\text{max}}$, the area above the fluorescence curve (Table 4). Pi treatment resulted a slightly slower rate of fluorescence induction (Figure 8F) and initiated a 30% increase in the area above the fluorescence induction curve (Table 4).

**DISCUSSION**

**DCMU and Class 1 treatments:**

**Time-resolved fluorescence decays:**

Fluorescence decay kinetics are related to QA$^-$ re-oxidation kinetics and can be used to deduce the redox state of the PQ pool (Krause and Weis, 1991). Furthermore, the rate of QA$^-$ oxidation is dependent upon the rate of e$^-$ transfer to the QB(PQ) pool and this in turn can be affected by the redox state of the PQ pool (Robinson and Crofts, 1983). When all QA is oxidized before the single turnover flash, QA$^-$ oxidation kinetics have been de-convoluted to yield three decay components attributed to PS2 species with QB bound before the flash, QB not bound before the flash and QB-non-reducing centers (Cao and Govindjee, 1990).

However, under conditions where QA is not completely oxidized before the flash a number of redox species would be present and it is not surprising that the fluorescence decays, in this case, can be approximated by 2 components rather than 3 (Table 3). Despite the fact that QA was likely to have been initially reduced (particularly in the case of class 1 treatments), the rate of oxidation of QA$^-$ after a single turnover flash should still be affected by the redox state of the PQ pool. Moreover, decreases in the rate of QA$^-$ oxidation should affect the contribution
and/or the half-times of the 2 components of the decays. Indeed, all class 1 treatments resulted in a loss of the μsec component of QA⁻⁻ re-oxidation kinetics and a 100% contribution (α₁=1.0) of the slower msec half-time decay component (Table 3) indicating that QA⁻⁻ oxidation had decreased due to reduction of the PQ pool. The loss of the μsec decay component observed after treatment with DCMU, which prevents binding of QB to PS2 and, therefore, prevents oxidation of QA⁻ by QB, further confirms that class 1 treatments resulted in a decrease in the rate of QA⁻⁻ oxidation after a single turnover flash (Table 3). The reduction of the PQ pool implied by the decreased rate of QA⁻⁻ oxidation after class 1 treatments is consistent with the observation of fluorescence changes indicating the occurrence of a state 1 to 2 transition since PQ reduction would be required to activate the kinase responsible for the state 1 to 2 transition.

**Time-resolved fluorescence induction:**

The reduction state of inter-system electron carriers can also be determined by time-resolved fluorescence induction kinetics on the msec time scale. The area bounded by the fluorescence rise and its asymptote is a measure of the pool size of electron acceptors of PS2 which is equivalent to the pool of oxidized PQ available for photochemistry (Schreiber, 1986; Horton and Bowyer, 1991; Krause and Weis, 1991). A faster rise (smaller area above the curve) is indicative that a smaller proportion of the total pool is being oxidized and is available to accept electrons from PS2 (Krause and Weis, 1991; Bennoun, 1982). Support for this interpretation comes from the observation that the addition of DCMU resulted in a rapid increase in the rate of QA reduction (Figure 8 D) and decreased the area above the induction curve, Aₘₐₓ, to 2% of the dark control level (Table 4). Class 1 treatments (NH₄⁺, anaerobiosis, CCCP) also resulted in faster rates of fluorescence induction (Figure 8 A, B, C) and decreased the Aₘₐₓ by 96, 97, and 64% respectively (Table 4). Combined with evidence from fast fluorescence decays, these data support the hypothesis that class 1 treatments result in a reduction of the PQ(cyt b₆f) pool.
An increase in the reduction of the PQ pool, as measured by either fluorescence induction or fluorescence decays, as a result of treatment with NH₄⁺, anaerobiosis, or CCCP, is consistent with the fact that each of these treatments appeared to cause a state 1 to 2 transition (Chapter 2). Reduction of the PQ(cyt b₆f) pool is required to activate the protein kinase responsible for the state 1 to 2 transition (Allen, 1992). An increase in the reduction of the PQ(cyt b₆f) pool, as a result of increased respiratory carbon flow, also provides indirect evidence that respiratory carbon flow can affect electron transport to PQ via an NAD(P)H-PQ oxidoreductase (Godde and Trebst, 1980).

Class 2 treatments (Pi and NO₃⁻ assimilation):

**Time-resolved fluorescence decays:**

In dark control Pi-limited cells, the 44% contribution of the µsec component of the QA⁻ decay was significantly smaller than the 64% contribution in N-limited control cells. This may indicate that the PQ pool was more reduced in Pi-limited control cells than in N-limited control cells. However, the ratio of PS2/PS1 fluorescence at 77K was higher in Pi-limited cells than in N-limited cells (Table 2, Chapter 2) suggesting that variations in fluorescence decay characteristics may arise from differences in PETC component stoichiometry in cells adapted to N- and Pi-limitation. Pi treatment of Pi-limited cells resulted in no significant change in the amplitudes of either the µsec or msec QA⁻ decay components although an increase in the half-time of the msec component was observed (Table 3). The observation that Pi treatment had no effect on the contributions of the µsec or msec decay components implies that the rate of QA⁻ oxidation was relatively unaffected compared to class 1 treatments and, therefore, that the redox state of the PQ pool was also relatively unaffected.

Treatment of N-limited cells with NO₃⁻ resulted in a decrease in the contribution of the µsec q(t) decay component from 64 to 48%, an increase in the half-time of the µsec decay component and an increase in the contribution of the msec decay component from 36 to 52%
(Table 3). Although the contribution of the μsec QA⁻ decay component decreased after NO₃⁻ treatment, the extent of this change was much smaller than that observed after class 1 treatments or treatment with DCMU. This suggests that increases in PQ reduction during NO₃⁻ assimilation were minor compared to class 1 treatments. The observation of only minor changes in PQ reduction, implied by fluorescence decay measurements after treatment with NO₃⁻, is consistent with both saturation pulse and 77K fluorescence analysis which indicated that a state 1 to 2 transition had not occurred (Chapter 2). Slight discrepancies may have resulted from actinic effects of repetitive single turnover flashes for signal averaging.

**Fluorescence inductions:**

Treatment with Pi slightly increased the area above the fluorescence induction curve and indicated a minor oxidation of the PQ pool during Pi assimilation (Table 4). Comparison of induction and μsec decay data suggests that Pi treatment had relatively little effect on the reduction of the PQ(cyt b₆f) pool. This is consistent with the observation that a state 1 to 2 transition was not induced by treatment with Pi (Chapter 2).

Treatment with NO₃⁻ resulted in an 82% decrease in the area above the fluorescence induction curve (Figure 8, Table 4) implying that the pool of oxidized e⁻ acceptors became more reduced. This is inconsistent with the minor changes in fluorescence decay kinetics observed and the absence of a state 1 to 2 transition (Chapter 2). The inconsistency between induction and decay measurements in cells assimilating NO₃⁻ suggests that additional factors may affect QA⁻ oxidation and reduction kinetics after treatment with NO₃⁻.

One possible reason for the increase in PQ reduction observed after treatment with NO₃⁻ is that illumination with a multiple turnover flash may affect the redox state of intersystem e⁻ acceptors. Electron flow can occur from PS2 to NADP within 20 msec (Lawlor, 1987). Thus, inhibition of electron transfer from PS1 to NADP would also affect the rate of fluorescence induction of the msec time scale. If inhibition of electron transfer from PS1 to NADP were due to a substrate-related phenomenon during NO₃⁻ assimilation, then reduction of
the NADP(H) pool would be expected. However, in N-limited *S. minutum*, the NADP(H) pool became more oxidized during dark NO$_3^-$ assimilation (Vanlerberghe et al., 1992). Alternatively, however, it is possible that inhibition of electron transfer from PS1 to NADPH might occur as a result of Fd/FNR limitation due to super-complex formation. Precedence for reversible super-complex formation involving Fd and FNR comes from the observation that FNR, LHC2 kinase, and cyt b$_6$f have been shown to co-purify when isolated from thylakoid membranes (Joliot et al., 1993, 1989; Gal et al., 1990a; Hodges et al., 1987). It has been proposed that electrons for NO$_2^-$ reduction may come from NADPH via Fd and reverse action of FNR (Paneque et al., 1967). Although very little is known about the regulation of FNR association in the dark, precedent for super-complex association suggests that FNR and nitrite reductase may associate during NO$_3^-$ assimilation. This, in turn, could decrease the association and/or availability of Fd/FNR for PS1 oxidation and result in reduction of the intersystem electron transport chain which would be further enhanced by repetitive averaging. The decrease in $A_{\text{max}}$ observed during NO$_3^-$ assimilation may not be due to direct reduction of PQ by NAD(P)H-PQ oxidoreductase activity but, rather, may reflect the accumulation of reductant at PS1 due to Fd/FNR limitation.

**SUMMARY**

The fluorescence induction and decay data presented above indicate that class 1 treatments (NH$_4^+$, anaerobiosis, CCCP), which stimulated respiratory carbon flow and resulted in a state 1 to 2 transition, resulted in an increase in the rate of fluorescence induction and a decrease in the rate of fluorescence decay. Both of these results were consistent with an increase in the reduction of the PQ pool. This would provide a mechanism by which the LHC2 kinase could be activated and result in a state 1 to state 2 transition. In addition, reduction of the PQ pool, as a result of class 1 treatments, implies that a mechanism exists which can reduce
the PQ pool in the dark and provides indirect evidence for NAD(P)H-PQ oxidoreductase activity. A link between an increase in the reduction of the NAD(P)H pool and an increase in the reduction of the PQ pool would provide strong evidence for interaction between respiratory carbon flow and the state transition which can regulate photosynthetic NADPH/ATP production ratios. This link will be examined in the next chapter.

Treatment with either NO$_3^-$ or Pi resulted in negligible changes in room temperature or 77K fluorescence after 20 minutes of acclimation indicating that a state 1 to 2 transition did not occur (Chapter 2). The rate of fluorescence decay on the µsec time scale was also relatively unaffected in either of these treatments in comparison to the affects of DCMU or class 1 treatments (Table 3) suggesting that the PQ pool was not greatly reduced. Millisecond induction kinetics confirmed that the PQ pool was not reduced by Pi treatment. In the case of NO$_3^-$ assimilation, illumination with the multiple turnover flash may have actinic effects which obscured the effect of NO$_3^-$ treatment on PQ redox in the dark. No changes in room temperature or 77K fluorescence were observed during NO$_3^-$ assimilation (Chapter 2), which was consistent with the minor changes in fluorescence decay but inconsistent with induction data. Such variability highlights the complexity of factors contributing to the fluorescence characteristics of cells assimilating NO$_3^-$.

When examining long term induction phenomena, it is obvious that extreme care must be taken, particularly when using induction kinetics to determine rates of QA reduction and oxidation and to deduce the redox state of the intersystem electron transport chain. Because light is used to induce and to measure fluorescence, inaccuracy is inevitable in the determination of QA reduction and oxidation kinetics unless the actinic effects of the measuring beam are corrected for (see Appendix 2).
CHAPTER 4: THE EFFECTS OF CLASS 1 AND CLASS 2 TREATMENTS ON THE REDOX STATE OF THE PYRIDINE NUCLEOTIDE POOL:

INTRODUCTION

It was proposed in Chapter 1 that respiratory carbon flow may poise the PETC to decrease the NADPH/ATP production ratio by initiating a state 1 to 2 transition. An increase in the ratio of reduced/oxidized pyridine nucleotides resulting from increased respiratory carbon flow would reduce the PQ(cyt b6f) pool via an NAD(P)H-PQ oxidoreductase (Godde and Trebst, 1980) and result in a state 1 to 2 transition. Treatment of nutrient-limited *S. minutum* with NH$_4^+$, anaerobiosis, CCCP, NO$_3^-$, or Pi has been shown to result in a rapid increase in the rate of respiratory carbon flow as measured by starch degradation and/or CO$_2$ efflux (see chapter 2). However, only the first three treatments (NH$_4^+$, anaerobiosis or CCCP) resulted in changes consistent with the reduction of PQ and the occurrence of a state 1 to 2 transition (chapters 2 and 3). Selective use of these treatments provides an ideal system to test the implications of the model for interaction between respiratory carbon flow and state 1 to 2 transitions. Specifically, this system will allow an examination of whether an increase in the NAD(P)H/NAD(P) ratio is a requirement for an increase in PQ reduction and a state 1 to 2 transition.

An increase in the cellular NADPH/NADP ratio has been correlated with the occurrence of a state 1 to 2 transition in the dark by several workers (Bulte et al., 1990; Gans and Rebeille, 1990; Mohanty et al., 1990). In *Chlamydomonas reinhardtii*, Gans and Rebeille (1990) presented evidence consistent with the occurrence of a state 1 to 2 transition after treatment of darkened cells with CCCP (uncoupler) or a combination of antimycin A and SHAM (full inhibition of METC electron transport to either cytochrome or alternative oxidase). Both treatments were proposed to result in an activation of respiratory carbon flow due to the release of ADP-limitation of PK and activation of glycolysis. Furthermore, both treatments could be
shown to increase NADPH/NADP but not NADH/NAD ratios, suggesting that the former ratio was important in initiating a state 1 to 2 transition (Rebeille and Gans, 1988; Gans and Rebeille, 1990; Bulte et al., 1990). Mohanty et al. (1990) provided evidence consistent with the occurrence of a state 1 to 2 transition after treatment of darkened, N-limited *S. minutum* with NH$_4^+$ but not NO$_3^-$. Mohanty et al. (1990) also suggested that the state 1 to 2 transition was due to an increase in the NADPH/NADP ratio although increases in both the NADH/NAD and NADPH/NADP ratios were shown to occur in darkened N-limited *S. minutum* after treatment with NH$_4^+$ (Vanlerberghe et al., 1992). In all studies to date, an increase in the cellular NADPH/NADP ratio was proposed to result in an increase in PQ reduction via NAD(P)H-PQ oxidoreductase activity in the thylakoid membrane which, in turn, resulted in a state 1 to 2 transition.

Several pieces of evidence suggest, however, that NADH may be as important as, if not more important than, NADPH as a substrate for the thylakoid NAD(P)H-PQ oxidoreductase. First, isolated NAD(P)H-PQ oxidoreductase has been shown to utilize NADH with higher efficiency than NADPH (Godde and Trebst, 1980). Second, NADH is commonly associated with catabolic respiratory processes and is used primarily for the generation of ATP while NADPH is primarily associated with reductive biosynthesis (Stryer, 1988). Therefore, in the absence of reductant utilizing reactions such as NO$_3^-$ assimilation, it would be surprising if an increase in the NADH/NAD ratio did not result as a consequence of increased respiratory carbon flow. It is equally possible, then, that increases in the NADH/NAD ratio may interact with NAD(P)H-PQ oxidoreductase to reduce the PQ(cyt b$_{6f}$) pool and activate the kinase responsible for a state 1 to 2 transition.

Although an interaction between NADPH and the PQ(cyt b$_{6f}$) pool is consistent with the previous work (Mohanty et al., 1990; Bulte et al., 1990; Gans and Rebeille, 1990), no studies have comprehensively tested the implications of the hypothesis that an increase in the NADPH/NADP but not the NADH/NAD ratio is an absolute requirement for a reduction of the PQ pool and the occurrence of a state 1 to 2 transition. The focus of this chapter will be to
determine whether regulation of the PETC poising for the NADPH/ATP production ratio is accomplished by increases in the NADPH/NADP and/or NADH/NAD ratios. It is hypothesized that class 1 treatments, which increase respiratory carbon flow, PQ reduction, and cause state 1 to 2 transitions, will correlate with increases in reduced/oxidized pyridine nucleotide ratios. On the other hand, class 2 treatments (which increase respiratory carbon flow but have lesser affects on PQ reduction and do not appear to result in a state 1 to 2 transition) will not increase the ratio of reduced/oxidized pyridine nucleotides.

**Techniques to measure pyridine nucleotides:**

To test this model, it was necessary to accurately measure changes in both the NADH/NAD and NADPH/NADP ratios, which in turn required measurement of NAD, NADH, NADP and NADPH. Gans and coworkers measured only NAD and NADP and calculated the pools of NADH and NADPH by subtracting the oxidized pool from an estimate of the total pyridine nucleotide pool (Bulte et al., 1990; Gans and Rebeille, 1990; Rebeille and Gans, 1988). Although this procedure can accurately measure the quantities of oxidized pyridine nucleotides, resolution of changes in reduced pools, which contribute only a small amount to the overall pool size, can be difficult. In particular, the contribution of the NADH pool to the total NAD(H) pool in most green algae and higher plants is less than 10% (Vanlerberghe et al., 1992; Huppe et al., 1992; Bulte et al., 1990; Takahama et al., 1980; Bonzon et al., 1983; Matsumura-Kadota et al., 1982; Muto and Miyachi, 1981). Resolution of a 50% change in the NADH pool (which would constitute only a 5.6% change in the NAD pool) would be limited by experimental error. To accurately measure changes in the reduced pyridine nucleotide pools in the present work, simultaneous measurements of oxidized and reduced pyridine nucleotides were undertaken using rapid, parallel sampling in which one sample was quenched in acid (destroys reduced pyridine nucleotides) and the other in base (destroys oxidized pyridine nucleotides). Highly specific enzymatic cycling assays allowed resolution of changes in NADP, NADPH, NAD and NADH (Passonneau and Lowry, 1974).
Measurements of all pyridine nucleotides using this method have been made in *S. minutum* after treatment with NH$_4^+$, NO$_3^-$ (Vanlerberghe et al., 1992) and Pi (Gauthier and Turpin, 1993). The purpose of this chapter was to measure NAD, NADP, NADH and NADPH after treatment with anaerobiosis and CCCP to provide a complete pyridine nucleotide data set for analysis. The complete data set was then used to test the positive and negative implications of the hypothesis that increases in respiratory carbon flow, which increase the NADH/NAD or NADPH/NADP ratios, will result in an increase in PQ reduction and a state 1 to 2 transition.

The advantages of the present study are twofold. First, the simultaneous measurement of all four forms of pyridine nucleotide (NAD, NADH, NADP, NADPH) allowed accurate resolution of changes in both NADH/NAD and NADPH/NADP ratios. Second, unlike previous work (Bulte et al., 1990; Rebeille and Gans, 1988; Gans and Rebeille, 1990; Mohanty et al., 1990) which has used only a small (*n* ≤ 2) data set to correlate increases in the NADPH/NADP ratio with an increase in PQ reduction, the combination of data sets from all five treatments in the present study allowed a more rigorous examination of the model for interaction between respiratory carbon flow and state transitions.

**MATERIALS AND METHODS**

**Experimental:**

Experimental conditions were the same as those described in chapter 1. Cells were concentrated to 20 μg Chl mL$^{-1}$ and dark-adapted for 20 minutes before the initiation of sampling.

**Pyridine nucleotide determinations:**

**Sampling:** Pyridine nucleotides were determined essentially as described previously (Vanlerberghe et al., 1992). Duplicate cell samples (200 μL) were killed simultaneously by
injection into 900 µL of acid (ChlCl₃:MeOH:1N HCl, 7.5:17.5:1, v/v) or alkali (ChlCl₃:MeOH:1N KOH, 7.5:17.5:1, v/v). All samples were kept on ice until the experiment was complete. Basic samples were then heated for 5 minutes at 60 °C to ensure complete decomposition of oxidized pyridine nucleotides. Acid treated samples retain oxidized pyridine nucleotides with decomposition of reduced forms. All samples were subsequently neutralized with KOH or HCl (for acid- and base-treated samples, respectively) and mixed with 400 µL NaF (12.5 mM). After centrifugation, the aqueous layer was removed and concentrated to less than 500 µL in a Speed Vac centrifuge to ensure removal of MeOH from the samples. Sample volume was then adjusted to 1 mL with distilled H₂O.

Enzymatic cycling: Enzymatic cycling of pyridine nucleotides was performed as described previously (Vanlerberghe et al., 1992; Passonneau and Lowry, 1974). In cycling, the nucleotides act as catalysts for an enzymatic dismutation between two substrates. The nucleotide concentrations are far below the $K_M$ values of the two enzymes and the reaction rates (and, consequently, the final products) are proportional to the initial nucleotide concentrations. One of the products is determined after several thousand cycles. This technique allows extremely small physiological concentrations of pyridine nucleotides ($10^{-14}$ mol) to be "amplified" via cycling into a measurable quantity of an end-product. By using enzymes which are extremely specific for either the phosphorylated or non-phosphorylated forms of pyridine nucleotides, it is possible to accurately measure NADH, NAD, NADP and NADPH.

Pyridine nucleotide samples were stored in liquid N₂ for less than 24 hours before cycling. Cycling assays (final volume = 120 µL) consisted of 100 µL assay reagent and up to 20 µL of sample. The sample volumes used for each nucleotide were 2.5 (NAD), 10 - 20 (NADH), 7.5 (NADP) and 5 µL (NADPH). Phosphorylated pyridine nucleotides were cycled using an assay mix (0.2M Tris, pH 8.4;10 mM lactate; 50 mM ammonium acetate; 5 mM 2-OG (BMC 127850) and 0.3 mM ADP) containing glutamate dehydrogenase (BMC 127078, 90 U).
and G6P dehydrogenase (BMC 127663, 70 U). Non-phosphorylated pyridine nucleotides were cycled in an assay mix (0.1M Tris, pH 8.0; 1 mM G6P; 5 mM 2-OG (BMC 127850); 30 mM ammonium acetate; and 0.1 mM ADP) containing lactate dehydrogenase (Sigma L7755, 120 U) and glutamate dehydrogenase (BMC 127078, 180 U). All enzymes were treated with activated charcoal prior to cycling. Samples were cycled simultaneously with standards for one hour at 37 °C. Standards were 0 to 1 pmol NADP(H) and 0 to 1.5 pmol NAD(H) of authentic pyridine nucleotides (BMC 775754, 107735, 128031, 107824). Standard curves always had an $r^2$ of $\geq$ 0.98. The product of the NADP(H) assay was 6-phosphogluconate while the product of the NAD(H) assay was pyruvate. Cycling products were stored in liquid N2 until measurement.

Products of the cycling assays were measured using standard coupled enzymatic assays and a dual wavelength spectrophotometer (ZFP22, Sigma, Berlin, FRG) as described previously (Quick et al., 1989; Wirtz et al., 1980, see also Appendix 3). All metabolite measurements were normalized to chlorophyll.

Fluorescence:

Room temperature fluorescence was measured as described in Chapter 1.

RESULTS

For clarity and completeness, the results presented here will summarize the effects of all 5 treatments on both phosphorylated and non-phosphorylated pyridine nucleotides. These data include original research by this author and other published work from Dr. Turpin's lab. Please refer to figure legends for the original source of data. Results presented are the average of a minimum of 3 separate experiments; variation around each point was less than 5%.
NADP, NADPH, and the NADPH/NADP ratio:

1) NH₄⁺:

NADPH levels changed less than 5% for the first 1.5 minutes and less than 12% for the following 3.5 minutes after treatment with NH₄⁺ (Figure 9A). After 10 minutes, NADPH levels decreased by 25%. In contrast, NADP decreased by 12% within 5 seconds and by 20% within 30 seconds of treatment with NH₄⁺. NADP decreased to 36% of the control level within 2 minutes (Figure 9A). The NADPH/NADP ratio increased by 20% within 30 seconds and reached a peak of 1.6-fold the dark control ratio within 2 minutes (Figure 9D). The NADPH/NADP ratio then declined to a plateau level of approximately 40% greater than the dark control within 5 minutes after treatment.

2) Anaerobiosis:

Anaerobiosis doubled NADP within 5 seconds and reduced NADPH by 23% (Figure 9B). NADP oscillated to one half the control level within 1 minute, increased to 30% above the control after 2 minutes, and then returned to a level 5 to 10% below control levels within 3 minutes. NADPH mirrored this oscillation with a 20% decrease after 1 minute, a 10% decrease after 2 minutes and a return to a level 10% greater than the control (Figure 9B). The NADPH/NADP ratio oscillated to a level less than one half the control within 5 seconds, increased to a level 70% greater than the control within 1 minute, dropped to 30% of the control level within 2 minutes, and then continued to oscillate at a level slightly higher than the control for the remainder of the experiment (Figure 9E).

Re-supply of oxygen to anaerobic cells: If oxygen was re-supplied to dark anaerobic cells, NADPH increased by 12% within 30 seconds and 20% within 2 minutes of re-supply. NADP decreased approximately 5% within 1 minute of re-supply (data not shown). The NADPH/NADP ratio increased by approximately 15% within 30 seconds of re-supply and 25% within 3 minutes of re-supply before returning to control levels within 10 minutes of re-supply (Figure 9E, inset).
Figure 9: The effect of class 1 treatments on NADP, NADPH and the NADPH/NADP ratio in *S. minutum*. Figures A-C: NADPH, closed circles; NADP, open circles. Figures D-F, NADPH/NADP ratio. N-limited cells (30 μg Chl mL⁻¹) were dark adapted for 20 minutes before beginning sampling. Cells were treated at time 0 with: A,D: NH₄⁺; B,E: anaerobiosis; C,F: CCCP. Figure 5E inset, the effect of O₂ re-addition after 20 minutes of anaerobic adaptation. NH₄⁺ data reproduced with permission from Vanlerberghe et al. (1992).
3) CCCP:

The data in Figure 9C illustrate the identical trends of 9 such experiments. Within 5 seconds of treatment with CCCP, NADP increased by 50%, NADPH decreased by 15% and the ratio of NADPH/NADP dropped by almost half. NADP reached a maximum some 2.5-fold greater than the dark control level within 1 minute of treatment while NADPH decreased gradually to approximately 30% of dark control levels within 5 minutes (Figure 9C). Within 30 seconds, the NADPH/NADP ratio dropped to 20% of its original level and remained lower than 15% of the original level for the remainder of the experiment (Figure 9F).

4) NO₃⁻:

Treatment with NO₃⁻ resulted in a 240% increase in NADP, a 30% decrease in NADPH and 70% decrease in the NADPH/NADP ratio within 5 seconds (Figure 10A, C). Within 30 seconds, this ratio decreased to a level 2.1-fold less than the dark control and remained at this level for the first 20 minutes. NADPH retained the 30% decrease for approximately 2 minutes before recovering to a level slightly higher than the dark control level within 20 minutes.

5) Pi:

Within 20 seconds of treatment with Pi, NADPH peaked at a level 60% greater than the dark control level whereupon it decreased back to a level slightly below the dark control within 5 minutes (Figure 10B). NADP dropped immediately upon treatment with Pi and reached a level approximately half that of the control within 5 minutes. Within 5 seconds of treatment, the NADPH/NADP ratio increased by 1.5-fold and reached a plateau at approximately twice the dark control level after 2.5 minutes (Figure 10D).

NAD, NADH, and the NADH/NAD ratio:

1) NH₄⁺:

Within 5 seconds of treatment with NH₄⁺, NADH increased 2-fold, NAD decreased by 9% and the NADH/NAD ratio increased by 2.3-fold (Figure 11A). NADH increased to a level
Figure 10: The effect of class 2 treatments on NADP, NADPH and the NADPH/NADP ratio in *S. minutum*. Figures A and B: NADPH, closed circles; NADP, open circles. Figures C and D, NADPH/NADP ratio. Cells (30 μg Chl mL⁻¹) were dark adapted for 20 minutes before beginning sampling. Cells were treated at time 0 with: A,C: NO₃⁻; B,D: Pi. NO₃⁻ and Pi treatments were made to N- and Pi-limited cells, respectively. Data represents the mean of 3 experiments. Figures reproduced with permission from Vanlerberghe et al. (1992) and Gauthier and Turpin (1993).
Figure 11: The effect of class 1 treatments on NAD, NADH and the NADH/NAD ratio in *S. minutum*. Figures A-C: NADH, closed circles; NAD, open circles. Figures D-F, NADH/NAD ratio. N-limited cells (30 µg Chl mL⁻¹) were dark adapted for 20 minutes before beginning sampling. Cells were treated at time 0 with: A,D: NH₄⁺; B,E: anaerobiosis; C,F: CCCP. Figure 7E inset, the effect of O₂ re-addition after 20 minutes of anaerobic treatment. NH₄⁺ data reproduced with permission from Vanlerberghe et al. (1992).
4 times that of the control within 1 minute and remained at a level 3-fold greater than the dark control for the remainder of the experiment. The NADH/NAD ratio reached a maximum of 4.8-fold greater than the dark control level after 1 minute. The ratio gradually decreased to a level 3-fold greater than the dark control within 15 minutes (Figure 11D).

2) Anaerobiosis:

NAD and NADH levels were relatively unaffected for the first 20 seconds after initiation of anaerobiosis (Figure 11B). After 2 minutes, NADH increased 2.5-fold and NAD was 10% smaller than the dark control. After 5 minutes, NADH decreased and remained at a level 2-fold greater than the control for the remainder of the experiment. Anaerobiosis had no effect upon the NADH/NAD ratio until 30 seconds after treatment whereupon the ratio increased by 25%. After 2 minutes, this ratio reached a maximum which was 2.8-fold greater than the dark control and then returned to a plateau level of approximately 2.4-fold greater than the control (Figure 11E).

Re-supply of anaerobic cells with oxygen: Re-supplying steady-state anaerobic cells with O2 decreased NADH by 60% within 5 seconds of re-supply. NADH recovered to control levels after 3 minutes. NAD increased by approximately 5% within 30 seconds of O2 re-supply (data not shown). The NADH/NAD ratio was halved within 5 seconds of O2 re-supply and returned to control levels within 3 minutes of oxygen re-supply (Figure 11E, inset).

3) CCCP:

Within 5 seconds of uncoupling with CCCP, NAD decreased 12%, NADH increased 2.4-fold and the NADH/NAD ratio increased 2.7-fold (Figure 11C, F). NAD remained 10 to 15% lower than the dark control for the remainder of the experiment while NADH increased to 3.5-fold the control levels within 1 minute. The NADH/NAD ratio peaked at a level approximately 4-fold greater than the dark control ratio within 1 minute of treatment and
remained more than 3-fold greater than the control for at least 8 minutes after treatment (Figure 11F).

4) NO$_3^-$:

NAD and NADH and the NADH/NAD ratio were relatively unchanged for the first minute after treatment with NO$_3^-$ (Figure 12A). NADH increased by 60% within 2 minutes and continued to increase to approximately 1.8-fold greater than dark control levels within 15 minutes of treatment. NAD decreased by approximately 10% after 1.5 minutes (Figure 12A). The NADH/NAD ratio increased to 1.7-fold control levels within 1.5 minutes and remained above this level for the remainder of the experiment (Figure 12C).

5) Pi:

Treatment with Pi resulted in a 20% decrease in NAD within 5 seconds after which time NAD recovered to a level 20% greater than the control within 5 minutes (Figure 12B). NADH increased 1.5-fold after 20 seconds, dropped to control levels within 1.5 minutes, and then gradually increased to a level 30% greater than the control within 5 minutes of treatment. The NADH/NAD ratio oscillated with a 40% increase within 20 seconds, a 10% decrease after 1.5 minutes and then reached a plateau at a level 5 to 10% greater than the dark control within 2.5 minutes (Figure 12D).

Fluorescence:

Re-supply of O$_2$ to darkened, steady-state anaerobic cells resulted in a rapid decrease in F, the level of fluorescence induced by the low intensity measuring beam. Within 5 seconds, F decreased by 40% and within 1 minute F recovered to a level close to dark aerobic control levels (Figure 13). The fluorescence induced by a saturating flash (F$_{M}$) required approximately 5 to 10 minutes to recover (Figure 13).
**Figure 12:** The effect of class 2 treatments on NAD, NADH and the NADH/NAD ratio in *S. minutum*. Figures A and B: NADH, closed circles; NAD, open circles. Figures C and D, NADH/NAD ratio. Cells (30 µg Chl mL⁻¹) were dark adapted for 20 minutes before beginning sampling. Cells were treated at time 0 with: A,C: NO₃⁻; B,D: Pi. NO₃⁻ and Pi treatments were made to N and Pi-limited cells, respectively. Data represents the mean of 3 experiments. Figures reproduced with permission from Vanlerberghe et al. (1992) and Gauthier and Turpin (1993).
**Figure 13:** The effect of O$_2$ re-addition on room temperature fluorescence from cells (3 µg Chl mL$^{-1}$) that were anaerobically adapted for 20 minutes. Up arrow indicates removal of O$_2$ and onset of anaerobiosis, down arrow indicates re-addition of O$_2$. $F_0$, minimal control level of fluorescence; $F_M$, maximal control level of fluorescence induced by a saturating flash; $F$, fluorescence induced by the fluorescence measuring beam after treatment; $F_M'$, maximal level of fluorescence induced by a saturating flash after treatment.
DISCUSSION

Very little is known about the kinetic properties of the NAD(P)H-PQ oxidoreductase. It is not clear if this enzyme is a rate-limiting enzyme subject to allosteric regulation, or if it is an equilibrium enzyme. All of the following analysis assumes that the NAD(P)H-PQ oxidoreductase is an equilibrium enzyme and is regulated by changes in the product/substrate ratios.

NADP, NADPH, and the NADPH/NADP ratio:

Class 1 Treatments (NH$_4^+$, anaerobiosis, CCCP):

Treatment of N-limited *S. minutum* with NH$_4^+$, anaerobiosis, and CCCP resulted in an increase in respiratory carbon flow which was correlated with PQ reduction and a subsequent state 1 to 2 transition (Chapters 2 and 3). Although these treatments consistently increased PQ reduction, they had widely varying effects upon the NADPH/NADP ratio (Figure 9 D-F). Treatment with NH$_4^+$ decreased NADP and NADPH to 1/2 and 9/10 of their original levels, respectively (Figure 9A). This resulted in an increase in the NADPH/NADP ratio by 1.6-fold within 2 minutes (Figure 9D). In the case of anaerobiosis, large oscillations in NADP and NADPH were transitory in nature only lasting approximately 2 minutes (Figure 9B). The NADPH/NADP ratio dropped rapidly, increased to a level greater than the control and then returned to equilibrium within 2 minutes. (Figure 9E). After uncoupling with CCCP, NADP increased 2.6-fold while NADPH dropped to 2/5 of its original level (Figure 9C). This resulted in an 80% decrease in the NADPH/NADP ratio within 30 seconds and a 10-fold decrease within 5 minutes (Figure 9C). On the other hand, uncoupling nutrient-sufficient *Chlamydomonas reinhardtii* with CCCP was reported to cause a 45% increase in the contribution of NADPH to the total NADP(H) pool corresponding to an increase in the NADPH/NADP ratio (Bulte et al., 1990; Gans and Rebeille, 1990; Rebeille and Gans, 1988). The reasons for the difference between NADPH/NADP ratios observed in the present study and previous work are unclear. It
is possible, though, that the nutrient status of the alga may affect the path of carbon flow after uncoupling to NAD- or NADP-utilizing enzymes and hence result in different effects on the NADPH/NADP ratio. It is also possible that methodology may account for these differences. The advantage of the present work is that it measures NADPH and NADP separately to determine the NADPH/NADP ratio while the measurements of Gans and coworkers (Rebeille and Gans, 1990; Bulte et al., 1990; Gans and Rebeille, 1988) were based on measurements of oxidized pyridine nucleotides and suffer the limitations previously discussed.

**Class 2 Treatments (NO₃⁻ and Pi):**

Treatment of *S. minutum* with NO₃⁻ or Pi also resulted in an increase in respiratory carbon flow but, in contrast to the three treatments outlined above, these two treatments resulted in relatively minor changes in PQ reduction and did not appear to cause a state 1 to 2 transition (Chapters 2 and 3). However, these two treatments did not have consistent effects on the NADPH/NADP ratio (Figure 10C, D). Treatment with NO₃⁻ caused a doubling of NADP and a small transient (< 2 min.) drop in NADPH (Figure 10A). The overall effect upon the NADPH/NADP ratio was a 2-fold decrease within 2 minutes of treatment (Figure 10C). On the other hand, treatment with Pi halved NADP, doubled NADPH (Figure 10B), and resulted in a 2-fold increase in the NADPH/NADP ratio within 2 minutes of treatment (Figures 10D).

**Increases in the NADPH/NADP ratio do not appear to be correlated with PQ reduction:**

From the previous discussion it appears that class 1 and class 2 had no consistent effect upon the NADPH/NADP ratio. Pi treatment did not result in a state 1 to 2 transition but increased the NADPH/NADP ratio while CCCP treatment resulted in a state 1 to 2 transition but decreased the NADPH/NADP ratio. These observations suggest that NADPH may not be responsible for reducing PQ and the resulting state 1 to 2 transitions.
NAD, NADH, and the NADH/NAD ratio:

Class 1 Treatments (NH₄⁺, anaerobiosis, CCCP):

Treatments with NH₄⁺, anaerobiosis, and CCCP all resulted in relatively rapid decreases in NAD, increases in NADH, and an overall increase in the NADH/NAD ratio of greater than 2.8-fold within 2 minutes of treatment (Figure 11). Treatment with NH₄⁺ resulted in a 2.3-fold increase in the NADH/NAD ratio within 5 seconds of treatment and a level 4.7-fold higher than the dark control within 1 minute (figure 11D). The effects of anaerobiosis were slightly delayed and no effect on the NADH/NAD ratio was observed until 30 seconds after treatment whereupon the level increased to 2.8-fold the dark control level within 2 minutes (Figure 11E). Within 5 seconds of CCCP treatment, NADH increased 2.4-fold, NAD decreased 9%, and the NADH/NAD ratio reached a level 2.7-fold larger than the dark control level (Figure 11C, F). Within 2 minutes, the NADH/NAD ratio peaked at 4-fold the dark control level (Figure 11F).

The observation that large increases in the NADH/NAD ratio resulted after all class 1 treatments (which were correlated with increased PQ reduction and a state 1 to 2 transition) implies that NADH may be responsible for PQ reduction via the NAD(P)H-PQ oxidoreductase.

Re-supply of O₂ to anaerobic cells: One implication of the proposal that increases in the NADH/NAD ratio result in reduction of PQ and a state 1 to 2 transition is that re-oxidation of PQ should be correlated with a decrease in the NADH/NAD ratio. Re-supply of O₂ to steady-state anaerobic cells would allow oxidation of PQ by removing substrate limitation for the terminal oxidase for the CRETC. Indeed, within 5 seconds of O₂ re-supply, the level of fluorescence induced by the measuring beam (F) decreased by 40% and completely recovered within 1 minute (Figure 13). F has been shown to be a good estimate of the oxidation state of QA and hence the PQ pool (Chapter 2). The observed decrease in F, therefore, is consistent with an oxidation of QA(PQ) due to the removal of substrate limitation. The difference between F_M' and F_M indicates the level of non-photochemical quenching due, in part, to a state 1 to 2 transition (Chapter 2). Only after F had recovered (implying PQ oxidation) did F_M'
increase, indicating that the LHC2 kinase had been deactivated and a state 2 to state 1 transition had occurred (Figure 13). The rapid oxidation of PQ was correlated with a slow increase in the NADPH/NADP ratio (Figure 9E, inset) and a rapid 60% oxidation of the NADH/NAD ratio (Figure 11E, inset). If NADPH were responsible for reducing PQ, one would not expect the observed accumulation of reductant as the PQ pool was oxidized. Instead, oxidation of PQ would be expected to result in increased flow of accumulated pools of reductant to PQ. The observed decrease in the NADH/NAD ratio is consistent with this hypothesis although it is highly likely that the METC, which would also be O_2-limited, would contribute significantly to the 60% decrease in the NADH/NAD ratio. An increase in the NADPH/NADP ratio after O_2 re-supply to anaerobic cells is inconsistent with NADPH being responsible for the reduction of PQ and provides more evidence to suggest that NADH is responsible for PQ reduction and the state 1 to 2 transition observed during class 1 treatments.

Changes in the NADH/NAD ratio after CCCP treatment:

The increase in the NADH/NAD ratio observed in *S. minutum* after treatment with CCCP contrasts with data from nutrient-sufficient *Chlamydomonas reinhardtii* where no change in the NADH/NAD ratio was observed upon uncoupling with CCCP (Gans and Rebeille, 1988; Rebeille and Gans, 1990; Bulte et al., 1990). The discrepancy between the results from work with *S. minutum* and *C. reinhardtii* likely stems from the inaccuracy inherent in the method used to calculate NADH levels in work with *C. reinhardtii* (Bulte et al., 1990; Gans and Rebeille, 1988; Rebeille and Gans, 1990). This method involves calculation of NADH as the difference between the total NAD(H) pool and measured levels of NAD. Since NADH contributes less than 10% to the total pool, the 240% increase in NADH, seen in *S. minutum* after treatment with CCCP (Figure 11C), would only cause a 12 to 15% decrease in the NAD pool. Experimental error in the measurement of the NAD pool could easily obscure changes of this magnitude and result in no observed change in the contribution of NADH to the total NAD(H) pool in *C. reinhardtii* (Bulte et al., 1990; Rebeille and Gans, 1990; Gans and Rebeille,
1988). Direct measurement of both NADH and NAD, however, has allowed an accurate resolution of large changes in the NADH/NAD ratio after CCCP treatment of *S. minutum* (Figure 9C).

Overall, the observation that class 1 treatments resulted in an increase in the NADH/NAD ratio but had no consistent effect on the NADPH/NADP ratio implies that NADH may be responsible for reduction of the PQ pool and a state 1 to state 2 transition.

**Class 2 Treatments (Pi and NO₃⁻):**

Treatment with Pi resulted in rapidly oscillating changes in the NAD(H) pool for the first 2 minutes after treatment with Pi (Figure 12 B, D). After 2.5 minutes of Pi treatment, however, the NADH/NAD ratio reached a plateau at a level which was only 10% higher than the control and remained at this level for the first 20 minutes of treatment (Figure 12D). Pi treatment was not observed to result in a large reduction of the PQ pool or cause a state 1 to 2 transition. The observation that the NADH/NAD ratio was relatively unaffected while the NADPH/NADP ratio increased after Pi treatment is supports the contention that NADH is responsible for the reduction of the PQ pool and a state 1 to state 2 transition.

NO₃⁻ treatment resulted in changes in the NAD(H) pool which were slower and of smaller magnitude than changes induced by class 1 treatment. NADH, NAD and the NADH/NAD ratio were relatively unchanged for the first minute after treatment with NO₃⁻. Subsequently, NAD decreased, NADH increased, and the NADH/NAD ratio peaked at a level approximately 1.7-fold the dark control level (Figure 12A, C). The increased NADH/NAD ratio, observed during NO₃⁻ treatment, is not completely consistent with the hypothesis that NADH is responsible for PQ reduction and a state 1 to 2 transition. Such inconsistency highlights the complexity of the case of NO₃⁻ assimilation. NO₃⁻ resulted in only minor changes in qNP, the F686/F717 77K fluorescence ratio and fluorescence decay kinetics which suggested that PQ reduction was only marginally effected and that a state 1 to 2 transition had not occurred (Chapter 2 and 3). The situation was complicated, however, because induction
kinetics were faster during NO$_3^-$ assimilation, suggesting that PQ reduction might have occurred. The increases in the NADH/NAD ratio observed (1.7-fold) were intermediate between those observed for class 1 treatments (2.8 - 4.7-fold) and the other class 2 treatment, Pi addition (1.1-fold). There are several plausible explanations for the inconsistencies noted between increases in the NADH/NAD ratio during NO$_3^-$ assimilation and the proposed interaction between increased NADH/NAD ratios, PQ reduction and a state 1 to 2 transition.

It is possible that NO$_3^-$ may represent an intermediate between the class 1 and class 2 definition. This, however, does not explain why only small changes in 77K fluorescence were observed suggesting that a state 1 to 2 transition had not occurred. Alternatively, it is possible that a threshold increase in the NADH/NAD ratio is required to increase the electron flow rate from NADH to PQ such that it becomes limited by terminal oxidase activity and results in PQ reduction. It has been suggested that the terminal oxidase is the rate-limiting step in the cyanobacterial respiratory electron transport chain, which shares PQ with the PETC (Myers, 1986; Peltier and Schmidt, 1991). If the amount of NADH present in dark control cells were well below the $K_M$ (NADH) of the CRETC, electron flow would not be limited and PQ would remain mostly oxidized. Increases in NADH, though, could increase the number of electrons flowing to the terminal oxidase and result in a buildup of reduced PQ once the terminal oxidase became saturated with electrons from PQ. The amount of NADH required to saturate the terminal oxidase and result in significant reduction of PQ might be greater than the increase noted after treatment with NO$_3^-$ but less than that observed after class 1 treatments. Finally, it is possible that increases in the chloroplastic NADH/NAD ratio after treatment with NO$_3^-$ are compartmentalized. In such a scenario, the observed increases in the NADH/NAD ratio would result from mitochondrial activity and would not reflect increases in the chloroplast. Since increases in chloroplastic NADH/NAD ratio would be required to increase PQ reduction, a lack of change in chloroplastic NADH/NAD ratios during NO$_3^-$ treatment would be consistent with the observations that a state 1 to 2 transition had not occurred. These issues will be dealt with in more depth in Chapter 6.
SUMMARY

The present work has the advantage of employing a large number of different types treatments (n=5) and a consistently measured pyridine nucleotide data set from the same organism to test a proposed model for interaction between respiratory carbon flow and state transitions. Using treatments which rigorously tested the implications of this model, it appeared that increases in the NADPH/NADP ratio could not be correlated with increases in PQ. Thus, during class 1 treatments, the NADPH/NADP ratio was observed to increase (NH4+), oscillate (anaerobiosis) and decrease (CCCP). Similarly, during class 2 treatments, the NADPH/NADP ratio either decreased (NO3-) or increased (Pi). This led to the contention that NADPH was not responsible for PQ reduction and the occurrence of a state 1 to 2 transition. On the other hand, accurate measurement of both NADH and NAD allowed resolution of increases in the NADH/NAD ratio which could, for the most part, be correlated with PQ reduction. Class 1 treatments resulted in large increases in the NADH/NAD ratio of 4.7-fold (NH4+), 2.8-fold (anaerobiosis) and 3.8-fold (NH4+). In addition, Pi treatment (class 2) caused a rapid oscillation followed by almost no change in the NADH/NAD ratio after 2.5 minutes. Since class 1 treatments resulted in PQ reduction and a state 1 to 2 transition and class 2 treatments did not, these results were consistent with increases in NADH being responsible for PQ reduction and a state 1 to 2 transition. Intermediate increases in the NADH/NAD ratio during NO3 assimilation were somewhat inconsistent with this model and highlighted the complexity of the factors involved during NO3 assimilation. Several plausible explanations were proposed to explain these inconsistencies and these will be dealt with in a later chapter.

The observation that increases in NADH/NAD ratios were largely correlated with an increase in PQ reduction suggests that the physiological significance of the CRETC may be twofold. First, it would appear that the CRETC functions to recycle reductant for respiratory carbon flow in the chloroplast much as the METC in the mitochondria recycles reductant from TCA cycle activity. Second, the rate of e flow through the CRETC can affect the redox state of the PQ(cyt b6f) pool. This, in turn, appears to result in a state 1 to 2 transition and poises
the PETC for a decrease in the NADPH/ATP production ratio. It would appear, then, that the CRETC may also function to allow changes in respiratory carbon flow status to communicate with the PETC and down-regulate the NADPH/ATP production ratio.
CHAPTER 5: INTERACTION BETWEEN RESPIRATORY CARBON FLOW AND THE STATE TRANSITION AFTER UNCOUPLING WITH CCCP

INTRODUCTION

Chapters 2 through 4 involved developing and testing a general model which correlated an increase in the reduction state of the pyridine nucleotide pool with the occurrence of a state 1 to 2 transition. Treatments which increased the rate of respiratory carbon flow resulted in changes in fluorescence characteristics which were classified into two groups. Class 1 treatments (NH$_4^+$, anaerobiosis or CCCP) resulted in large changes in steady-state fluorescence and PS2/PS1 fluorescence ratios indicative of a state 1 to 2 transition and these changes were correlated with reduction of the PQ pool. Class 2 treatments (NO$_3^-$ or Pi) resulted in minimal changes in fluorescence indicating that a state 1 to 2 transition had not occurred. Changes in PQ reduction, resulting from Class 2 treatments, were much smaller than those observed after class 1 treatment. Increases in the NADPH/NADP ratio were not correlated with a reduction of the PQ(cyt b$_{6f}$) pool and a state 1 to 2 transition suggesting that increases in the NADH/NAD ratio might be the factor responsible for reduction of the PQ(cyt b$_{6f}$) pool. In support of this contention, class 1 treatments resulted in increases in the NADH/NAD ratio of 2.8-fold or greater while Pi treatment (class 2) resulted in only a minimal (10%) increase after 2 minutes of treatment. Some inconsistency was introduced by the observation that NO$_3^-$ assimilation resulted in an increase in the NADH/NAD ratio which was intermediate in magnitude between that of class 1 treatments and Pi treatment. Several plausible explanations were suggested for this inconsistency and these will be dealt with in more detail in the subsequent chapter.

In order to fully understand how interactions occur between respiratory carbon flow and the state transition, it is necessary to understand how respiration is activated. This understanding can provide an insight into the specific mechanism responsible for the increases in cellular NADH/NAD ratios observed during class 1 treatments and may help explain the smaller increases observed after class 2 treatments. In green algae it appears that much of glycolytic
carbon flow occurs in the chloroplast. Indeed, it has been proposed that starch is metabolized to the level of 3-PGA in the chloroplast before being exported to the cytosol. In support of this contention, the NAD- and NADP-utilizing forms of GAPDH were observed to be localized 86 and 100%, respectively, in isolated chloroplasts of *Chlamydomonas reinhardtii* (Klein, 1986). Although it is possible that other minor pathways may produce NADH in the chloroplast, I hypothesize that chloroplastic NAD-GAPDH is the major source of the NADH that results in PQ reduction.

The regulation of respiratory carbon flow in *S. minutum* has been extensively studied and detailed metabolite data exist for the kinetics of changes in respiratory metabolites and cofactors after treatment with NH$_4^+$ (Turpin et al., 1990; Vanlerberghe et al., 1992), anaerobiosis (Vanlerberghe et al., 1989), Pi (Gauthier and Turpin, 1993), and NO$_3^-$ (Feil and Turpin, unpublished; Vanlerberghe et al., 1992). However, changes in key respiratory metabolites and cofactors have not been resolved after treatment with CCCP in *S. minutum*. The purpose of the present chapter was to examine the metabolic sequence of events leading from activation of respiratory carbon flow to the reduction of PQ and initiation of a state 1 to 2 transition in the specific case of CCCP uncoupling. This information was then used to determine the enzymes potentially responsible for NADH production and PQ reduction in the chloroplast. In the subsequent chapter, this information will be compiled with available data from the other 4 treatments to develop a more complete model for the regulatory sequence responsible for activation of a state transition by respiratory carbon flow.

**MATERIALS AND METHODS**

**Experimental:**

Refer to Chapter 1. The algal suspension was aerobically dark adapted for 30 minutes before the initiation of sampling.
**Starch degradation:**

Aliquots for determination of starch degradation were taken over a 3 hour period as described in Vanlerberghe et al. (1990). Long sampling periods were required because the large initial pool size of starch in these cells made it impossible to accurately measure changes over short time periods. Cells were used directly from the chemostat (1.2 μg mg⁻¹ Chl). Samples (100 μL) were removed at 20 minute intervals, frozen in liquid N₂, and lyophilized. Free contaminating glucose was fully oxidized and all starch solubilized by resuspending the freeze dried sample in 400 μL of 0.02N NaOH, autoclaving for 100 minutes (121°C, 14 psi), vortexing, and autoclaving for an additional 100 minutes. Solubilized starch was then degraded to glucose by treating samples with α-amylase (20 units, Sigma A-1278) and amyloglucosidase (2 units, Sigma A-3042) for 15 hours at 55°C in 200 mM Na-acetate (pH 5.0). Amyloglucosidase and α-amylase were dialyzed in 200 mM Na-acetate (pH 5.0) for 24 hours prior to use. Glucose was determined using standard metabolite analysis (see below).

**Metabolites:**

Cells were harvested and concentrated to 40 μg Chl mL⁻¹. Samples (1 mL) were killed in 10% HClO₄ (final v/v) and rapidly frozen in liquid N₂. Samples were then slowly thawed on ice, neutralized with 5M KOH/1M triethanolamine, and centrifuged. The supernatant was brought up to 1.5 mL with dH₂O. All neutralized samples were stored in liquid N₂ until analysis. All metabolites were measured using standard, coupled enzymatic assays (Quick et al., 1989; Wirtz et al., 1980; see also Appendix 3) and a dual wavelength spectrophotometer (ZFP22, Sigma Instruments, FRG). All assay reagents were obtained from Boehringer Mannheim Co.

**Gas exchange:**

**CO₂ release:**

The rate of CO₂ release from cells (5 μg Chl mL⁻¹) was measured in an open gas exchange system with an infra red gas analyzer (IRGA, ADC 225 MK3, Analytical
Development Co. Ltd., Hoddesdon, England). CO₂-free air was bubbled at a constant rate of 200 mL·min⁻¹ through cells contained in a sealed cuvette. The air from the cuvette was returned to the IRGA where any CO₂ release from the cells was detected. The rate of CO₂ release was calculated from the chlorophyll content of the cuvette, the flow rate, and measured concentration of CO₂. Subsequently, 10 μM CCCP was injected via a serum-stoppered port and increases in the rate of CO₂ release were monitored. 90 ppm CO₂ in N₂ was used as a standard.

**O₂ consumption:**

Net oxygen exchange after uncoupling with CCCP was measured using a Clark type oxygen electrode (Hansatech Ltd., King's Lynn, England). Cells (1.2 μg Chl mL⁻¹) were dark-adapted 20 minutes prior to placing them in the darkened oxygen electrode chamber. Rates of control respiratory O₂ consumption were measured for 3 minutes prior to uncoupling with CCCP.

**Fluorescence:**

Fluorescence was measured as described previously in Chapters 1 and 2.

**RESULTS**

**The effect of CCCP on key respiratory metabolites:**

The data reported are the mean of 3 separate experiments. The variation about each data point was less than 10% and in most cases was due to slight concentration differences between experiments. In all cases, the trend observed in the mean data set was apparent in each replicate. Time points for fast sampling were 5, 20, 35, 50, 75, and 90 seconds.
Starch degradation: Dark aerobic starch breakdown occurred at a rate of 12.3 µmol glucose equivalents mg⁻¹ Chl h⁻¹. The rate of starch breakdown in dark CCCP treated cells was 25.0 µmol gluc mg⁻¹ Chl h⁻¹ (Figure 14).

Adenylates: Uncoupling of S. minutum with CCCP resulted in complementary changes in ATP, ADP and AMP (Figure 15A). Within 5 seconds, the concentration of ATP decreased 2-fold and reached a minimum within 15 seconds of uncoupling. ATP recovered slightly after 2 minutes but remained below control levels for the duration of the experiment. ADP and AMP increased 2- and 5-fold above the control level within 1 minute. ADP gradually returned to control levels while AMP recovered to a level approximately 2-fold greater than the control after 40 minutes. The adenylate energy charge dropped from 0.8 to 0.4 within 15 seconds and recovered only marginally for the duration of the experiment (Figure 15B).

PEP and Pyr: Within 5 seconds of CCCP treatment, pyruvate increased 100% and PEP decreased 40% which resulted in a 3.2-fold decrease in the PEP/PYR ratio (Figure 16A, B). PEP leveled off at a tenth of its original concentration within 5 minutes while Pyr dropped back to control levels and subsequently increased for the remainder of the experiment. The PEP/PYR ratio did not recover for the duration of the experiment (Figure 16B).

F6P, FBP, TP: The levels of F6P, FBP and TP were unaffected for the first 20 seconds after treatment with CCCP (Figure 17 A). After a lag of 20 seconds, F6P decreased slowly from 160 to 40 nmol mg⁻¹ Chl with an approximate half-time of 5 minutes. After a similar lag, FBP increased to a level 4-fold greater than the control with a half-time of 5 minutes. Triose phosphates (TP) increased 2- to 3-fold with the same kinetics as FBP. After 20 seconds, the ratio of FBP/F6P increased slowly over the course of the experiment from 2 to 30 (Figure 17B).
Figure 14: The effect of CCCP treatment on the long term rate of starch degradation in *S. minutum*. Cells were dark adapted for 20 minutes before beginning sampling. Arrow indicates treatment with CCCP. Data represent the mean of 3 experiments. Rate of starch degradation before and after treatment with CCCP was 12.3 and 25.0 µmol gluc equiv mg⁻¹ Chl h⁻¹, respectively.
Figure 15: The effect of CCCP treatment on the cellular concentration of adenylates in *S. minutum*. Cells were dark adapted for 20 minutes before beginning sampling. Arrow indicates treatment with CCCP. Data represent the mean of 3 experiments. A. ATP, open circles; ADP, closed circles; AMP, open squares. B. Adenylate energy charge, $\frac{[ATP]+0.5[ADP]}{[ATP]+[ADP]+[AMP]}$
Figure 16: The effect of CCCP treatment on the cellular concentration of pyruvate (Pyr) and phospho enol pyruvate (PEP) in *S. minutum*. Cells were dark adapted for 20 minutes before beginning sampling. Arrow indicates treatment with CCCP. Data represent the mean of 3 experiments. A. Pyr, open circles; PEP, closed circles. B. PEP/Pyr ratio.
Figure 17: The effect of CCCP treatment on cellular levels of fructose bisphosphate (FBP), fructose-6-phosphate (F6P) and triose phosphate (TP) in *S. minutum*. Cells were dark adapted for 20 minutes before beginning sampling. Arrow indicates treatment with CCCP. Data represent the mean of 3 experiments. A. FBP, closed circles; TP, open circles; F6P, open squares. B. FBP/F6P ratio.
**G6P, G1P:** G6P decreased 4-fold with the same lag and kinetics observed in F6P (Fig 18A). After addition of CCCP, G1P increased slightly but returned to near control levels within 5 minutes. The ratio of G1P/G6P tripled with a half-time of 5 minutes (Figure 18B).

**6PG:** Treatment with CCCP had no effect on 6PG levels (data not shown).

**PEP/TP, ATP/ADP, NADH/NAD, \( \Gamma = \frac{[\text{PEP}][\text{ATP}][\text{NADH}]}{[\text{TP}][\text{ADP}][\text{NAD}]} \):** Within 5 seconds of CCCP treatment, the PEP/TP ratio decreased 25% and decreased steadily to a level which was only 1% of the initial control value within 5 minutes (Table 5). Similarly, the ATP/ADP ratio decreased by 70% within 5 seconds of CCCP treatment and remained at or below this level for the first 20 minutes after treatment with CCCP (Table 5). In contrast, the NADH/NAD ratio increased 2.6-fold after 5 seconds of CCCP treatment and continued to increase for the first 2 minutes after treatment (Table 5, see also Figure 11E, Chapter 4). The overall effect of the individual ratio changes was to decrease the mass action ratio (\( \Gamma \)) by 43% within 5 seconds and by 94% within 2 minutes. The mass action ratio continued to decline for 20 minutes after treatment with CCCP (Table 5).

**Gas exchange:**

**CO₂ release:** The average (n=3) rate of dark CO₂ release measured was 137 \( \mu \text{mol CO₂ mg}^{-1} \text{ Chl h}^{-1} \). After CCCP treatment CCCP, this rate increased within 1 minute to 257 \( \mu \text{mol CO₂ mg}^{-1} \text{ Chl h}^{-1} \), an approximately 2-fold increase (Figure 19). After 15 minutes, the rate of CO₂ release slowly decreased and reached control levels within 45 minutes.

**O₂ exchange:** The dark rate of O₂ consumption was 151.2 \( \mu \text{mol O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1} \). After 2 minutes of CCCP treatment, the rate of O₂ exchange doubled to 343.4 \( \mu \text{mol O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1} \).
**Figure 18:** The effect of CCCP treatment on cellular levels of glucose-6-phosphate (G6P) and glucose-1-phosphate (G1P) in *S. minutum*. Cells were dark adapted for 20 minutes before beginning sampling. Arrow indicates treatment with CCCP. Data represent the mean of 3 experiments. A. G6P, open circles; G1P, closed circles. B. G1P/G6P ratio.
Table 5: The effect of CCCP on the PEP/TP, ATP/ADP, NADH/NAD and [PEP][NADH][ATP]/[TP][NAD][ADP] ratios compared to the dark aerobic control. Values were normalized to the dark aerobic control.

<table>
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<tr>
<th>Treatment</th>
<th>PEP/TP</th>
<th>ATP/ADP</th>
<th>NADH/NAD</th>
<th>[PEP][NADH][ATP]/[TP][NAD][ADP]†</th>
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<tr>
<td>+ CCCP</td>
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<td>0.01</td>
<td>0.33</td>
<td>2.00</td>
<td>0.005</td>
</tr>
</tbody>
</table>

† An approximation of the mass action ratio of the reaction sequence TP + NAD + ADP + Pi → (1,3 bisPGA, 3PGA, 2-PGA) → PEP + NADH + ATP + H⁺

* Actual values were 1.33, 3.75, 0.45 and 0.225 for PEP/TP, ATP/ADP, NADH/NAD and mass action ratio, respectively.
Figure 19: The effect of CCCP treatment on the rate of dark CO$_2$ efflux in *S. minutum* as measured by an open gas exchange IRGA system. Cells (3.5 μg Chl mL$^{-1}$, pH 6.0) were dark adapted for 45 minutes to allow equilibration before treatment with CCCP. Cells were treated at time 0 with 10 μM CCCP.
Within 7 minutes, the rate of $O_2$ consumption increased to a level greater than 4-fold the control rate (Figure 20).

**Fluorescence measurements:**

**Steady-state saturation pulse analysis:** The effect of CCCP treatment was to initially quench $F_0$, the level of fluorescence measured by the low intensity measuring beam (Figure 21A). After 2 minutes, $F_0$ began to increase and reached a level 1.5-fold greater than the dark control within 11 minutes. Similarly, $q_{NP}$ was initially decreased by 15% relative to the dark control (Figure 21B). After 2 minutes, $q_{NP}$ began increasing relative to the dark control and reached a maximum value of 0.23. Treatment with CCCP resulted in an initial increase in $q_p$ relative to the dark control. After 2 minutes of CCCP treatment $q_p$ decreased slowly by 40% (Figure 21B).

**77K fluorescence:** The initial effect of CCCP treatment was to slightly increase the $F_{686}/F_{717}$ ratio of fluorescence measured at 77K (Figure 21C). The $F_{686}/F_{717}$ ratio began to decrease only after 2 minutes of treatment with CCCP. Within 8 minutes, the $F_{686}/F_{717}$ ratio had decreased from 2.35 to 1.55.

**DISCUSSION**

**Metabolic changes observed after uncoupling with CCCP:**

After treatment with CCCP, the rate of starch degradation doubled (Fig 14). The kinetics of the increase in starch degradation could not be resolved by the technique used because the amount of degraded starch was small compared to the large starch reserve in nutrient-limited cells. The rate of $CO_2$ release, however, increased by approximately 2-fold within 1 minute (Figure 17), suggesting that the rate of respiratory carbon flow was enhanced
Figure 20: The effect of CCCP treatment on the rate of dark, O₂ consumption in S. minutum measured using a Hansatech oxygen electrode. Cells (3 μg Chl mL⁻¹) were dark adapted for 20 minutes to allow equilibration before treatment with CCCP.
Figure 21: The effect of CCCP treatment on A. room temperature saturation pulse analysis (see also Figure 5C, Chapter 2), B. Photochemical ($q_p$) and non-photochemical ($q_{NP}$) quenching coefficients calculated from A, and C. 77K fluorescence $F_{686}/F_{717}$ ratios.
within 1 minute. The following discussion will outline a model to explain a) how respiratory
carbon flow is activated by treatment with CCCP and b) specifically how this increase in
respiratory carbon might affect the chloroplastic NADH/NAD ratio, increase the redox state of
the PQ(cyt b6f) pool, and result in a state 1 to 2 transition. A detailed model for this interaction
is presented in Figure 22.

**Activation of respiratory carbon flow:**

Based on what is known about the use of intermediates in the study of metabolic control
(Rolleston, 1972) and specific studies on the activation of the key respiratory enzymes PK and
PFK in *S. minutum* (Turpin et al., 1990; Vanlerberghe et al., 1989; Botha and Turpin, 1990a;
Lin et al., 1989), the following mechanism was hypothesized for the activation of respiratory
carbon flow after treatment with CCCP.

**Activation of "lower glycolysis" and respiratory control:** In isolated mitochondria and *in vivo*, it has been observed that respiratory O2 consumption by the METC can be controlled by
the availability of ADP (Lambers, 1985; 1990). Adenylate control, however, may function at
one of two levels. It appears that both mechanisms may operate *in vivo* in a manner which
seems to be tissue-specific and varies with developmental stage and substrate availability
(Lambers, 1990). In the first case, the rate of e- flow through the METC is directly limited by
ADP because ADP-limitation of the ATP synthase increases the proton motive force and
restricts e- transport to O2 (Day and Lambers, 1983). The second possibility is that the rate of
respiratory carbon supply is ADP-limited and this in turn limits reductant supply to the METC
(Azcon-Bieto et al., 1983). Pyruvate kinase (PK), in particular, has been observed to function
far from equilibrium; this fact has been deduced from estimates of substrates and products *in vivo* (Rolleston, 1972; Turner and Turner, 1980; Douce, 1985). In addition, experimental
evidence suggests that PK is ADP-limited *in vivo* (Turpin et al., 1990; Vanlerberghe et al.,
Figure 22: The proposed mechanism for interaction between respiratory carbon flow and poising of the PETC via the state 1 to 2 transition after uncoupling with CCCP. See text for details and list of abbreviations for abbreviations.
The initial effect of CCCP treatment was to dissipate the mitochondrial proton gradient and prevent chemiosmotic production of ATP (Figure 22, 1). This, in turn, resulted in a 50% decrease in ATP within 5 seconds with concurrent 2- and 3-fold increases in ADP and AMP (Figure 15A). Changes in adenylate pools served to decrease the adenylate energy charge from 0.8 to 0.6 within 5 seconds (Figure 15B). These changes in adenylates appeared to release respiration from adenylate control because the rates of respiratory O₂ consumption and CO₂ efflux rapidly increased after CCCP treatment (Figures 19 and 20). If ADP were directly limiting the rate of e⁻ flow through the METC, this should have resulted in a rapid decrease in the NADH/NAD ratio followed by a decrease in pyruvate as carbon flow was activated to respond to "draw-down" from the TCA cycle. However, the rapid increase in ADP was accompanied by a 3-fold increase in the NADH/NAD ratio (See Figure 11C, Chapter 3), implying that direct ADP-limitation of the METC was not occurring. On the other hand, a 2-fold increase in pyruvate, a 40% decrease in PEP and a 3.2-fold decrease in the PEP/PYR ratio were observed within 5 seconds of CCCP treatment (Figure 16A and B). The decrease in substrate for PK (PEP) and increase in product (Pyr) which occurred immediately after treatment with CCCP may constitute a "cross-over" point (Chance and Williams, 1965; Williamson, 1966) and implies that respiration is activated by a release of ADP-limitation on pyruvate kinase activity (Figure 22, 2). The observation of increased carbon flow through PK (measured as an enhancement of CO₂ efflux and the NADH/NAD ratio), despite the fact that the increased ratio of products/substrates would make the reaction less thermodynamically feasible, is a characteristic of rate-limiting or regulatory enzymes (Rolleston, 1972). The estimated concentration of ADP in vivo (33 μM, Turpin et al., 1990) is below the k_M for both the plastidic (k_M =200 μM) and cytosolic (k_M= 50 μM) isozymes of PK (Lin et al., 1989) and is consistent with ADP-limitation of PK in vivo in S. minutum. On the basis of metabolite changes after uncoupling with CCCP, it appears that adenylate control functions at the level of ADP-limitation of PK in N-limited S. minutum which confirms studies done with this alga during anaerobiosis and NH₄⁺ assimilation (Turpin et al., 1990; Vanlerberghe et al., 1989).
GAPDH: The enzymatic reactions governing the conversion of TP to PGA and PEP (NAD-GAPDH, PGA kinase, phosphoglyceromutase and enolase) are thought to be close to equilibrium under normal conditions (Turner and Turner, 1980; Douce, 1985; Stryer, 1988). The rate of flow through these enzymes, then, is thought to be governed largely by the concentrations of cofactors, substrates and products (Rolleston, 1972; Stryer, 1988). Thermodynamically, the rate of flow through equilibrium enzymes is related to the ratio of products/substrates, or the mass action ratio. In general, the smaller the mass action ratio for an equilibrium enzyme, the greater the flow through the enzyme (for thermodynamic rationale, see the next chapter). The mass action ratio for NAD-GAPDH is difficult to determine because levels of 1,3 bisPGA are extremely low \textit{in vivo} and, consequently, GAPDH and PGA kinase are generally considered as a combined reaction (Rolleston, 1972; Takahama et al., 1981). The mass action ratio for the combined reaction is \[ G = \frac{[PGA][ATP][NADH][H^+]}{[TP][ADP][NAD][Pi]} \]. However, it was not possible to measure PGA after treatment with CCCP and PEP was used as an approximation of the PGA component of the mass action ratio. Thus, the mass action ratio reflected the combined reactions from TP to PEP. This interpretation was justified by the observation that, of other cases in which respiratory carbon flow changed, the magnitude and kinetics of decreases in PEP were mirrored by changes in PGA consistent with these reactions being close to equilibrium (Turner and Turner, 1980; Turpin et al., 1990; Vanlerberghe et al., 1990). The mass action ratio was further approximated by removing the $[H^+]$ component from the equation.

Within 5 seconds of CCCP treatment, the PEP/TP ratio decreased by 25% and the ATP/ADP ratio decreased by 70% (Table 5). The rapid decrease in PEP observed after treatment with CCCP (Figure 16A) initially decreased the PEP/TP ratio. Rapid decreases in ATP and increases in ADP after treatment with CCCP (Figure 15A) would both have contributed to the reduction of the ATP/ADP ratio within 5 seconds (Table 5). Thus, despite
the fact that a 2.6-fold increase in the NADH/NAD ratio occurred within 5 seconds, the mass action ratio for TP conversion to PEP (Γ) had almost halved (Table 5). The PEP/TP ratio continued to decline until it reached a value which was only 1% of the dark control within 5 minutes of CCCP treatment (Table 5). The continued decline of this ratio was subsequently maintained by increases in TP that resulted from PFK activation after 30 seconds (see discussion below). The continued decline of the PEP/TP ratio had the effect of decreasing Γ by 79% after 30 seconds and by 95% after 2 minutes, despite the fact that the NADH/NAD ratio continued to increase as a result of CCCP treatment (Table 5). The 100-fold decrease in the mass action ratio for carbon flow from TP to PEP after 5 minutes of treatment with CCCP was, therefore, driven decreases in the ATP/ADP and PEP/TP ratios. In turn, decreases in the PEP/TP ratio were driven first by activation of PK (within 5 seconds) and subsequently by activation of PFK (within 30 seconds, see below).

The flow through equilibrium enzymes is thermodynamically related to the mass action ratio such that decreases in the mass action ratio increase the rate of flow (See next chapter; see also Rolleston, 1972; Stryer, 1988). An increase in carbon flow from TP to PEP would be thermodynamically favoured by decreased levels of PEP and ATP and increased levels of TP and ADP. In turn, increased carbon flow via NAD-GAPDH should result in increased NADH production and an increased NADH/NAD ratio. NAD-GAPDH was shown to be 86% localized in the chloroplast in *Chlamydomonas reinhardtii* (Klein, 1986). Although little is known about the localization of NAD-GAPDH in *S. minutum*, similarities between FBPase isozymes and PFK between *S. minutum* and *Chlamydomonas* suggest that glycolytic compartmentation is similar between these two algae (Botha and Turpin, 1990a, b). Assuming that the same enzyme localization occurs in *S. minutum*, the increase in carbon flow implied by the increased mass action ratio should result in an increase in the chloroplastic NADH/NAD ratio (Figure 22, 4). Thus, it is proposed that carbon flow via NAD-GAPDH would increase within 5 to 15 seconds of uncoupling because of a decrease in the product of this reaction (PGA) resulting from observed decreases in PEP (Figure 16A). Since NAD-GAPDH produces NADH, this would
also contribute to the increase in the cellular NADH/NAD ratio observed (Figure 11C, Chapter 3).

**Upper glycolysis (Phosphofructokinase):** Within the first 30 seconds after CCCP treatment, very little change occurred in either F6P, FBP, or TP levels. After 30 seconds there was a 75% decrease in the level of F6P and a 2- and 4-fold increase in FBP and TP, respectively (Figure 17). These changes were similar to the "cross-over" effect observed for PK and are consistent with the contention that PFK is an important regulatory enzyme in glycolysis (Turner and Turner, 1980; Turpin et al., 1990; Botha et al., 1988). In vivo levels of PEP have been shown to strongly inhibit PFK in *S. minutum* (Botha and Turpin, 1990; Turpin et al., 1990) and other organisms (Botha et al., 1988; Knowles et al., 1990; Garland and Dennis, 1980; Kelly and Latzko, 1977). The 40% decrease in PEP levels observed within 5 seconds could, therefore, be important in the activation of PFK and the top half of glycolysis. Increases in the FBP/F6P ratio occurred after the drop in PEP (compare Figures 16 and 17) and suggested that activation of PK was responsible for the activation of PFK (Figure 22, 3). Pi has also been shown to be a potent activator of *S. minutum* PFK (Turpin et al., 1990; Botha and Turpin, 1990a). It is possible that PFK was further activated by the uncoupler-mediated decline in ATP and consequent increase in Pi. One consequence of an activation of PFK activity is an increase in TP levels. An increase in TP would further activate NAD-GAPDH by augmenting increases in the TP/PGA ratio brought about by activation of PK (see previous discussion; see also Figure 22, 4).

The lack of change in 6PG after treatment with CCCP could imply that the majority of carbon flow from starch occurs via glycolysis and not through the oxidative pentose phosphate pathway.

**Phosphoglucomutase:** Phosphoglucomutase catalyses the conversion of G1P, a direct product of starch degradation, to G6P and appears to be an equilibrium enzyme (Douce, 1985).
G6P, G1P, and the G1P/G6P ratio remained relatively unchanged for the first 30 seconds after treatment with CCCP whereupon G6P decreased approximately 4-fold, serving to triple the G1P/G6P ratio within 10 minutes (Figure 18B). The 30 second lag in activation of phosphoglucomutase suggests that an increase in the rate of starch degradation occurred only after PK and PFK were activated.

**The kinetics of PQ reduction and the state 1 to 2 transition:**

In the chloroplast, the first effect of uncoupling with CCCP was an immediate decrease in F (the level of fluorescence induced by the measuring beam after treatment) and an increase in $F'_M$ (Figure 21A). This resulted in a decrease in $q_{NP}$ from dark control levels and a slight increase in $qp$ (Figure 21B). The speed with which $q_{NP}$ relaxed suggests that the quenching was caused mostly by $q_E$ which is related to the thylakoid $\Delta pH$ (Horton and Hague, 1988). On the basis of thermoluminescence (Bennoun, 1982) and room temperature fluorescence measurements (Ting and Owens, 1993), it has been suggested that chloroplastic respiratory electron transport from NAD(P)H to O$_2$ may result in proton translocation across the thylakoid membrane and a trans-thylakoid $\Delta pH$. The rapid release of $q_{NP}$ observed after uncoupling with CCCP (Figure 21B) is consistent with the occurrence of a proton gradient in the dark due to CRETC activity in aerobically adapted control cells. The slight increase in $qp$ observed indicated that $QA^-$ became more oxidized immediately after treatment and suggests that thylakoid electron transport may have been limited by the trans-thylakoid $\Delta pH$ in the dark (Figure 21B).

Two minutes after treatment with CCCP, $F'_M$ began to decrease while F began to increase. F increased 2-fold which resulted in a concurrent decrease in $qp$ with a half-time of approximately 4 minutes (Figure 21A, B). Since CCCP entered the cell and uncoupled proton gradients within 5 seconds, it is unlikely that the changes in F were related solely to the photochemical effects of CCCP on PS2 (ADRY effects, Renger, 1972). Instead, it is more
likely that changes were correlated with an increase in PQ reduction due to an increase in the chloroplastic NADH/NAD ratio. Thus, changes in PQ reduction occurred with a half-time of approximately 4 minutes. $F'_M$ decreased by 20% within 20 minutes ($t_{1/2} = 7$ minutes) which resulted in an increase in $q_{NP}$ (Figure 21B). It is likely that the majority of this increase in $q_{NP}$ resulted from an increase in $q_T$ (a state 1 to state 2 transition) since uncoupling would have released $q_E$ associated with the thylakoid $\Delta pH$.

A determination of the kinetics of metabolic change makes it possible to more accurately resolve the kinetics of a state transition. It was observed that changes in the F686/F717 ratio (figure 21C) were offset slightly (1 to 2 minutes) from changes in PQ redox indicated by increases in $F$ and decreases in $q_p$ (Figure 21A B) but occurred with similar kinetics once initiated. This implied that once PQ was reduced, activation of the LHC2 kinase and reallocation of LHC2 occurred within 1 to 2 minutes. These state transitions kinetics are considerably faster than those reported for green algae and higher plants ($t_{1/2} = 5 - 7$ minutes, Williams and Allen, 1987). Although some of this change may occur because thylakoids are less stacked in N-limited cells (Plumley and Schmidt, 1991), it is equally possible that the kinetics of PQ reduction determine the kinetics of a state transition. Unless PQ redox changes occur immediately, calculation of $t_{1/2}$ using initiation of treatment as a starting point will be an overestimate of the actual kinetics of the state transition.

The relationship between the NADH/NAD ratio and PQ redox changes after treatment with CCCP:

In chapter 4 it was proposed that increases in the NADH/NAD ratio would result in reduction of PQ. A 3-fold increase in the NADH/NAD ratio occurred within 5 seconds of treatment with CCCP (Figure 11C, Chapter 4). Since the kinetics of PQ reduction showed a 2 minute lag after changes in the NADH/NAD ratio, it is unlikely that the cellular ratio is responsible for increasing the reduction of PQ. Changes in the rate of carbon flow through NAD-GAPDH were approximated by the kinetics of changes in the mass action ratio for the
conversion of TP to PEP (Table 5, see discussion above). The full activation of NAD-GAPDH appeared to occur as a result of a combination of decreases in the ATP/ADP and PEP/TP ratios which were initiated first by PK activation and subsequently by PFK activation. On the basis of changes in the mass action ratio, the half-time for increases in carbon flow through NAD-GAPDH (and increased chloroplastic NADH production) was between 5 and 30 seconds (Table 5). Changes in F and qp (indicating PQ reduction) did not begin to occur until approximately 2 minutes after treatment with CCCP (Figure 21A, B). This suggests that increased flow through NAD-GAPDH and increased chloroplastic NADH/NAD ratios might be responsible for reduction of the PQ(cyt b6f) pool and the transition from state 1 to state 2.

SUMMARY

Metabolite changes accompanying uncoupling with CCCP treatment were examined to determine the mechanism responsible for the activation of respiratory carbon flow and the metabolic sequence of events leading to reduction of PQ and the initiation of the state 1 to 2 transition. The immediate effect of uncoupling was to decrease ATP and increase ADP and AMP levels. This was accompanied by rapid increases in the rate of CO2 efflux and O2 consumption, indicating that an increase in respiratory carbon flow had occurred. Long-term rates of starch degradation doubled and, combined with gas exchange data, suggested that an increase in respiratory carbon flow occurred as a result of CCCP treatment. Increases in ADP were correlated with a rapid increase in Pyr and a decrease in PEP consistent with the activation of PK by increasing ADP availability. Decreases in F6P and increases in FBP and TP were observed to occur within 30 seconds of CCCP treatment and were consistent with the activation of PFK. The lag in changes in the FBP/F6P ratio after the activation of PK was consistent with the activation of PFK activity by decreases in PEP (brought about by PK activation). Activation of PK and PFK resulted in a subsequent decrease in G6P and an increase in the G1P/G6P ratio,
implying that activation of starch degradation occurred as a result of "draw-down" of carbon via glycolysis.

In addition, uncoupling with CCCP was observed to greatly decrease the combined mass action ratio for the reactions resulting in conversion of TP to PEP (all equilibrium reactions). This decrease in the mass action ratio occurred initially as a result of decreases in the ATP/ADP ratio and decreases in PEP resulting from activation of PK. Subsequently, the mass action ratio was further decreased by increases in TP resulting from activation of PFK. Decreases in the mass action ratio can be related to increases in the flow through equilibrium enzymes. It was proposed that increased flow through NAD-GAPDH (one of the enzyme reactions included in the combined mass action ratio) could result in increased NADH production. On the basis of enzyme localization studies in *Chlamydomonas*, it was proposed that NAD-GAPDH activity was localized predominantly in the chloroplast (Klein, 1986). Assuming that a similar enzyme localization occurred in *S. minutum*, it was proposed that increases in NAD-GAPDH activity would lead to an increase in the chloroplastic NADH/NAD ratio. Increases in carbon flow via NAD-GAPDH, indicated by decreases in the mass action ratio, were followed by increases in PQ reduction and resulted in a state 1 to state 2 transition. The slight lag between increases in carbon flow via NAD-GAPDH and PQ reduction implied that increases in the chloroplastic NADH/NAD ratio via NAD-GAPDH activity were responsible for PQ reduction and a state 1 to state 2 transition.
CHAPTER 6: DEVELOPMENT OF A COMPREHENSIVE MODEL FOR THE INTERACTION BETWEEN RESPIRATORY CARBON FLOW AND POISING OF THE STATE TRANSITION

INTRODUCTION

The primary hypothesis of the present work is that increases in the NADH/NAD ratio are responsible for increased PQ reduction and the occurrence of a state 1 to 2 transition. In support of this contention, class 1 treatments resulted in increases in the NADH/NAD ratio of 2.8-fold or greater while Pi treatment (class 2) resulted in only a minimal (10%) increase after 2 minutes of treatment. However, the observation that NO₃⁻ treatment resulted in an increase in the NADH/NAD ratio which was intermediate in magnitude between that of class 1 treatments and Pi treatment was inconsistent with the contention that increases in the NADH/NAD ratio resulted in PQ reduction and a state 1 to 2 transition. Several plausible explanations were suggested for this inconsistency including a) the fact that a threshold increase in the NADH/NAD ratio might be required to result in increased PQ reduction and b) the increases in the cellular NADH/NAD ratio observed did not reflect changes in the chloroplastic NADH/NAD ratio and, therefore, did not affect PQ reduction. This chapter will focus on the second possibility. The rationale for this approach will be developed below.

Reduction of PQ in the thylakoid membrane requires that the chloroplastic NADH/NAD ratio increases as a result of respiratory carbon flow. It is quite possible that increases in the mitochondrial NADH/NAD ratio would not directly affect chloroplastic NADH/NAD ratios or affect the redox state of PQ. This is because it is unlikely that reductant would be shuttled from the mitochondria (where NADH can be efficiently oxidized either by the cytochrome or alternative pathway of the METC) to the chloroplast where chlororespiratory flow has been estimated to comprise only 10 to 20% of the full respiratory electron transport capacity of a cell (Bennoun, 1982; Peltier et al., 1987). This suggests that chloroplastic respiratory carbon flow must be responsible for the increase in PQ reduction observed after treatment with class 1 effectors.
The interpretation of changes in cellular pyridine nucleotide ratios is limited by the fact that changes in compartmental ratios may not be reflected by changes in cellular levels. The outer membranes of the chloroplast and mitochondrion are impermeable to pyridine nucleotides. Movement of redox potential between compartments appears to be limited to indirect pyridine nucleotide shuttles such as the proposed malate/oxaloacetate shuttle or triose phosphate/phosphoglycerate shuttle (Heber, 1974; Ebbighausen et al., 1985; 1987). Preliminary estimates of the NADH/NAD ratio in the cytosol and mitochondria suggest that the ratio is much higher in the mitochondria than in the cytosol (Kromer and Heldt, 1991) and implies that gradients in reduced pools of pyridine nucleotides may be maintained between cellular compartments.

To date, no method exists to rapidly fractionate green algal cells making it impossible to directly measure compartmental NADH/NAD ratios. However, on the basis of enzyme localization work in the chloroplasts of the green alga Chlamydomonas reinhardtii (Klein, 1986), some information is known about the compartmentation of NADH and NADPH producing and consuming enzymes. In turn, information about the activity of these enzymes can be determined by examining changes in the pools of substrates and products of each enzyme (Turpin et al., 1990; Rolleston, 1972).

One major source of NADH in the chloroplast is from the activity of NAD-GAPDH which catalyzes the conversion of TP to PGA. 86% of NAD-GAPDH was localized in the chloroplast in the green alga Chlamydomonas reinhardtii (Klein, 1986). In the previous chapter, it was proposed that increases in NAD-GAPDH activity could lead to an increase in chloroplastic NADH/NAD ratios and result in PQ reduction. The purpose of the present chapter is to examine this hypothesis in more detail in order to develop a consistent and detailed model for the interaction between respiratory carbon flow and the PETC in vivo. This involved testing the hypothesis that class 1 treatments should result in a large increase in respiratory carbon flow via chloroplastic NAD-GAPDH whereas class 2 treatments should not. To do this, a large metabolite data set was assembled from previously published work from this lab and
analyzed to compare general trends in metabolite and cofactor ratios between class 1 and class 2 treatments. Thermodynamic considerations of key respiratory enzymes revealed some large differences between class 1 and class 2 treatments.

**RESULTS**

In the interests of clarity, previously published data will be summarized herein. Table footnotes indicate the source of data.

**Kinetics of changes in ADP, Pyr/PEP and FBP/F6P:**

Table 6 is a summary of the effects class 1 and class 2 treatments on ADP, Pyr/PEP and FBP/F6P ratios over 20 minute period.

**Class 1 treatments:**

**NH$_4^+$:** Treatment of N-limited cells with NH$_4^+$ resulted in a 1.6-fold increase in ADP and a 6.6-fold increase in the Pyr/PEP ratio within 5 seconds. The increase in ADP was maintained for 30 seconds but decreased to control levels within 2 minutes. The Pyr/PEP ratio remained 7-fold higher than the dark control for at least 20 minutes after treatment. The FBP/F6P ratio increased by 66% within 5 seconds and achieved an 8.5-fold increase within 5 minutes (Table 6). The TP/PGA ratio doubled within 5 seconds and increased to 12.4-fold the dark control within 5 minutes after treatment with CCCP (Table 6).

**Anaerobiosis:** Anaerobiosis resulted in a 1.8-fold increase in ADP and 2-fold increase in the Pyr/PEP ratio within 30 seconds. ADP remained at this level while the Pyr/PEP ratio increased to a level 4-fold greater than the control within 5 minutes. The FBP/F6P ratios was unchanged for the first 2 minutes after which the ratio increased and reached 3-fold the dark control ratio.
Table 6: A comparison of the effects of class 1 and class 2 treatments on changes in key respiratory intermediates and their ratios. Pi treatment was made to Pi-limited cells, all other treatments were made to N-limited cells. All values are normalized to the respective dark, aerobic control value.

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<th>ADP</th>
<th>Pyr/PEP</th>
<th>FBP/F6P</th>
<th>PGA/TP</th>
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* The actual values in N-limited cells were 108.3 (± 10.4) nmol mg⁻¹ Chl, 0.53 (± 0.26), 0.86 (± 0.65) and 18.0 (± 10.7) for ADP, Pyr/PEP, FBP/F6P and PGA/TP, respectively.

** These values are approximated from the PEP/TP ratio because PEP and PGA remain in equilibrium.

*** The actual values in Pi-limited cells were 57 nmol mg⁻¹ Chl, 2.5, 2.0, 1.25 for ADP, Pyr/PEP, FBP/F6P and PGA/TP, respectively.
within 5 minutes. (Table 6). The TP/PGA ratio doubled within 30 seconds and increased 9-fold within 5 minutes of anaerobic treatment (Table 6).

**CCCP:** The kinetics of changes in ADP, and the Pyr/PEP and FBP/F6P ratios are summarized in Table 6 and have already been described (see results, Chapter 5). The TP/PGA (=TP/PEP) ratio tripled within 30 seconds and increased 87-fold within 5 minutes of treatment with CCCP (Table 6)

**Class 2 treatments:**

**NO₃⁻:** ADP increased by 15% within 5 seconds of treatment with NO₃⁻ and then recovered to a level which was 15% less than the dark control for the remainder of treatment. The Pyr/PEP ratio was relatively unaffected until 2 minutes where upon it increased by 50% and reached a maximum of 2.3-fold the dark control level within 20 minutes. The FBP/F6P ratio increased by 60% within 5 seconds and tripled within 2 minutes (Table 6). The TP/PGA ratio doubled within 5 seconds and increased 3.5-fold within 5 minutes of treatment with NO₃⁻ (Table 6).

**Pi:** Treatment with Pi resulted in a 30% increase in ADP within 30 seconds whereupon ADP decreased but remained at a level 10% greater than the dark control for at least 20 minutes. Within 5 seconds of treatment, Pyr/PEP ratios increased by 50%. However, within 2 minutes, the Pyr/PEP ratio declined to half the dark control level and remained lower than the control for the next 20 minutes. The FBP/F6P ratio decreased to half the control level within 5 seconds and remained lower than the control for 5 minutes. After 20 minutes, the FBP/F6P ratio recovered and reached a level 250% greater than the dark control (Table 6). The TP/PGA ratio increased 50% within 5 seconds of treatment with Pi and then declined to dark control levels within 30 seconds after treatment with Pi (Table 6).
PGA/TP, NADH/NAD, ATP/ADP and \( \Gamma \) ([PGA][NADH][ATP] / [TP][NAD][ADP]):

Table 7 presents a summary of the effects of class 1 and class 2 treatment on the ratios of PGA/TP, NADH/NAD and ATP/ADP after 5 minutes of treatment. These ratios were then used to make an approximation of \( \Gamma \), the mass action ratio for the conversion of TP to PGA by NAD-GAPDH and PGA kinase.

**Class 1 treatments (NH\(_4^+\), anaerobiosis and CCCP):**
The PGA/TP ratio decreased by 92, 89, and 97\% within 5 minutes of treatment with NH\(_4^+\), anaerobiosis and CCCP, respectively. Similarly, the ATP/ADP ratio decreased by 20, 64, and 75\% after treatments with NH\(_4^+\), anaerobiosis and CCCP. On the other hand, the NADH/NAD ratio increased by 4-, 2.8-, and 3.6-fold after 5 minutes of treatment with NH\(_4^+\), anaerobiosis and CCCP, respectively. The mass action ratio for the conversion of TP to PGA decreased by 71, 90, and 99\% after 5 minutes of treatment with NH\(_4^+\), anaerobiosis and CCCP, respectively (Table 7).

**Class 2 treatments (NO\(_3^-\) and Pi):**
After 5 minutes of treatment with NO\(_3^-\), the PGA/TP ratio decreased by 71\%, the NADH/NAD ratio increased by 70\% and the ADP/ATP ratio increased by 23\%. This decreased the combined mass action ratio (for conversion of TP to PGA) by 38\% within 5 minutes (Table 7). All 4 ratios remained relatively unchanged after 5 minutes of treatment with Pi (Table 7).

**Effects of illumination:**
Illumination resulted in a 70\% increase in the PGA/TP ratio, a 30\% increase in the ATP/ADP ratio and a 24\% increase in the NADH/NAD ratio (Table 7). In turn, this resulted in a 2.8-fold increase in the combined mass action ratio.
Table 7: The effect of illumination and treatments which increase the rate of respiratory carbon flow on the PGA/TP, ATP/ADP, NADH/NAD and [PGA][NADH]/[ATP]/[TP][NAD][ADP] ratios compared to the dark aerobic control. Values were taken 5 minutes after treatments and were normalized to the dark aerobic control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGA/TP</th>
<th>ATP/ADP</th>
<th>NADH/NAD</th>
<th>[PGA][NADH]/[ATP]/[TP][NAD][ADP]</th>
</tr>
</thead>
<tbody>
<tr>
<td>illuminated, aerobic **</td>
<td>1.72</td>
<td>1.28</td>
<td>1.24</td>
<td>2.78</td>
</tr>
<tr>
<td>dark, aerobic control (N or Pi-limited)</td>
<td>1.00***</td>
<td>1.00***</td>
<td>1.00***</td>
<td>1.00 †</td>
</tr>
</tbody>
</table>

**Class 1 treatments:**

+ NH$_4^+$
+ anaerobiosis
+ CCCP

**Class 2 treatments:**

+ NO$_3^-$
+ Pi

* An approximation of the mass action ratio of the reaction sequence TP + NAD + ADP + Pi → (1,3 bisPGA) → 3PGA + NADH + ATP

** Values from N-limited cells, data for Pi-limited cells not available.

*** Actual values for N-limited cells were 18.0 (± 10.7), 4.16 (± 0.8) and 0.059 (± 0.024) for the PGA/TP, ATP/ADP and NADH/NAD ratios, respectively. Actual values were 1.25, 0.96, 0.125 and 0.125 for PGA/TP, ATP/ADP, NADH/NAD and the mass action ratio in Pi-limited cells.

† Actual value for $\Gamma$ in the dark N-limited control was 4.37 (± 1.53). This corresponds to a $\Delta G'$ value of -2.13 kcal/mol calculated from $\Delta G'$ = RT ln($\Gamma/K_{eq}$). $\Delta G''$ for this reaction is -3.0 kcal mol$^{-1}$ (Stryer, 1988). Values for $K_{eq}$ calculated from $K_{eq} = 10^{-\Delta G''/2.36}$ (Stryer, 1988).

†† This is an approximation and represents the PEP/TP ratio (see Chapter 5).

††† These values were not significantly different from the dark aerobic control values.
DISCUSSION

Activation of respiratory carbon flow: the effect of class 1 and 2 treatments on ADP, and the Pyr/PEP, FBP/F6P and PGA/TP ratios:

Class 1 treatments (NH$_4^+$, anaerobiosis or CCCP):

The changes in key respiratory metabolites previously observed with CCCP treatment were remarkably similar to the changes in metabolites observed after treatment with NH$_4^+$ and anaerobiosis. After treatment with NH$_4^+$ or anaerobiosis, an increase in ADP was closely correlated to an increase in the Pyr/PEP ratio (Table 6) indicating that respiratory flow was increased by activation of PK due to relief of ADP-limitation of PK (Turpin et al., 1990; Vanlerberghe et al., 1989). In turn, the FBP/F6P ratio increased after to increases in the Pyr/PEP ratio suggesting that the decrease in PEP resulting from PK activation served to activate PFK in all class 1 treatments (Turpin et al., 1990; Vanlerberghe et al., 1989). In all cases, the PGA/TP ratio appeared to decrease initially as a result of PK activation (which decreased PGA) and this decrease was further augmented by PFK activation (which increased TP) indicated by increases in the FBP/F6P ratio (Table 6).

Class 2 treatments (NO$_3^-$ or Pi):

Increases in ADP were not correlated with an increase in the Pyr/PEP ratio or PK activation after treatment with NO$_3^-$ or Pi. ADP increased slightly and then decreased by 15% within 30 seconds of treatment with NO$_3^-$ while the Pyr/PEP ratio was unchanged until 2 minutes after treatment with NO$_3^-$ after which the ratio doubled within 5 minutes (Table 6). These observations were inconsistent with an activation of PK by relief of ADP-limitation. On the other hand, the FBP/F6P ratio increased by 60% within 5 seconds of NO$_3^-$ treatment and corresponded to a 2-fold decrease in the PGA/TP ratio (Table 6). It has been suggested that respiratory carbon flow through the oxidative pentose phosphate pathway is activated by a decrease in the cellular NADPH/NADP ratio after treatment with NO$_3^-$ (Vanlerberghe et al., 1992; Figure 8A). Thus, it is thought that respiratory carbon flow is activated in response to the
reductant demands for NO₃⁻ reduction rather than a release of ADP-limitation of PK (Vanlerberghe et al., 1992). PK activation occurred after PFK activation and it is thought that PK may be activated, in this case, by a decline in glutamate, which is an inhibitor of PK in vivo (Turpin et al., 1990).

After treatment with Pi, there was an 18% increase in ADP and a 50% increase in the Pyr/PEP ratio within 5 seconds. However, the Pyr/PEP ratio subsequently dropped rapidly and reached a level which was only 30% of the dark control level within 2 minutes of treatment with Pi (Table 6). The FBP/F6P ratio dropped relative to the dark control for the first 5 minutes of treatment with Pi and only increased by 2.5-fold within 20 minutes (Table 6). The fact that neither PK or PFK appeared to be activated is consistent with the observation that the PGA/TP ratio remained relatively unchanged by Pi treatment for the first 20 minutes (Table 6). Although the activation of respiration by Pi treatment is not well understood, it is thought that respiratory carbon flow may be controlled by the ATP requirements of a plasmalemma H⁺-ATPase which is activated to maintain intracellular pH and provide the proton motive force to power Pi uptake (Gauthier and Turpin, 1993). It has also been proposed that cells respond to Pi-limitation by developing enzymatic pathways to bypass glycolytic enzymes which require Pi (Theodorou et al., 1990). If these enzyme bypasses were functioning in cells re-supplied with Pi, the rate of carbon flow would increase but flow through NAD-GAPDH (which requires Pi) might remain minimal. Thus, the minimal change in the PGA/TP ratio observed after Pi treatment might reflect the operation of Pi conserving carbon shunts during the initial period of Pi assimilation.

**The effect of class 1 and 2 treatments on the combined mass action ratio for NAD-GAPDH and PGA kinase:**

The enzymatic reaction governing the conversion of TP to PGA are described by the following equations:
The enzymes responsible for the conversion of TP to 3-PGA (NAD-GAPDH and PGA kinase) are thought to be close to equilibrium and, hence, are freely reversible (Turner and Turner, 1980; Douce, 1985; Stryer, 1988). The rate of flow through "equilibrium" reactions is dependent to a large extent on the extent to which they are displaced from equilibrium and this is described by the free energy of the reaction (ΔG'). ΔG' is related to the ratio of the equilibrium constant for a reaction $K_{eq}$ and the mass action ratio, $\Gamma$, by the equation $\Delta G' = RT \ln(\Gamma/K_{eq})$. Since reactions are thermodynamically favoured when ΔG' is negative, as the mass action ratio (Γ) for an equilibrium enzyme decreases the flow through the enzyme increases (Rolleston, 1972).

In the previous chapter it was proposed that increases in respiratory carbon flow via the NAD-GAPDH would increase the chloroplastic NADH/NAD ratio and result in PQ reduction. This proposal was made assuming that localization of NAD-GAPDH in $S. \ minutum$ is similar to that observed in $Chlamydomonas$, where 86% of NAD-GAPDH was localized in the chloroplast. Furthermore, it was hypothesized that increases in NAD-GAPDH activity in the chloroplast would be correlated to increases in NADH/NAD ratios and would result in PQ reduction in class 1 but not class 2 treatments. In order to examine this hypothesis rigorously, it was necessary to determine if increases in flow through NAD-GAPDH occurred. This was done by a thermodynamic consideration of the effects of changes in key respiratory metabolites.

The mass action ratio for NAD-GAPDH is difficult to determine because levels of 1,3-bisPGA are extremely low in vivo and consequently, GAPDH and PGA kinase are generally considered as a combined reaction (Rolleston, 1972; Takahama et al., 1981). The mass action ratio for the combined reaction is $\Gamma = \frac{[PGA][ATP][NAD][H^+]}{[TP][ADP][NAD][Pi]}$. However, because [Pi] and
were not actually measured, the mass action ratio was approximated as
\[
\Gamma = \frac{[PGA][ATP][NADH]}{[TP][ADP][NAD]}. \]
Although compartmentation may certainly affect the accuracy of
this approximation, it was useful to demonstrate certain trends in vivo.

Effects of Class 1 treatments on the mass action ratio:
The mass action ratio decreased by 70, 90 and 99% after 5 minutes of class 1 treatment with NH$_4^+$, anaerobiosis or CCCP, respectively (Table 7). During all class 1 treatments, large decreases in the PGA/TP and ATP/ADP ratios were responsible for the decrease in the mass action ratio for TP to PGA conversion despite increases in the NADH/NAD ratio. Decreases in the TP/PGA ratio occurred as a result of activation of both PK and PFK (see previous discussion). In turn, large decreases in the mass action ratio after 5 minutes of class 1 treatments would thermodynamically favour an increase in flow from TP to PGA (Rolleston, 1972). In particular, decreases in this combined mass action ratio suggest that carbon flow through NAD-GAPDH would increase as a result of class 1 treatments. Assuming that 86% of NAD-GAPDH is chloroplastic, this increase in activity would serve to enhance NADH production in the chloroplast and could be responsible for the reduction of the PQ pool observed after class 1 treatments.

Effects of Class 2 treatments on the mass action ratio:
On the other hand, the mass action ratio for conversion of TP to PGA was not significantly affected by treatment with either Pi or NO$_3^-$ (Table 7). This suggests that the flow of carbon from TP to PGA during Pi assimilation was not thermodynamically favoured in comparison to the dark control and that flow through NAD-GAPDH and PGA kinase was relatively unchanged within 5 minutes of either class 2 treatment. In the absence of an increase in flow through NAD-GAPDH, chloroplastic NADH/NAD ratios should also be relatively unaffected and would be consistent with the absence of large changes in PQ reduction or a state
1 to 2 transition during class 2 treatments. The intermediate changes in cellular NADH/NAD ratios observed after NO$_3^-$ treatment were likely to reflect increases in mitochondrial ratios due to TCA cycle activity. Thus, the case of Pi treatment strongly supports the contention that class 2 treatments should not result in an increase in the chloroplastic NADH/NAD production ratio via increased flow through NAD-GAPDH. With the exception of fluorescence induction data, the case of NO$_3^-$ treatment also appears consistent with the model suggesting regulation of the state 1 to 2 transition by increases in chloroplastic NAD-GAPDH activity.

**Does ATP play a role in the regulation of the state transition?:**

Bulte et al. (1990) have suggested that, in addition to the effects of PQ reduction, decreases in [ATP] play an important role in the regulation of state transitions. Although they did not speculate as to the exact role of ATP, these authors indicated that oxidation of PQ was not sufficient for the recovery of a state 1 to state 2 transition, and that increases in [ATP] were also required. These authors used far red light and DCMU to oxidize PQ in cells that were treated with CCCP or ATPase inhibitors. However, it is quite possible that NAD(P)H might reduce PQ in the light via the NAD(P)H-PQ oxidoreductase (see literature review section on H$_2$ photoevolution, p. 23). Since the redox state of PQ was only inferred, and not directly measured by Bulte et al. (1990), it is likely that oxidation of PQ is indeed sufficient for the recovery of a state 1 to state 2 transition and that PQ remains reduced under conditions where the ATP pool is decreased from control.

From the present work, it would appear that ATP does not have a direct role in regulating the state transition. Instead, it seems that decreases in the ATP/ADP ratio release respiratory carbon flow from ADP limitation at the level of PK. This indirectly results in an increase in carbon flow via NAD-GAPDH and production of NADH in the chloroplast. The increased chloroplastic NADH/NAD ratio resulting from increases in carbon flow via NAD-GAPDH then results in reduction of PQ via the NAD(P)H-PQ oxidoreductase and activates the kinase responsible for the state 1 to 2 transition. Hence, if the ATP/ADP ratio remained low
upon illumination, PK would remain activated, chloroplastic NADH production would continue and PQ would remain reduced in the light. Thus, I suggest that decreases in ATP per se do not directly affect the redox state of PQ, but rather form part of a signal transduction pathway which leads to an increase in chloroplastic NADH/NAD ratios.

The physiological significance of interaction between respiratory carbon flow and photosynthesis via the CRETC in vivo:

The present study has clearly shown that interaction occurs between respiratory carbon flow and the PETC in the dark. Specifically, NAD(P)H-PQ oxidoreductase activity appeared to allow NADH produced by respiratory carbon flow (via NAD-GAPDH) to reduce the PQ(cyt b6f) pool and result in a state 1 to 2 transition. It has been proposed that the CRETC may be physiologically significant in vivo in darkened cells because it may maintain a transthylakoid proton gradient in the dark which allow rapid induction of ATP production upon illumination (Peltier et al., 1987), and it may allow oxidation of reductant produced by starch degradation in the dark (Bennoun, 1982). The observation that CRETC activity also appears to allow regulation of the state 1 to 2 transition by changes in respiratory carbon flow through NAD-GAPDH suggests that an additional important function for the CRETC may be to allow communication between respiration and photosynthesis in both the dark and the light.

In the present study, experiments were undertaken in the dark to reduce the complexity of processes contributing to both biochemical and physiological measurements of respiratory carbon flow, PQ reduction and the state 1 to 2 transition. Although interactions between respiratory carbon flow and the PETC were observed in the dark, the ramifications for interaction between respiratory carbon flow and photosynthesis in the light are considerable.

First, the regulation of the state transition in the dark by respiratory carbon flow (via the CRETC) may be important to poise the light harvesting reactions of cells for subsequent illumination. Class 1 treatments were shown to result in large decreases in the combined mass action ratio for the conversion of TP to PGA (Table 7). On the other hand, illumination
resulted in a 2.7-fold increase in the combined mass action ratio (Table 7) consistent with thermodynamic enhancement of photosynthetic carbon fixation which requires carbon flow in the opposite direction to glycolysis. It is conceivable that the large changes in the mass action ratio brought about by class 1 treatments could make photosynthetic carbon flow less thermodynamically favourable and reduce carbon flow in the photosynthetic direction upon illumination. This, in turn, could result in over-reduction of PS2 due to NADP-limitation of the PETC. Over-reduction of PS2 may cause photodamage unless NADPH production could be down-regulated. The state 1 to 2 transition occurring during class 1 treatments in the dark could, therefore, provide a mechanism to photoprotect PS2 upon illumination under these conditions. In addition, the state 1 to 2 transition would decrease the amount of LHC2 associated with PS2 and could enhance the amount of cyclic $\epsilon^-$ flow and ATP production which would directly increase the mass action ratio and tend to favour carbon flow in the photosynthetic direction. These proposals could be examined using illumination of class 1 treated cells in the presence of inhibitors of the NAD(P)H-PQ oxidoreductase (e.g. rotenone). If control- and inhibitor-treated cells were treated with class 1 or class 2 treatments and subsequently illuminated, the presence of the inhibitor should increase the susceptibility of class 1 treated cells to photodamage upon illumination in comparison to control and class 2 treated cells.

Perhaps more importantly, however, the CRETC would also allow direct interaction between respiratory carbon flow and the PETC in the light. Increases in respiratory carbon flow occur in the light in response to increased biosynthetic requirements (Weger et al., 1989) and in response to decreased ATP/ADP ratios (Turpin and Weger, 1990). CRETC activity would allow increases in NADH production (which would specifically signal an increase in respiratory carbon flow) to directly regulate the poising of the PETC for linear and cyclic electron transport via the state transition in the light. As mentioned above, this regulation may be important to allow photoprotection of PS2 if respiratory carbon flow were to affect photosynthetic carbon flow. Preliminary research has indicated that the 5 treatments used in the present study also
result in increases in respiratory carbon flow in the light (Holmes and Turpin, unpublished). Furthermore, class 1 treatments also appear to result in a state 1 to 2 transition in the light while \( \text{NO}_3^- \) treatment does not. This is clearly a fruitful area for future research to extend the present study's observations about the interactions between respiratory carbon flow and the PETC.

In conclusion, it appears that regulation of the PETC NADPH/ATP production ratio by respiratory carbon flow through NAD-GAPDH may be physiologically significant. NAD-GAPDH utilizes metabolites which are shared by both the reductive reactions of the Calvin cycle and the oxidative reactions of glycolysis. The status of glycolytic carbon flow through this enzyme can affect TP/PGA ratios and is likely to affect photosynthetic carbon fixation in illuminated cells. The significance of the CRETC may be that it allows NADH produced by NAD-GAPDH to signal the status of glycolytic carbon flow to the PETC and, in turn, could allow the PETC to compensate via the state 1 to 2 transition.
CHAPTER 7: GENERAL SUMMARY AND CONCLUSIONS:

The interaction between photosynthesis and respiration has previously been studied mainly with respect to the regulation of respiration by photosynthesis upon illumination. However, little is known about the regulation of photosynthesis by respiration. The fact that respiration has both a catabolic function and is necessary to provide carbon skeletons for biosynthesis implies an intimate co-regulation with photosynthesis.

On the basis of previous studies on the mechanism of the state transition and the fact that NAD(P)H-PQ oxidoreductase has been observed to affect PQ(cyt b$_{6f}$) pool reduction in vitro, a hypothesis was developed to examine the interaction between respiratory carbon flow and the regulation of the NADPH/ATP production ratio by the PETC (via the state transition). It was hypothesized that increases in respiratory carbon flow that lead to increases in the ratio of reduced/oxidized pyridine nucleotides would result in reduction of the PQ(cyt b$_{6f}$) pool and a state 1 to 2 transition. In contrast, increases in respiratory carbon flow which did not lead to increases in reduced/oxidized pyridine nucleotide ratios were not expected to change PQ(cyt b$_{6f}$) redox or result in a state 1 to 2 transition.

Five treatments were shown to increase respiratory carbon flow in the green alga, Selenastrum minutum. Dark assimilation of NH$_4^+$ or NO$_3^-$ by N-limited cells, anaerobiosis, uncoupling with CCCP and Pi assimilation by Pi-limited cells all resulted in a 2- to 10-fold increase in respiratory carbon flow as measured by respiratory CO$_2$ release and/or starch degradation. These treatments were then sub-divided into two classes on the basis of their ability to cause a state 1 to 2 transition. Treatment with NH$_4^+$, anaerobiosis or CCCP resulted in large perturbation of room temperature and 77K fluorescence parameters indicative of a state 1 to 2 transition and were termed class 1 treatments. Class 1 treatments were also observed to decrease the potential quantum yield of linear e$^-$ transport consistent with poising of the PETC for down-regulation of NADPH/ATP production ratio. NO$_3^-$ assimilation (N-limited cells) and Pi assimilation (Pi-limited cells) were designated as class 2 treatments because they resulted in
only minor changes in room temperature and 77K fluorescence parameters indicating the absence of a state 1 to 2 transition. In turn, the potential quantum yield of linear electron transport was relatively unaffected by either of these treatments.

On the basis of the original hypothesis, it was expected that class 1 treatments would increase PQ(cyt b$_6$f) pool reduction whereas class 2 treatments would not. The reduction of the PQ(cyt b$_6$f) pool was deduced from measurements of time-resolved fluorescence decay and induction kinetics. The rate of fluorescence decay was significantly decreased and the rate of fluorescence induction was greatly increased indicating that the PQ(cyt b$_6$f) pool had been reduced by class 1 treatments. On the other hand, fluorescence decay and induction parameters were relatively unaffected by Pi treatment (class 2) suggesting that PQ reduction was unaffected. Similarly, NO$_3^-$ treatment resulted in only minor changes in fluorescence decay kinetics. These observations were consistent with the original hypothesis. However, NO$_3^-$ treatment resulted in a large increase in the rate of fluorescence induction. The magnitude of changes in induction kinetics during NO$_3^-$ assimilation were inconsistent with changes in decay kinetics and the absence of a state 1 to 2 transition during NO$_3^-$ assimilation. It was proposed that a decrease in Fd/FNR association with PS1 due to super-complex formation with nitrite reductase could result in reduction of the PETC upon induction due to Fd/FNR limitation of PS1 in NO$_3^-$ treated cells.

On the basis of these observations, it was hypothesized that class 1 treatments would result in large increases in the reduced/oxidized ratio of pyridine nucleotides whereas class 2 treatments would have a much smaller effect. The present study used a highly specific method to measure pyridine nucleotides that allowed accurate measurement of changes in both the NADPH/NADP and NADH/NAD ratios. Increases in the NADPH/NADP ratio were not correlated with an increase in PQ(cyt b$_6$f) pool reduction or a state 1 to 2 transition in the present study. Large increases in the NADH/NAD ratio occurred after all class 1 treatments and were correlated with PQ reduction and a state 1 to 2 transition. Only small changes in the NADH/NAD ratio were observed during Pi assimilation. These results suggested that increases
in the NADH/NAD ratio were responsible for reduction of the PQ pool and a state 1 to 2 transition. NO₃⁻ assimilation, however, resulted in an intermediate increase in the NADH/NAD ratio which was inconsistent with the observed absence of a state 1 to 2 transition during NO₃⁻ assimilation.

One plausible explanation for the inconsistency posed by the increased NADH/NAD ratio during NO₃⁻ assimilation was that, due to compartmentation, increases in the NADH/NAD ratio might not occur in the chloroplast and would not affect PQ(cyt b₆f) pool reduction. It was proposed that class 1 treatments resulted in increases in chloroplastic NADH/NAD ratios whereas class 2 treatments did not. This possibility was explored by comparing the mechanism by which respiratory carbon flow was activated during class 1 and 2 treatments. In all class 1 treatments, respiration appeared to be activated by increases in ADP which released PK from ADP limitation. PFK was then subsequently activated by decreases in PEP due to PK activation. In turn, all class 1 treatments resulted in large decreases in the mass action ratio for the conversion of TP to PGA via NAD-GAPDH and PGA kinase despite increases in the cellular NADH/NAD ratios. NAD-GAPDH has been shown to be localized 86% in the chloroplast of *Chlamydomonas reinhardtii* (Klein, 1986). Assuming a similar enzyme localization in *S. minutum*, the increase in carbon flow through NAD-GAPDH implied by a decreased mass action ratio strongly suggested that this enzyme was responsible for an increase in chloroplastic NADH/NAD ratios, reduction of PQ and the occurrence of a state 1 to 2 transition.

On the other hand, activation of respiratory carbon flow after class 2 treatments did not involve release of PK from ADP-limitation. Instead, class 2 treatments resulted in a decrease (NO₃⁻) or only a minor increase in ADP (Pi). The mass action ratio for TP to PGA conversion was not significantly affected by either treatment, consistent with only minor changes in PQ reduction and absence of a state 1 to 2 transition during class 2 treatments. On the whole, these results are consistent with the hypothesis that NADH produced by NAD-GAPDH was responsible for PQ reduction and a state 1 to 2 transition.
The observation that state 1 to 2 transitions occurred in the dark in response to increases in respiratory carbon flow implies that regulation of photosynthesis by respiratory carbon flow through the CRETC may be physiologically significant in the light. First, the state 1 to 2 transition would allow for photoprotection of PS2 in class 1 treated cells upon illumination. Photoprotection of PS2 might be necessary due to changes in glycolytic flow which, in turn, could affect carbon flow in the photosynthetic direction upon illumination. Second, increases in respiratory carbon flow which resulted in increases in chloroplastic NADH/NAD ratios in the light could potentially directly regulate the redox state of the PQ(cyt b6f) pool and the activation of a state 1 to 2 transition. Preliminary studies of the effect of class 1 and 2 treatments in the light suggest that this may be a fruitful area for future research.

In conclusion, the present study clearly indicates that interaction occurs between respiratory carbon flow and poising of the PETC for NADPH/ATP production ratios via the state 1 to 2 transition. This interaction is likely to be a necessary consequence of the fact that both anabolic (Calvin cycle) and catabolic (glycolysis) processes occur in the same compartment (the chloroplast) and share common intermediates. The significance of the CRETC may be that it allows NADH produced by NAD-GAPDH to signal the status of glycolytic carbon flow to the PETC and, in turn, could allow the PETC to compensate via the state 1 to 2 transition.
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APPENDIX 1: APPROXIMATION OF QNP AND QP

As can be seen in Figure 23, the level of fluorescence measured by the measuring beam in the dark was not a true $F_0$ (qp=1, qNP=0) because it was quenched relative to the level of $F_0$ measured at low light intensities in S. minutum. Similarly, the true $F_M$ (qp=0, qNP=0) occurred at low light intensity and implies that $F_M$ measured in the dark in this alga is quenched. However, in the interests of simplicity, calculations of qp and qNP were made relative to the dark control assuming that qp=1 and qNP=0.

Approximation of qNP and qp from room temperature PAM traces

1) qNP:

according to van Kooten and Snel (1990):

$$q_{NP} = 1 - \frac{(F_M' - F_0')}{(F_M - F_0)}$$

$$= \frac{[(F_M - F_M') - (F_0 - F_0')]}{(F_M - F_0)}$$

$$q_P = \frac{(F_M' - F)}{(F_M' - F_0')}$$

where $F_M$ is the fluorescence intensity after a saturating flash in dark aerobic control cells, $F_M'$ is the fluorescence intensity after a saturating flash in treated cells, $F_0$ is the level of fluorescence induced by the measuring beam in dark aerobic cells (qp = 0), $F_0'$ is the true minimal level of fluorescence induced by after treatment (qp = 0) and F is the actual fluorescence intensity measured at any time t.

Since it is not possible to accurately measure true $F_0'$ in the dark under conditions where QA is reduced (i.e. qp ≠ 0)

assume that the true $F_0' \leq F_0$ if qT or qE occurs. That is, \((F_0 - F_0') \geq 0\).

\[\therefore\] a minimum estimate of the amount of qNP and qp occurring would be:
\[ q_{NP} = \frac{(F_M - F_M')(F_M - F_0)}{F_M - F_0} \]

\[ q_P = \frac{(F_M' - F)(F_M' - F_0)}{F_M' - F_0} \]
Figure 23: The effect of light intensity on the measurement of room temperature fluorescence parameters, \( F_0 \), the minimal level of fluorescence measured by the 1.6 kHz measuring beam and \( F_M \), the maximal level of fluorescence induced by a saturating flash. Cells were adapted for 10 minutes to each light intensity. \( F_M \) was the maximal level of fluorescence obtained with a 50 msec multiple turnover flash at each light intensity. \( F_0 \), was the minimal level of fluorescence induced by the 1.6 kHz measuring beam within 5 seconds of darkening. N.B. Illumination with far red light immediately before \( F_0 \) measurement had no effect of the value of \( F_0 \) obtained.
APPENDIX 2: CORRECTION OF TIME-RESOLVED FLUORESCENCE DECAYS FOR THE ACTINIC EFFECTS OF THE MEASURING BEAM

INTRODUCTION

The decay of variable fluorescence from a maximal level of fluorescence, \( F_M \) to a minimal level of fluorescence, \( F_o \), can be used to determine the rate of re-oxidation of \( Q_A^- \) (Cao and Govindjee, 1990; Krause and Weis, 1991). In this method, all \( Q_A \) (but not \( Q_B \) or PQ) is reduced by the means of a high intensity single turnover flash. The decline of fluorescence, which reflects the re-oxidation of \( Q_A^- \) to \( Q_A \), is measured by a weak, modulated "non-actinic" measuring light in the following "dark" period. In cells where all \( Q_A \) is oxidized before the flash, \( Q_A^- \) oxidation kinetics have been deconvoluted to yield three decay components which are attributed to \( Q_A^- \) oxidation by \( Q_B \) in PS2 centers a) with \( Q_B \) bound before the flash and b) with \( Q_B \) not bound before the flash and c) by recombination between \( Q_A^- \) and the S2 state of the water oxidizing complex in centres that are unable to transmit electrons to the \( Q_B \) pool (PS2 \( \beta \) centres) (Cao and Govindjee, 1990; Etienne et al, 1990).

Because the rate of re-oxidation of \( Q_A^- \) can be affected by the redox state of the PQ pool, it is also possible to infer the reduction state of the PQ pool from fluorescence decay kinetics. However, if the PQ pool is reduced before the single turnover flash, the \( Q_A \) pool will also be somewhat reduced due to redox equilibrium with the PQ pool. Thus, PS2 will exist in a number of redox conformations and \( Q_A^- \) will not be completely oxidized either before or after the single turnover flash. Despite this added complexity, the rate of \( Q_A^- \) oxidation will still be affected by PQ redox and the rate of \( Q_A^- \) oxidation will decrease as the PQ pool becomes reduced. The accurate determination of the rate of \( Q_A^- \) re-oxidation, however, is dependent upon the fact that the measuring beam itself is non-actinic, that is, that the measuring beam does not cause \( Q_A \) reduction and/or have any effect upon \( Q_A^- \) re-oxidation kinetics (Krause and Weis, 1991). In measurements of fluorescence decay to date (Cao and Govindjee, 1990; Etienne et al., 1990; Gleiter et al., 1992; Robinson and Crofts, 1983; Schreiber, 1986;
Govindjee et al., 1992) no attempt has been made to examine the actinic effects of the low intensity measuring beam used to measure the kinetics of the fluorescence decay.

It has already been observed that even the low intensity 1.6 kHz measuring beam used for steady-state saturation pulse measurements had actinic effects which were observed as a rise in fluorescence after treatment with DCMU (Figure 5, Chapter 2). However, resolution of fluorescence decay in the μsec time scale requires that the frequency of the measuring beam be increased from 1.6 to 100 kHz. In turn, this increases the light intensity of the measuring beam and increases the likelihood that the measuring beam will have actinic effects. In cells where the QA pool was already reduced, it was, therefore, quite likely that an increase in light intensity of the MB would further reduce the QA pool. Thus, it was anticipated that resolution of QA⁻ re-oxidation kinetics after class 1 treatments simply using fluorescence decay measurements would be difficult. That is, accurate measurement of the rate of QA⁻ re-oxidation would be complicated by the fact that the measuring beam would be contributing to QA reduction in addition to being used to measure QA⁻ oxidation. We have examined this question by measuring the actinic effects of the higher frequency measuring beam after both class 1 and class 2 type treatments.

MATERIALS AND METHODS

Refer to Chapter 2, methods for time-resolved fluorescence decays.

RESULTS

Uncorrected fluorescence decays:

Figure 24 shows the effect of class 1 and class 2 treatments on fluorescence decays that have been measured essentially as previously described (Schreiber, 1986; Etiene et al., 1990;
Cao and Govindjee, 1990; Govindjee et al., 1992). The 100 kHz measuring beam was used for a duration of 40 msec to allow resolution of the kinetics of fluorescence decays in cells treated with either NH$_4^+$ or anaerobiosis. Decays shown were normalized to $F_{\text{vmax}} = F_{\text{m}} - F_{\text{o}}$ (control) and $F_{\text{vmax}}' = F_{\text{m}'} - F$ (treated cells) where $F_{\text{m}}$ and $F_{\text{m}'}$ were the values extrapolated to $t=0$ in fitted decay curves. With the exception of anaerobically treated cells, all the decays were observed to reach a quasi steady-state plateau within 5 to 10 milliseconds which was higher than the initial $F_{\text{o}}$ level (Figure 24). The quasi-stationary level reached in cells treated with class 1 treatments (NH$_4^+$ or CCCP) or DCMU was considerably higher than that reached by dark control cells (Figure 24 A, B, D). Class 2 treatments (NO$_3^-$ or Pi) resulted in quasi steady-state levels which were only slightly higher than control cells (Figure 24 E, F). The difference between the plateau and the initial $F_{\text{o}}$ (control) or $F$ (treated) induced by the measuring beam remained for as long as the measuring beam remained at 100 kHz.

Fluorescence decays shown in Figure 24 were transformed to $q(t)$ decays as described (Chapter 3) and curve-fitted to asymptote to the $F_{\text{o}}$ (control) or $F$ (treated) measured previous to the single turnover flash. The dark control decay was de-convoluted to a sum of two exponentials with a fast component ($T_1 = 288$ µsec) comprising 65% of the decay ($\alpha_1 = 0.65$), and a slower component ($T_2 = 34.5$ msec) comprising 35% of the decay ($\alpha_2 = 0.35$) (see Table 8). DCMU treatment resulted in a loss of the µsec decay component and the appearance of a rapid ($T_1 = 7$ µsec) rise component as indicated by the negative amplitude of $\alpha_1$ (Cao and Govindjee, 1990). The half-time of the msec component, $T_2$ was increased tenfold over the dark control (Table 8). NH$_4^+$ assimilation increased the $t_{1/2}$ of both the µsec and msec $q(t)$ decay components ($T_1$ and $T_2$) by approximately 2- and 4.5-fold and increased the contribution of the msec decay component from 35 to 90% (Table 8). Anaerobiosis resulted in complete loss of the fast component of the decay and a 100% contribution of the msec decay component (Table 8). Uncoupling with CCCP decreased the contribution of the µsec component from 65 to 42% and increased the contribution of the msec component from 35 to 58% (Table 8). The
Figure 24: The effect of treatments which increase respiratory carbon flow on uncorrected time-resolved fluorescence decay kinetics. Decays were measured using a 40 msec duration, 100 kHz measuring beam and were not corrected for the actinic effect of the measuring beam. Open circles: dark, aerobic control cells; closed circles: cells treated with A. NH$_4^+$; B. anaerobiosis; C. CCCP; D. DCMU; E. NO$_3^-$; F. Pi. Treatments A-E and F were made to N-limited and Pi-limited cells (3-5 µg Chl/mL), respectively. The single turnover flash ($t_{1/2}$=8 µsec) was initiated 3 msec after the measuring beam was switched from 1.6 to 100 kHz. Decays were normalized to $F_{v \text{ max}} = F_M - F_O$ (control) or $F_{v \text{ max}}' = F_M' - F$ (treated). All curves were an average of 8 measurements.
Table 8: The effect of DCMU and treatments which increased respiratory carbon flow on the amplitude ($\alpha_i$) and half-times ($T_i = T_i/0.69$) of the fast (µsec; $\alpha_1$ and $T_1$) and medium (msec; $\alpha_2$ and $T_2$) components of time-resolved $q(t)$ decays which were not corrected for the actinic effect of the 100 kHz measuring beam. Cells were adapted to treatments for 20 minutes before measurements of decays. Fluorescence decays (Figure 25) were transformed to $q(t)$ decays and then curve-fitted for either a single exponential or sum of two exponentials (see Materials and Methods).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\alpha_1$ (SE)</th>
<th>$\alpha_2$ (SE)</th>
<th>$T_1$ (µsec) (SE)</th>
<th>$T_2$ (msec) (SE)</th>
<th>(uncorrected for the actinic effect of the measuring beam)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark aerobic controls:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-limited cells</td>
<td>0.65 (.02)</td>
<td>0.35 (.02)</td>
<td>288.3 (23.14)</td>
<td>34.98 (8.28)</td>
<td></td>
</tr>
<tr>
<td>Pi-limited cells</td>
<td>0.45 (.01)*</td>
<td>0.55 (.01)*</td>
<td>258.8 (11.2)</td>
<td>51.4 (4.8)</td>
<td></td>
</tr>
<tr>
<td>+ DCMU</td>
<td>-1.17 (.03)*</td>
<td>.17 (.03)*</td>
<td>7.2 (2.92)*</td>
<td>392.9 (23.3)*</td>
<td></td>
</tr>
<tr>
<td>Class 1 treatments:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NH$_4^+$</td>
<td>0.1 (.01)*</td>
<td>0.9 (.01)*</td>
<td>658.0 (61.5)*</td>
<td>156.1 (11.24)*</td>
<td></td>
</tr>
<tr>
<td>+ anaerobiosis</td>
<td>0.0*</td>
<td>1.0*</td>
<td>0.0*</td>
<td>25.8 (2.26)</td>
<td></td>
</tr>
<tr>
<td>+ CCCP</td>
<td>0.42 (.01)*</td>
<td>0.58 (.01)*</td>
<td>98.7 (1.83)*</td>
<td>38.9 (2.19)</td>
<td></td>
</tr>
<tr>
<td>Class 2 treatments:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NO$_3^-$</td>
<td>0.48 (.01)*</td>
<td>0.52 (.01)*</td>
<td>332.0 (25.5)</td>
<td>47.1 (0.99)</td>
<td></td>
</tr>
<tr>
<td>+ Pi ††</td>
<td>0.48 (.04)</td>
<td>0.52 (.04)</td>
<td>197.3 (6.0)</td>
<td>118.3 (9.69)**</td>
<td></td>
</tr>
</tbody>
</table>

† In all cases where curves fitted a single exponential, the $r^2$ for the single exponential fit was greater than 0.98 and/or greater than the $r^2$ for the sum of two exponentials.

†† Pi treatment was made to Pi-limited cells, all other treatments were made to N-limited cells.

* Indicates that this value was significantly different from the N-limited dark aerobic control, i.e. the value was outside the 95% confidence interval as determined by a student t test.

** Indicates that this value was significantly different from the Pi-limited dark, aerobic control.
half-time of the μsec component (T₁) decreased 3-fold after CCCP treatment while T₂ was unaffected (Table 8). NO₃⁻ assimilation resulted in a decrease in the contribution of the fast decay by 17% (Table 8). Pi assimilation resulted in a 2.4-fold increase in T₂, the half-time of the msec decay component.

The actinic effects of the 100 kHz measuring beam

The actinic effect of the 100 kHz measuring beam was measured by disconnecting the single turnover flash unit and measuring only fluorescence induced by the change in frequency from 1.6 to 100 kHz. After 20 minutes of dark acclimation in control or treated cells, initiation of the 100 kHz measuring beam resulted in a fluorescence induction curve which reached a plateau within approximately 40 msec (Figure 25). The actinic effect of the 100 kHz measuring beam was minimal in N-limited dark control cells (Figure 25 A-E) but the 100 kHz measuring beam produced a distinct induction curve in Pi-limited control cells (Figure 25 F). The 100 kHz measuring beam resulted in significant fluorescence induction after DCMU or Class 1 treatment as compared to the dark control (Figure 25 A, B, C, D). Both NO₃⁻ and Pi treatment resulted in only minor changes in fluorescence induction as a result of the 100 kHz measuring beam compared to the dark control (Figure 25 E, F).

Correction factors for fast decays:

In all cases, the fluorescence decayed to the level of fluorescence induced by the measuring beam within 40 msec (data not shown). All decay curves were corrected by subtracting the induction curves from the observed fluorescence decay before transforming fluorescence data to q(t) oxidation data.

The effect of correcting fluorescence decays on q(t) decay parameters:

Correcting fluorescence decay curves for actinic effects of the measuring beam had no significant (as determined by a student T test) effect on the contribution of the q(t) μsec and
Figure 25: The effect DCMU and class 1 and 2 treatments on the level of fluorescence induced by the 100 kHz measuring beam. Open circles: dark, aerobic control cells; closed circles: cells treated with A. NH$_4^+$; B. anaerobiosis; C. CCCP; D. DCMU; E. NO$_3^-$; F. Pi. Treatments A-E and F were made to N-limited and Pi-limited cells (3-5 µg Chl mL$^{-1}$), respectively. The 1.6 kHz measuring beam was switched on at time 0 (large arrow, A.) and was switched to 100 kHz for a 40 msec duration as indicated by the small arrow. All curves were an average of 8 measurements.
msec decay components in N- or Pi-limited control cells or in cells treated with anaerobiosis, NO₃⁻ or Pi (Compare Table 8 and Table 3, Chapter 3). In cells treated with NH₄⁺ or CCCP correction of fluorescence decay curves resulted in a loss of α₁, the μsec q(t) decay component and 100% contribution of α₂, the msec decay component. Correction of fluorescence decays resulted in loss of the fluorescence rise component and a 100% contribution of α₂ after treatment with DCMU (Table 3 and 7). In all cases, correction of the fluorescence decay resulted in a large decrease in T₂, the half-time of the msec decay component ranging from 5 to 40-fold. T₁ decreased slightly after correction in all cases except Pi treatment although this decrease was not significant in the case of NO₃⁻ (Compare Tables 3 and 7).

DISCUSSION

Uncorrected fluorescence decays:

In uncorrected fluorescence decays from N- and Pi-limited control or treated cells, fluorescence decays reached a plateau that was higher than the initial F₀ level within 40 msec. This resulted in a slow q(t) component with half-times (T₂) which were greater than 25 msec for all treatments (Table 8). The steady state level reached after treatment with DCMU, NH₄⁺ and CCCP was considerably higher than the level reached in the dark control (Figure 24). This was consistent with a slower half-time of the msec component of the decay in each of these cases (Table 8) and suggested that the PQ pool might be more reduced under these circumstances. In addition, NH₄⁺ assimilation and anaerobiosis resulted in either a decrease or complete loss of the μsec decay component which supported the suggestion that the PQ pool had been reduced as a result of these treatments (Table 8).

Treatment with NO₃⁻ resulted in a 17% decrease in the contribution of the fast component of the uncorrected q(t) decay suggesting that the rate of QA oxidation was decreased and that the PQ pool had become slightly reduced (Table 8). Pi treatment resulted in no significant change in the contribution of either component but doubled the half-time of the
msec q(t) decay component (Table 8). The effects of NO₃⁻ or Pi treatment on uncorrected q(t) decays were significant and suggested some effect of each of these treatments on the oxidation kinetics of QA⁻ but were relatively minor compared to the effects of DCMU, NH₄⁺ or anaerobiosis. This observation is consistent with the hypothesis that class 1 treatments would reduce the PQ pool while class 2 treatments would not.

However, the half-time of the μsec decay component, T₁ decreased after the class 1 CCCP treatment suggesting that the rate of QA⁻ oxidation had increased and that oxidation of the PQ pool might have occurred. This was inconsistent with the observation that CCCP resulted in a state transition (Chapter 2). In order to resolve this apparent discrepancy and to determine whether the measuring beam was affecting the rate of fluorescence decay, the effect of the measuring beam was measured in the absence of the single turnover flash.

**Actinic effects of the measuring beam:**

It has been proposed that the PQ pool was reduced during class 1 treatments leading to reduction of the QA pool due to redox equilibrium between these two pools (Chapter 2). This suggested that the higher frequency (and intensity) measuring beam required to resolve fluorescence decay kinetics might also have actinic effects and affect QA reduction/oxidation kinetics. With the exception of anaerobically treated cells, the fluorescence decays reached a stable plateau level which was higher than F₀ (fluorescence level before the single turnover flash, control) or F (fluorescence level before the single turnover flash, treated) within 10 msec and remained close to this level as long as the measuring beam was on (Figure 24). In particular, the stable plateau was much greater than the original F level in cells treated with DCMU, NH₄⁺ and CCCP (Figure 24 A, C, D). This observation suggested that the measuring beam was actinic and resulted in QA reduction, preventing accurate resolution of QA oxidation kinetics from fluorescence decays. To test this hypothesis, the effect of the measuring beam in the absence of a single turnover flash was measured.
The actinic effect of the measuring beam was measured as the extent of fluorescence induced by conversion of the measuring beam from 1.6 to 100 kHz in the absence of the single turnover flash. A small induction curve occurred in N-limited control cells (Figure 25). The amount of fluorescence induced by the 100 kHz measuring beam after treatments with DCMU, NH$_4^+$ anaerobiosis and CCCP was particularly pronounced when compared to the dark control (Figure 25 A, B, C). These effects suggested that the 100 kHz measuring beam did, indeed, have an actinic effect in class 1 treated cells and caused reduction of the QA pool in excess of that due purely to redox equilibration between QA and a more reduced PQ pool. On the other hand, NO$_3^-$ and Pi resulted in very little increase in fluorescence induced by the 100 kHz measuring beam as compared to the respective dark controls suggesting that the measuring beam had a relatively small actinic effect (Figure 25 E, F). These results tend to confirm the proposal that the QA and PQ pool were relatively oxidized in class 2 treated cells while these pools were more reduced in class 1 treated cells.

The large increase in fluorescence induced by the 100 kHz measuring beam after DCMU and class 1 treatments was strongly correlated with the large difference between F and the quasi-plateau level reached in the fluorescence decays. That is, all decays from either control or treated cells decayed to the level of fluorescence induced by the measuring beam within 40 msec (data not shown). This suggested that the observed decay curve was actually a sum of QA$^-$ oxidation after the single turnover flash and QA$^-$ reduction due to actinic effects of the measuring beam. Thus, to resolve the kinetics of QA oxidation, the induction curve (due to the 100 kHz measuring beam alone = QA reduction) was subtracted from the observed decay curve (the effects of the single turnover flash in the presence of the 100 kHz measuring beam = sum of QA$^-$ oxidation and QA reduction).

**Effects of correcting fluorescence decays:**

In comparison to uncorrected curves where the fluorescence decayed to a quasi-plateau which was much higher than $F_0$ or F, correcting the fluorescence decay curves resulted in a
decay to 0 within 40 msec after all treatments (see Figure 8, Chapter 3). In addition, the half-time of the msec component of the q(t) decay was decreased in all cases to a half-time ranging from 3.5 to 8 msec (compare Table 3 and 8). This is much more consistent with the half-times of the msec components observed in nutrient sufficient *Chlamydomonas* (2-11 msec) (Govindjee et al., 1992) and in spinach and soybean (6-7 msec) (Cao and Govindjee, 1990).

One significant effect of correcting the fluorescence decay for the actinic effects of the measuring beam is that it changed the shape of the fluorescence decay curve in cells treated with CCCP, DCMU and NH₄⁺ and resulted in a loss of the rapid fluorescence decay component associated with the uncorrected decay (compare table 3 and 8).

The advantage of correcting the fluorescence decay for the actinic effects of the measuring beam is that it was possible to isolate $Q_A$ oxidation kinetics (as a result of the single turnover flash) from $Q_A$ reduction kinetics (due to the effects of the measuring beam). Once $Q_A$ oxidation kinetics were isolated, it was then possible to deduce the redox status of the PQ pool on the basis of slower $Q_A^-$ re-oxidation kinetics after a variety of treatments which increased the rate of respiratory carbon flow. It is obvious from the above discussion that extreme care must be taken when using fluorescence decays to determine rates of $Q_A$ reduction and oxidation and, further, to infer the redox state of the intersystem electron transport chain. By virtue of the fact that light is being used to a) induce and b) measure fluorescence, inaccuracy in determination of $Q_A$ reduction and oxidation kinetics is likely to occur unless the actinic effects of the measuring beam are corrected for.
APPENDIX 3: METABOLITE ASSAYS

Metabolite Assays:

Metabolite assays were enzymatically coupled to the reduction or oxidation of pyridine nucleotides and measured at 334 nm on a Sigma dual wavelength spectrophotometer (ZFP22, Sigma Instruments, FRG). All assay reagents were provided by Boehringer Mannheim Co.

**Pyr, PEP:** PEP and pyruvate were assayed using a 100 mM Tris buffer (pH 7.5) containing 5 mM MgCl₂, 2.5 mM NADH and 5 mM ADP. Pyruvate was measured as NADH oxidation due to pyruvate reduction to lactate and was started by lactate dehydrogenase (1U, BMC 127230). PEP was coupled to this reaction with pyruvate kinase (1.3 U, BMC 128155).

**Hexose phosphates and ATP (F6P, ATP, G6P, G1P):** Hexose phosphates and ATP were measured sequentially in an assay mixture (100 mM Tris, pH 8.1; 5 mM MgCl₂) containing 2.5 mM NADP and 10 mM glucose. G6P was coupled to NADP reduction via conversion of G6P to 6PG by G6PDH (.14 U, BMC 127663). F6P was coupled to this reaction using phosphoglucoisomerase (0.7 U, BMC 128 139). G1P was coupled to the G6P reaction with phosphoglucomutase (0.4, BMC 108375). ATP was measured by coupling glucose phosphorylation via hexose kinase (.42 U, BMC 127809) to the G6PDH reaction.

**ADP, AMP:** ADP and AMP were measured in a 50 mM Hepes buffer (pH 7.6, 10 mM KH₂PO₄; 2.5 mM NADH and 5 mM PEP and LDH (.4U BMC 127 230). PEP and ADP were converted to Pyr and ATP via pyruvate kinase (3 U, BMC 128 155) and coupled to pyruvate oxidation to lactate via lactate dehydrogenase. AMP was coupled to this reaction by conversion of AMP and ATP via myokinase (5U, BMC 107 506).
**TP and FBP:** Triose phosphate was measured in a 100 mM Tris (pH 8.1; 5 mM MgCl₂; 2.5 mM NADH). Triose phosphates were converted to glycerine 3-P using GDH/TPI (0.3 U, BMC 127787). FBP was coupled to this assay using aldolase (0.018 U, BMC 102652).

**6PG:** 6PG was measured by coupling 6PG oxidation to NADPH production in an assay mix containing 0.25 mM NADP and 100 mM Tris, pH 8.1 and 5 mM MgCl₂. The reaction was started by adding 6PGDH (0.2 U, BMC 108391).

**Glucose:** Glucose was measured in an assay mix containing 100 mM Tris (pH 8.1); 5 mM MgCl₂; 0.25 mM NADP, 2.5 mM ATP and G6PDH (0.14 U, BMC 127663). Glucose was phosphorylated to G6P by hexokinase (0.42 U, BMC 127809) and coupled to G6P oxidation by G6PDH.