STUDIES ON CARBON MONOXIDE AND DIOXYGEN BINDING TO CYTOCHROME P-450cam.

By

NIMAL RAJAPAKSE

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N. RAJAPAKSE

Department of Chemistry

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date 2nd January 1985.

ABSTRACT

Interest has remained very intense during the last two decades on the heme-containing monooxygenase system, cytochrome P-450. The P-450 hemoproteins are widely distributed in nature and engage in oxygenation of a wide variety of substrates according to the reaction,

$$R\text{-}H + O_2 + 2H^+ + 2e^- \longrightarrow R\text{-}OH + H_2O$$

where R-H represents an unactivated carbon-hydrogen bond.

Investigations on binding of small gas molecules such as CO and O_2 to the P-450 enzymes are important not only in understanding various aspects of monooxygenation but also in developing protein-free model systems that can mimic the catalytic properties of P-450.

This thesis describes gas binding studies carried out on cytochrome P-450cam. A procedure is given for growing the bacterium Pseudomonas putida strain 786 from which soluble, camphor hydroxylating P-450 enzyme is isolated and purified. The binding of CO to the stoichiometrically reduced substrate-free enzyme at different temperatures was studied using a standard spectrophotometric procedure. From these experimental data, the thermodynamic parameters ΔH° and ΔS° were calculated for the reaction,

$$(P\text{-}450)Fe(II) + CO \rightleftharpoons (P\text{-}450)Fe(II)\text{-}CO \ .$$

Attempts to determine such thermodynamic parameters for the binding of dioxygen to the substrate-bound P-450 enzyme were not successful.

On comparison of the determined thermodynamic parameters for the substrate-free system with the literature values for substrate-bound enzyme, hemoglobin, myoglobin and P-450 model systems, it is concluded that the substrate molecule was bonded in the immediate vicinity of the active-site thereby lowering the CO affinity to the substrate-bound system.

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LIST OF ABBREVIATIONS

The following abbreviations and common names are used in this thesis along with the standard abbreviations used commonly in chemical literature.

A	Absorbance
A_0	Observed absorbance at the beginning of an experiment
A_∞	Observed absorbance at the end of an experiment
Buffer P	50 mM phosphate buffer, pH 7.4
Buffer P-100	50 mM phosphate buffer, pH 7.4, 100 mM KCl
Buffer T	50 mM Tris buffer, pH 7.4,
Buffer T-50	50 mM Tris buffer, pH 7.4, 50 mM KCl
Buffer T-100	50 mM Tris buffer, pH 7.4, 100 mM KCl
Buffer T-600	50 mM Tris buffer, pH 7.4, 600 mM KCl
βME	β-mercaptoethanol
^{o}	Degrees Centigrade
cam or camphor	D-(+)-camphor
DNase 1	Deoxyribonuclease 1
DTT	Dithiothreitol
EXAFS	Extended X-ray absorption fine-structure spectroscopy
ENDOR	Electron nuclear double resonance
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
ΔH^o	Standard enthalpy change

Hb	Hemoglobin
max	Maximum
Mb	Myoglobin
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
P-450	Cytochrome P-450
P-450cam	Camphor hydroxylating P-450
P-450LM	Liver-microsomal P-450
RNase A	Ribonuclease A
RR	Resonance Raman
SDS	Sodium dodecyl sulphate
ΔS^{o}	Standard entropy change

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CHAPTER I

INTRODUCTION

I. Introduction

The hemoproteins which mediate transportation, storage and activation of dioxygen are vital for most living organisms. For example, in mammals, dioxygen is transported by hemoglobin, stored by myoglobin and reduced to water in the terminal step of respiration by cytochrome c oxidase. Also, decomposition and further activation of hydrogen peroxide formed in biological systems are undertaken by catalase and peroxidase while a number of other hemoproteins function as mono- and dioxygenases.

Amongst all these hemoproteins, cytochrome P-450 (P-450) is considered to be one of the most unusual and intriguing. The intense level of research conducted during the last 25 years since its discovery[1-5] reflects, in part, the importance of cytochrome P-450 as a drug-metabolizing enzyme, its central role in chemical carcinogenesis, and its broad substrate specificity.

The P-450 molecule consists of a single protein chain with a molecular weight of approximately 45,000 , joined to an iron protoporphyrin moiety (Figure I.1) via a thiolate ($-S^-$) ligand derived from a cysteine residue. The P-450 hemoproteins generally catalyze the splitting of molecular dioxygen and insertion of one oxygen atom into an unactivated C-H bond to form an alcohol, the other atom of oxygen being converted to a molecule of water (Reaction I.1).

$$RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O \qquad (I.1)$$

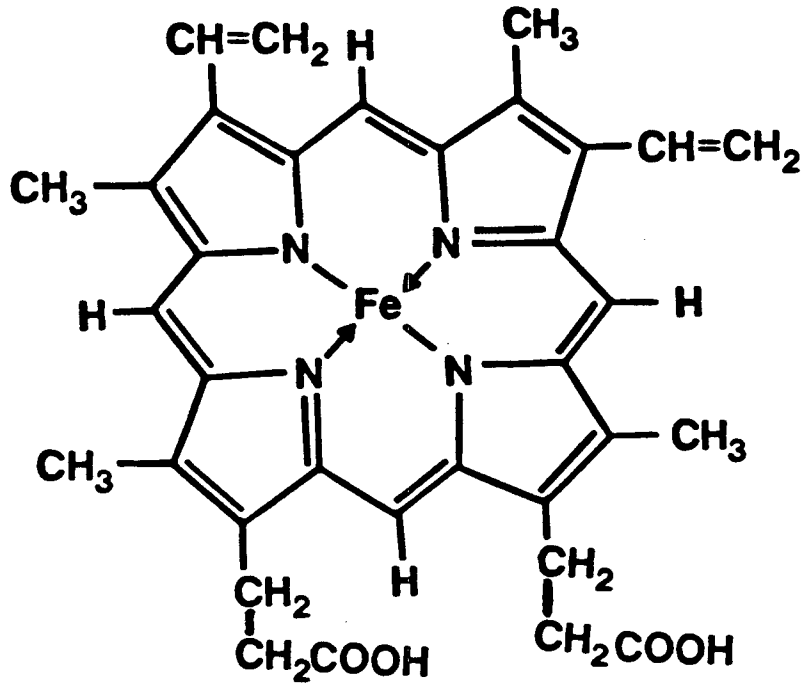
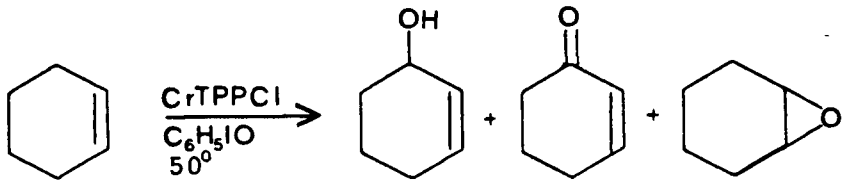


Figure I.1 The Structure of the Heme Unit.

Therefore, cytochrome P-450's are collectively known as monooxygenases, which are a class of enzymes that incorporate one atom of oxygen from molecular oxygen into a substrate. The ability of P-450 to carry out this hydroxylation reaction under physiological conditions with high stereo- and regiospecificity is in fact remarkable, when compared to the forcing reaction conditions and the number of different products that result, if the same hydroxylation reaction were to be carried out in the laboratory using non enzymatic, chemical reagents.[6]

eg.



Throughout the brief history of cytochrome P-450, numerous attempts to solubilize and purify the membrane-bound enzyme that is present in mammalian systems resulted in the isolation of an 'inactive' form of enzyme.[7] However, the discovery of soluble P-450 in the bacterial strain Peudomonas putida grown on D-(+)-camphor led to the isolation of pure enzyme. Therefore, in the present study, soluble camphor monooxygenase system, P-450cam, has been used as a biological model for mammalian P-450.

Reactions of organic compounds with dioxygen in the ground state are restricted by spin conservation.[8] This may be overcome by formation of metal-dioxygen complexes which in turn react with the organic substrate. However, the same

metal complexes also react directly with alkyl hydroperoxides to generate chain-initiating radicals.[8] Since alkyl hydroperoxides are ubiquitous in most hydrocarbon mixtures, the metal-hydroperoxide interactions tend to mask any reaction via 'oxygen activation'. Therefore it is of immense importance to develop catalytic systems that are capable of promoting selective oxidations, preferably using O_2 under mild reaction conditions. The study of cytochrome P-450 is thus of great importance not only in widening our knowledge of this remarkable biological system but also in developing protein-free catalytic systems that would mimic the O_2 activation capability of these enzymes; such systems bear an unlimited industrial potential.[9]

In addition to major unanswered questions about the mechanism of P-450 catalytic function, questions concerning the interactions of the substrates with the active-site of the enzyme remain equally challenging. In the present study, the attention was focused to gain some insight into active site-substrate interactions by studying the CO and O_2 binding to P-450cam. The thesis begins with an extensive literature survey in Chapter II, that introduces various aspects of monooxygenation mediated by P-450. Chapter III describes the details of experimental procedures. In Chapter IV, the results of experiments are discussed in comparison to other known hemoproteins and model systems, and some conclusions are drawn.

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CHAPTER II

LITERATURE REVIEW

II.1 Brief History of Cytochrome P-450

The presence in rat liver microsomes of a pigment with a peculiar carbon monoxide binding spectrum was first noted by Williams in 1955[1] while studying the oxidation-reduction kinetics of microsome-bound cytochrome b_5. This novel observation was not published until 1958, at which time Klingenberg[1] first reported the carbon monoxide-difference spectrum of this microsomal pigment (Figure II.1).

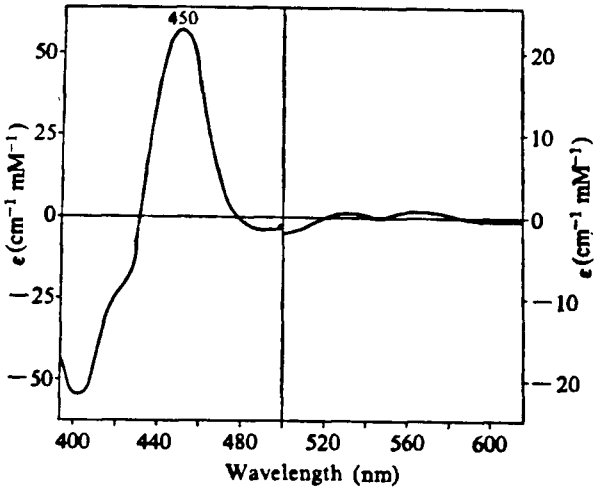


Figure II.1 The first published record of the carbon monoxide-difference spectrum of cytochrome P-450. (from ref.1).

Although the binding of carbon monoxide strongly suggested the presence of a heavy metal ion in the chromophore of the pigment, the electronic absorption spectrum showed no resemblance to any known coloured metalloprotein including hemoproteins. In 1962, Omura and Sato[2] presented conclusive spectral evidence for the hemoprotein nature of the pigment and confirmed that the pigment was a new b-type cytochrome. They also proposed a

'tentative' name "P-450" which meant "a pigment which absorbs at 450 nm".

The presence of α- and β- bands, in addition to intense Soret bands in the carbon monoxide-difference spectrum of the reduced pigment (Figure II.2), clearly revealed the hemoprotein nature of the new pigment. It also

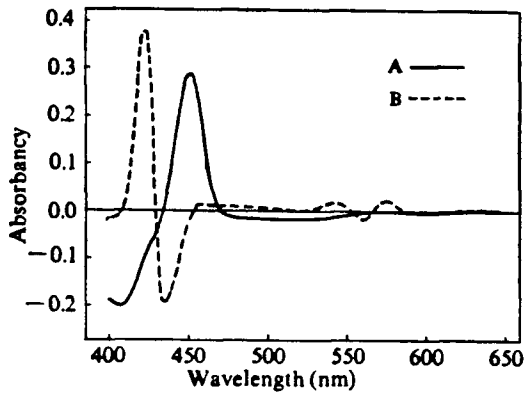
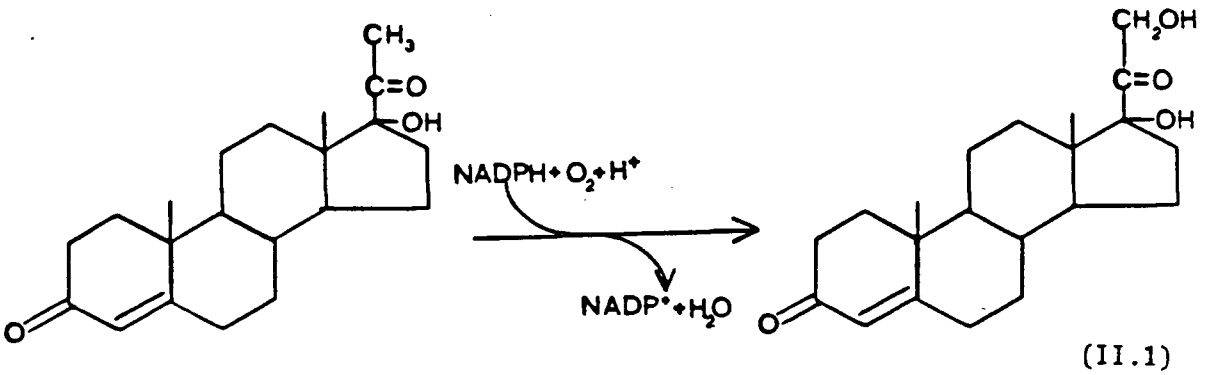


Figure II.2 The carbon monoxide-difference spectra of cytochrome P-450 (A) and P-420 (B).(from ref.1).

was observed that treatment of the pigment with detergents converted it quantitatively into a spectrally distinct solubilized form, with a prominent peak at 420 nm in the CO-difference spectrum.

Since the establishment of the hemoprotein nature of "P-450", the attention of many biochemists who were engaged in the study of physicochemical properties of hemoproteins became focused on this new b-type cytochrome. Utilizing the photochemical action spectrum technique, in 1963, Estabrook et al.[3] demonstrated for the first time the participation

of cytochrome P-450 in a steroid C-21-hydroxylation reaction catalyzed by the adrenal cortex microsomes (Reaction II.1). The photochemical action spectrum for the



(II.1)

17-Hydroxyprogesterone Cortexolone

light reversal of the CO-inhibited active monooxygenase enzyme present in these microsomes showed an intense absorption peak at 450 nm that was identical to the reduced CO-difference spectrum of the liver microsomes. In addition, Estabrook et al.[4] were able to demonstrate the participation of cytochrome P-450 in many other mixed function oxidase reactions including hydroxylations and oxidative dealkylation of drugs catalyzed by liver microsomes, confirming the physiological function of cytochrome P-450 to be monooxygenation. As the nature and function of cytochrome P-450 was elucidated, it was also shown that this enzyme could be induced by treating animals with various drugs.[5,6]

Although cytochrome P-450 was initially thought to be

present only in microsomes of animal tissue,[7] in a matter of a five-year period from the original discovery in microsomes, it was shown to be present in almost all forms of life.[8] In fact, cytochrome P-450 is distributed in nature from primitive bacteria to highly developed mammals, and participates in diversified metabolic reactions as the oxygen activating component of monooxygenase systems.

In 1967, Appleby[9] presented the first convincing evidence for the presence of P-450 in a bacterial species. The cytochrome P-450 found in the bacterium Rhizobium japonicum was not bound to membranous cell particles and hence could be partially purified by conventional procedures.[9] Shortly afterwards the camphor hydroxylating cytochrome P-450 was isolated from bacterium Pseudomonas putida [10] and purified to homogeniety,[11] and crystallized.[12] This source of the enzyme has provided the best material for studying the chemical and physicochemical properties of this class of enzymes.[8]

Since the establishment of the physiological function, the next challenge was to determine the mechanism of electron supply to cytochrome P-450. The difficulty in solubilizing the liver microsomal P-450 system, which was very tightly associated with the membranes, caused researchers to look for a more soluble form of P-450. The components of the 11-β-hydroxylase system of adrenal cortex mitochondria were finally separated by ionic treatment[13,14] into a P-450-containing membrane fragment and a soluble

NADH-P-450 reductase system, which was later separated by chromatography into an iron-sulfur protein and a flavoprotein. All of these components were required to reconstitute the 11-β-hydroxylase activity of the system, and the role of iron-sulfur protein was found to be the transferring of electrons from NADPH-linked flavoprotein to cytochrome P-450. In 1968, constituents of the camphor hydroxylating system were separated and identified as an iron-sulfur protein, a flavoprotein and cytochrome P-450 (Figure II.3).[15]

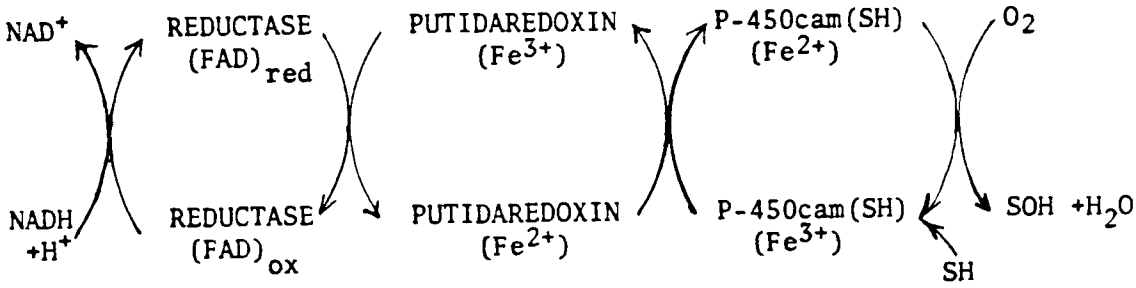


Figure II.3 Electron transfer reactions in bacterial system leading to camphor (SH) hydroxylation.

A major breakthrough towards the understanding of the reaction cycle of cytochrome P-450 occurred when Narasimhulu et al.[16], in 1965, observed the "substrate binding spectrum" of the C-21-hydroxylase system of adrenal cortex microsomes. When the substrate, 17-hydroxyprogesterone, was added to a suspension of adrenal cortex microsomes, the Soret peak was observed to shift to

a shorter wavelength, and the spectrum returned to the original position upon the addition of NADPH. The substrate-induced spectral change for the soluble cytochrome P-450 from Pseudomonas putida was observed[17] also and the association constant for camphor binding was calculated to be $(0.47\pm0.07)\ 10^{6}M^{-1}$ (Figure II.4).[18]

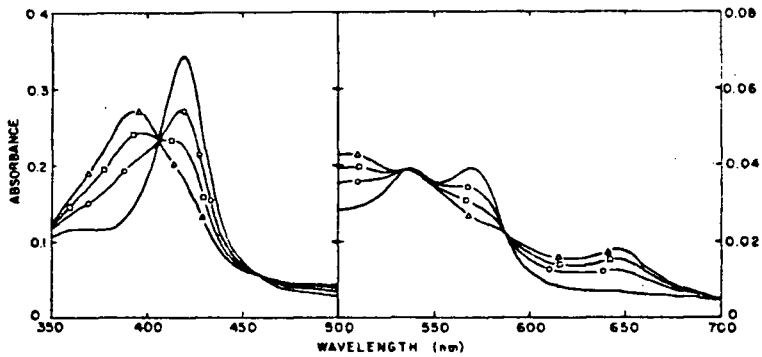


Figure II.4 The substrate-induced spectral changes for cytochrome P-450cam. Final camphor concentrations (μM) were, 0 (———) ; 2 (——0——) ; and 6 (——□——) ; and 20.5 (——Δ——),(from ref. 18).

The association reaction between P-450 and camphor changes the iron(III) from a low to high spin form as detected from EPR experiments,[19] and this in turn results in a significant change in the redox potential of the heme iron.[20] Thus, the first step in the reaction of this remarkable biocatalyst system became well understood.

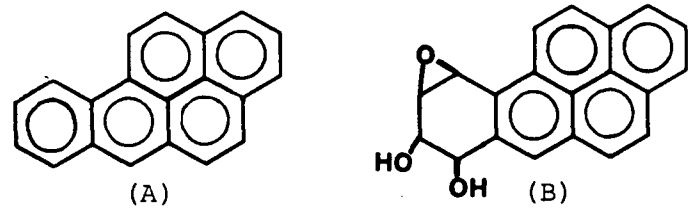
The microsomal fractions were shown to react with a wide variety of substrates using the photochemical action spectrum technique,[3] this being unusual behavior for a single enzyme species. Also, it was observed that the

cytochrome P-450 from microsomes of adrenal cortex and liver had essentially the same CO-difference spectrum, although the substrates were chemically very different.[21] However, the explanation of this observation was delayed until later separation and purification of microsomal cytochrome P-450. In 1975, it was shown that rabbit liver microsomal P-450 (P-450LM) purified to electrophoretic homogeniety contained as many as six forms of P-450LMs,[22-25] designated according to decreasing mobility in SDS-gel electrophoresis as P-450LM$_1$, P-450LM$_2$ and so on.[26] Some of these multiple forms (isozymes) have been characterized[27] and found to contain different C- and N-terminal residues and other significant structural differences.

II.2 Nature and Cytochrome P-450

All human beings and most other animals on earth are chronically exposed to numerous non-nutrient dietary chemicals, to drugs used in treatment or prevention of disease, and to an ever increasing number of man-made environmental pollutants. In order to sustain life in this potentially hazardous surroundings, nature has played a remarkable role in designing and activating relatively efficient ways of detoxification and excretion of these 'deadly' xenobiotics. Since its discovery in 1958, many forms of cytochrome P-450 have been shown to occur in almost all forms of life, functioning as a highly versatile oxygenating catalyst in diversified biochemical reactions. Cytochrome P-450 is known to be present in yeasts,[28] fungi,[9] plants,[29] bacteria,[30,31] insects,[32] fish, birds, reptiles, amphibians,[33,34] and mammals.[35] In mammals, cytochrome P-450s are found in microsomes of the liver, kidney, small-intestine, lung, adrenal cortex, skin, testis, placenta and several other tissues,[8] and are capable of metabolising drugs, food additives, anaesthetics, petroleum products, pesticides and carcinogens.[27] In this context, it is conceivable that the basic function of P-450 monooxygenase system is to convert the lypophylic xenobiotic compounds to more polar metabolites which in turn can be excreted from the body. However, the situation is by no means simple, since a number of cases have been reported in which xenobiotics are

converted to more hazardous metabolites.[36,37] For example, halogenated benzenes are converted to their arene oxides, which bind to cellular macromolecules causing cell damage, and polycyclic hydrocarbons are converted to true carcinogens (see Figure II.5).[38] Hence, it appears that



(A) (B)

Figure II.5 Benzopyrene (A) and its carcinogenic metabolite 7,8-diol-9,10-epoxide (B).

nature has failed to a certain extent in designing these enzyme systems by allowing a wider substrate specificity and thus causing the biotransformation of harmless chemicals to potential carcinogens.

The wide distribution of cytochrome P-450-containing monooxygenase systems from mammals to bacteria represents an interesting example of how this enzyme system evolved when the surroundings were changed. All components of the bacterial monooxygenase system exist in a 'primitive' soluble form and the more developed mitochondrial system consists of a soluble reductase component and a membrane-bound P-450; in the microsomes, all components are tightly membrane-bound and hence have acquired enough hydrophobicity to become incorporated into biomembranes.

II.3 Structural Considerations

It is of immense importance to elucidate the structure of the cytochrome P-450 enzymes in order to fully understand the mechanisms of oxygenation and dioxygen reduction so that the biological reactions can be mimicked. In this discussion of the structural aspects of cytochrome P-450, attention will be focused mainly on the bacterial P-450cam enzyme, since it is the most studied and most understood.

The heme prosthetic group in P-450 is bound to the polypeptide chain via an acid labile, non covalent linkage and hence the system is classified as a b-type cytochrome.[39] The cytochrome P-450 is composed of a single polypeptide chain and a single molecule of protoporphyrinatoiron(III) per molecule of protein.[39,40]

The immediate environment of the heme group attracted the attention of many investigators in late 1960's because of the abnormal spectral characteristics observed in substrate binding and CO-complexation. Since then, enough direct and compelling evidence has been gathered to indicate the presence of a sulfur atom in the first coordination sphere of the heme-iron, and the iron sulfur distance has been shown to be consistent with thiolate (RS^-) ligation in all four states of the enzyme.[41] In 1968, Miyake and Galor[42] were able to show using EPR experiments that the substrate-free ferric enzyme is predominantly in low spin (S=1/2) form. This observation was similarly found

in alkyl mercaptide complexes of ferric hemoglobin or ferric myoglobin.[43,44] The model studies of Chang and Dolphin[45] and others [46,47] were able to mimic the spectrum of carbonylated P-450 using simple iron(II)-porphyrins having mercaptide ion and CO ligation. Also the models showed a high energy, low intensity Soret band around 360 nm in the CO-complex which was not observed in the case of the enzyme CO-complex, due to the use of excess sodium dithionite reductant which obscured the absorptions below 380 nm. However, stoichiometrically reduced enzyme, when complexed with CO, gave the second Soret peak at 365 nm. The occurrence of split Soret bands, or "hyper spectra", are characteristic of iron porphyrins coordinated by ligands which can donate high electron density.[48] The measurement of changes in the proton concentration during the binding of CO,O_2 and exchange of O_2 by CO, indicated that the Fe-S linkage remained intact during the reactions.[49] Recent direct evidence from Resonance Raman (RR) spectroscopy[50] and extended X-ray absorption fine structure (EXAFS)[41] spectroscopy has confirmed thiolate ligation to heme-iron.

As in the case of other low spin ferric hemoproteins, the substrate-free cytochrome P-450 is believed to have a sixth ligand coordinated. A nitrogen or oxygen based residue has been proposed as the best candidate.[50] Although the problem still remains after extensive investigations, a water ligand has been implicated from the NMR relaxation

rate of water protons[51] and electron nuclear double resonance (ENDOR)[52] studies.

The amino acid sequence of cytochrome P-450cam consists of 412 amino acid residues with a calculated molecular weight of 46,820 including heme. Of these residues, 210 are hydrophobic, 98 are polar neutral, and 104 are ionic (54 acidic and 50 basic) amino acids.[53] There are eight cysteine residues with four in the NH_2-terminal half and four in the COOH-terminal half, and all of them are situated in the interior of the polypeptide chain.[53] The above data predict that the conformation of the P-450cam molecule consists of 46% α-helix, 16% β- pleted sheet, 21 β-turns and 38% random coil structure.[53]

Based on the nature and the number of amino acid residues present, it is conceivable that the prosthetic group of the enzyme is surrounded mainly by hydrophobic, non-aromatic amino acids. The hydrophobic pocket thus formed would have a cavity large enough for substrate binding and would also prohibit any amino acid group being close to the site of formation of the extremely powerful oxidizing agent. The three dimensional shape of P-450 molecule, which is under investigation in the laboratory of I.C.Gunsalus, has been described as a "donut with a tail".[54]

II.4 The Mechanism of Catalysis

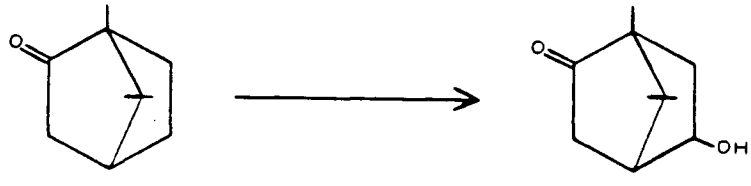
II.4.1 Types of Oxidation Reactions

Although the major interest lies in cytochrome P-450 enzymes that activate rather inert C-H bonds, the diversity of substrates and the variety of transformations that the enzyme can execute deserves a brief discussion. The mechanistic cycle will be described with particular reference to C-H hydroxylating systems, although with minor modifications it can be applied to other types of reactions as well.

Guengerich and MacDonald[55] classify the oxidative reactions of cytochrome P-450 into six categories.

(1). Carbon hydroxylation: The formation of an alcohol at a methyl, methylene or methine position.

Eg.



(2). Heteroatom release: The oxidative cleavage of the heteroatomic portion of a molecule. The molecule is hydroxylated adjacent to the heteroatom and the geminal hydroxy heteroatom intermediate thus formed loses the heteroatom to form a carbonyl compound.

Eg.

$$CH_3CH_2CH_2CH_2Br \longrightarrow CH_3CH_2CH_2CHBr(OH) \longrightarrow CH_3CH_2CH_2CHO$$

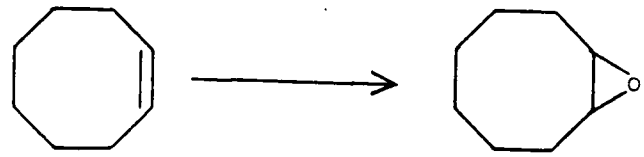
(3). Heteroatom oxygenation: Conversion of a heteroatom- containing substrate to its corresponding heteroatom oxide.

Eg.

$$Me_3N \longrightarrow Me_3N{=}O$$

(4). Epoxidation: Formation of an oxirane derivative from olefins and aromatic compounds.

Eg.



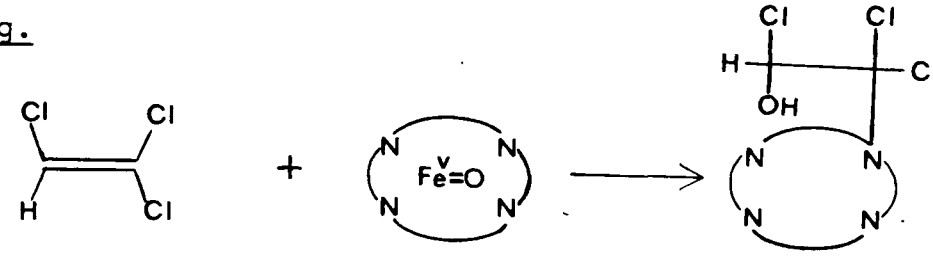
(5). Oxidative group transfer: 1,2-Carbon shift of a group with concomitant incorporation of oxygen as a carbonyl at C-1 position.

Eg.

$$Cl_2C{=}CHCl \longrightarrow CCl_3CHO$$

(6). Olefinic suicide destruction: Inactivation of the heme of cytochrome P-450 by an enzyme product or an enzyme intermediate.

Eg.



The common feature in all of these reactions is that one atom of oxygen, from the reductive scission of dioxygen, is being inserted into the substrate while the other is reduced to a water molecule. After consideration

of the observed stoichiometry, the results of labelled-oxygen experiments,the regioselectivity of the hydroxylation, and the number of electrons required from the reductase , a scheme has been constructed to illustrate the various steps of the oxygenase reaction cycle (Figure II.6).[56]

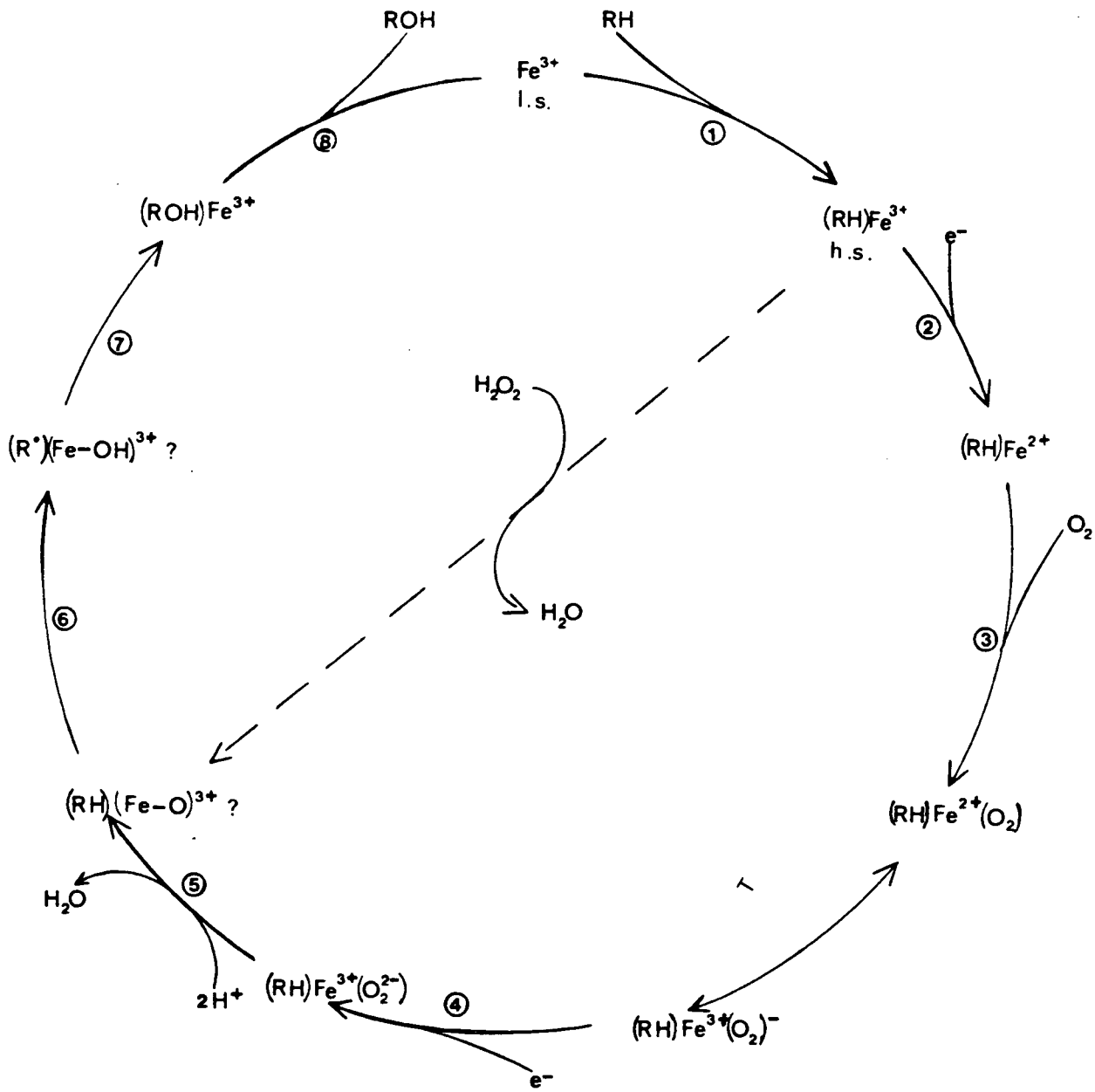


Figure II.6 The P-450 catalytic cycle.

II.4.2 The Catalytic Cycle

As in any enzymatic catalysis, the first step in the reaction cycle is the formation of an enzyme-substrate complex. This is followed by reduction of iron(III) to iron(II) which allows for dioxygen binding. A second reduction, that induces the splitting of dioxygen, forms the "active oxygen complex" and a molecule of water. Then the 'active oxygen' is inserted into a carbon-hydrogen bond, forming the alcohol product, which in turn is released as the enzyme is returned to the original ferric state.

II.4.2.1 Binding of Substrate

The associated change in the conformation due to the binding of the substrate to the enzyme causes the spin state of the iron(III) to shift from low spin (S=1/2), to high spin,(S=5/2).[19] This change has been observed by EPR spectroscopy[19] and by the measurement of the magnetic susceptibility.[18] The binding process proceeds at a high rate with a second order rate constant of $3.7\times10^{6}M^{-1}s^{-1}$ at 8^{o}.[57] The equilibrium constant in the presence of potassium ions has been estimated to be $4.7\times10^{5}M^{-1}$.[18] The main bonding forces are believed to be hydrophobic in nature, although there may be some dipole interactions between the substrate and the enzyme. This type of bonding is largely responsible for the observed increase in entropy on transfer of the lipophilic substrate from the aqueous phase into the hydrophobic environment of the active center.[58]

Although the precise location of the substrate is still unknown, inhibition experiments show the bonding site of camphor to be in the immediate vicinity of the oxygen bonding site.[59] The process of substrate binding could proceed as indicated in Figure II.7.

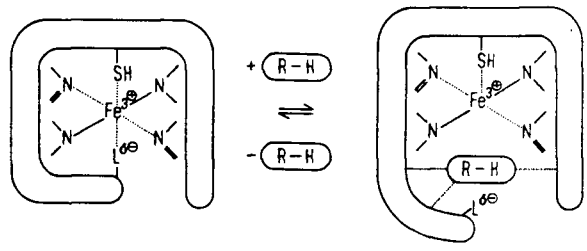


Figure II.7 Hypothetical scheme for the binding of substrates to cytochrome P-450. (from ref. 58).

The most significant change upon substrate binding is a shift in redox potential of the heme iron. The substrate-free cytochrome (low spin) has a reduction potential (E^0) of -270 mV while that of substrate-bound enzyme (high spin) is -170 mV.[20] Since the E^0 of putidaredoxin is -240 mV, the spin change in P-450 facilitates reduction by putidaredoxin, thereby enabling the successive binding of dioxygen.

II.4.2.2 First Reduction

The next step in the reaction cycle is the transfer of an electron to the cytochrome. Despite the considerable effort devoted to study the nature of this reaction, some questions still remain unanswered. Stopped-flow spectrophotometric studies[60] indicate this reduction to be

first order biphasic, but no rationale for this behavior has been presented. The rate constants calculated[60] indicate this step is not rate-limiting. Although the exact mechanism of electron channeling from putidaredoxin to the iron(III) center is not known, the putidaredoxin is thought to bind to a highly positively charged region of the P-450 protein, between the 340 and 412 positions from the NH_2 terminal end.

II.4.2.3 Binding of Dioxygen

The association reaction of the reduced substrate-bound enzyme with dioxygen, to form the ferrous-dioxygen complex, has been well established, owing to the fact that the complex is fairly stable at low temperatures.[27,61] The formation of oxy-cytochrome P-450cam, as determined from the stopped-flow technique, is first-order with respect to both dioxygen and the reduced enzyme, the second-order rate constant being 7.7×10^5 $M^{-1}s^{-1}$ at 4° and pH 7.4.[62] The dioxygen complex thus formed slowly decays even at low temperatures to give the oxidized enzyme. This process, known as the "autoxidation", and the structure and stability of dioxygen adduct, will be discussed in detail in Section II.6. The substrate-free enzyme-dioxygen complex undergoes the autoxidation reaction 10^2 times faster than the substrate-bound enzyme-dioxygen complex.[30] This indicates that the oxy-cytochrome P-450cam formed in the presence of camphor is not a simple adduct of dioxygen but a ternary complex of dioxygen,enzyme and camphor.

II.4.2.4 Second Reduction

The ability to by-pass the second, third and fourth steps (Figure II.6) of the enzymatic cycle by adding hydrogen peroxide to oxidized enzyme, and still obtain the hydroxylated substrate without any external electron supply, suggests that the nature of the two-electron reduced enzyme-oxo complex to be $Fe(III)-O_2^{-2}$ in nature.[63] In fact, the model studies indicate that the structure of this species may be high-spin ferric η^2-peroxide.[64,65] Unfortunately, the model Fe(III)-peroxo complexes, produced either synthetically [64] or electrochemically,[65] do not exibit the oxidizing power of the enzyme system possibly because of the absence of thiolate ligand.[65]

The rate of the second reduction agrees well with observed turn-over number for camphor hydroxylation, and hence appears to be the rate determining step in the catalytic cycle.[66,67]

II.4.2.5 Splitting of the Oxygen-Oxygen Bond

The step, where the bond between the two oxygen atoms is cleaved heterolytically, is least understood of the entire mechanistic scheme and has been the subject of much speculation. The conversion of the iron-peroxide complex to the "active oxygen" intermediate involves protonation, followed by heterolytic cleavage of the O-O bond with the production of a molecule of water. The remaining oxygen atom stays coordinated to the heme-iron. The overall charge on this two-atom unit, neglecting the contributions of the

porphyrin and thiolate, becomes +3, leading to designations such as $[Fe(III)-O]^{3+}$, or $[Fe(IV)-O^{-}]^{3+}$, or $[Fe(V)-O^{2-}]^{3+}$. Evidence has been gathered to demonstrate the occurrence of this "oxenoid" type intermediate by using ferric porphyrins with single oxygen atom donors like iodosylbenzene ,C_6H_5I-O [68] and peracids [27,69] to effect hydroxylations similar to that of the native P-450,NADPH,O_2 system.

It has been suggested that the presence of thiolate ligand may facilitate the splitting of the peroxide moiety by weakening it through charge repulsion by an electron-rich iron atom.[70] But no definitive explanation has been given to the fact that thiolate is not a required ligand for catalase and peroxidase, which undergo similar peroxy-iron intermediates in their reaction cycles.

II.4.2.6 Oxidation of Substrate

Since cytochrome P-450 is the only hemoprotein capable of hydroxylating an alkane at an unactivated C-H bond, the electrophilic iron-oxenoid species and its reaction with substrate are especially interesting.

If the cleavage of a C-H bond is a necessary step in the hydroxylation reaction, then a substrate free-radical would be the lowest energy intermediate that could exist in the strongly hydrophobic active site; any ionic species would be energetically unfavourable. Indeed, isotope effects, and regio- and stereo-selectivity of these reactions, all point towards such a mechanism.[27,71,72] Two elegant examples, that account for the existence of an

uncharged substrate intermediate have been discussed by Groves et al.[73] and Dolphin et al.[74]

A plausible mechanism of formation and hydroxylation of the substrate radical is outlined in Figure II.8.[71]

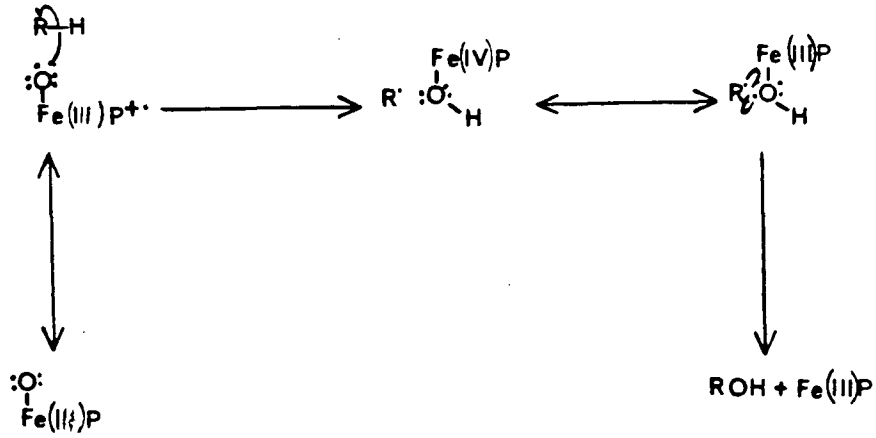


Figure II.8 Proposed mechanism for the formation and hydroxylation of substrate (RH) radical.

The abstraction of a hydrogen atom from substrate RH by the active iron-oxene complex forms an Fe(IV) hydroxide species and a substrate free-radical, $R^{\bullet}$; this in turn accepts the iron-bound hydroxy radical to form R-OH and regenerates Fe(III) enzyme ready for the next reaction cycle.

II.4.2.7 Dissociation of Product

The hydroxylated product apparently dissociates before a new cycle begins, since diols are not observed in alkane hydroxylations. The hydroxyl group of the product alcohol may be coordinated to iron, but this would make the reduction of the iron(III) center more difficult and also, even if the iron is reduced, would prohibit O_2 binding.

Thermodynamically, the binding of a polar molecule will be less favourable and another substrate molecule that is in abundance in the surroundings will be preferred; the high spin Fe(III)-substrate complex formed yields the way for another reaction cycle.

II.5 Electronic Spectroscopy

Electronic absorption spectroscopy has been used extensively to study the cytochrome P-450 system in relation to molecular structure, geometry, electronic configuration and oxidation state. Other spectroscopic techniques such as electron paramagnetic resonance (EPR), Mossbauer, circular dichroism (CD), magnetic circular dichroism (MCD), nuclear magnetic resonance (NMR) and resonance Raman (RR) have been used to confirm unambiguously the results obtained from electronic spectroscopy. Also, heavy reliance has been placed on comparisons with other known hemoproteins and low molecular weight iron-porphyrin complexes.

The P-450 hemoproteins from all known sources are remarkably similar in electronic absorption spectral properties.[27] The substrate-free ,ferric ("resting") state of the enzyme is typical of low spin ferric hemeproteins, with a Soret band at 416 nm and well defined α and β bands. Upon substrate binding, the hexacoordinate low spin form changes to pentacoordinate high spin form with a shift of the Soret band to 391 nm and disappearance of distinct bands in 500-600 nm region; however, a new low energy,low intensity band at 643 nm is formed. On reducing the substrate-free species in the absence of potential ligands such as CO or O_2, the Soret band moves to 408 nm and the α and β bands collapse forming a new peak at 542 nm. However, the most striking feature is observed when the reduced

enzyme is exposed to a CO atmosphere when a sharp, intense peak appears at 446 nm, an unusually high wavelength for a hemoprotein. The low energy band of the CO complex is observed at 540 nm with about the same intensity as the peak of the reduced species at 542 nm. The spectrum of the dioxygen complex is comprised of a Soret band at 418 nm and a low energy band at 552 nm. The small separation between Soret peaks of the reduced (408 nm) and O_2-complex (418 nm) created difficulties in studying the dioxygen binding as a function of gas pressure in efforts to determine the equilibrium constant (see Chapter IV).

Normal porphyrin spectra show three sets of bands classified according to orbital symmetry considerations. The bands appear in the visible, Soret and near UV regions.[48] These peaks are due to the well characterized π-π^* transitions. But some porphyrin-metal complexes show "hyper" spectra, with strong extra bands in the 300-800 nm region, a common spectral pattern exibited by a number of hyper porphyrins bearing two Soret bands (split Soret), one in the 380 nm region and the other in the 440-480 nm region. The CO and O_2 complexes of reduced P-450 species also exibit this feature. As demonstrated by the model studies[45,47] the presence of thiolate in an axial coordination position is required to mimic the P-450 spectrum, including the split Soret bands. Hansen et al.[48] using iterative extended Huckel (IEH) calculations were able to show that the split Soret bands arise from the

strong interaction of charge transfer transitions (from thiolate p- orbitals to porphyrin π^*-orbitals) with the porphyrin π-π^* transitions.

II.6 Interaction of Cytochrome P-450 with Carbon Monoxide and Dioxygen

The observation that the addition of carbon monoxide to sodium dithionite-treated liver microsomes resulted in an unusual Soret peak led to the discovery of cytochrome P-450 (see Section II.1) . Also, the biological function of this monooxygenase enzyme was determined by a study of inhibition of C-21-hydroxylase reaction of adrenal cortex microsomes via photochemical action spectrum of the CO complex.[3] Since these findings, many studies on CO and O_2 binding to reduced, native and reconstituted P-450 enzyme and to its model compounds have been reported.[61,75-80] The information gathered from such studies will be discussed and compared to the other heme-containing biomolecules such as hemoglobin (Hb), and myoglobin (Mb), which have been thoroughly studied and are well understood.

Although Hb and Mb contain the same prosthetic group as P-450, protohemin, they differ in many ways in their structure and properties. The difference in axial ligation (see Figure II.9) must play an important role.[81] It is interesting to note that Hb and Mb do not undergo a change in the oxidation state of the prosthetic groups in any stage of their normal biological functions.

In the case of P-450 and Mb, where only one heme unit per molecule interacts, the reaction of CO with the reduced heme unit is represented by reaction II.2,

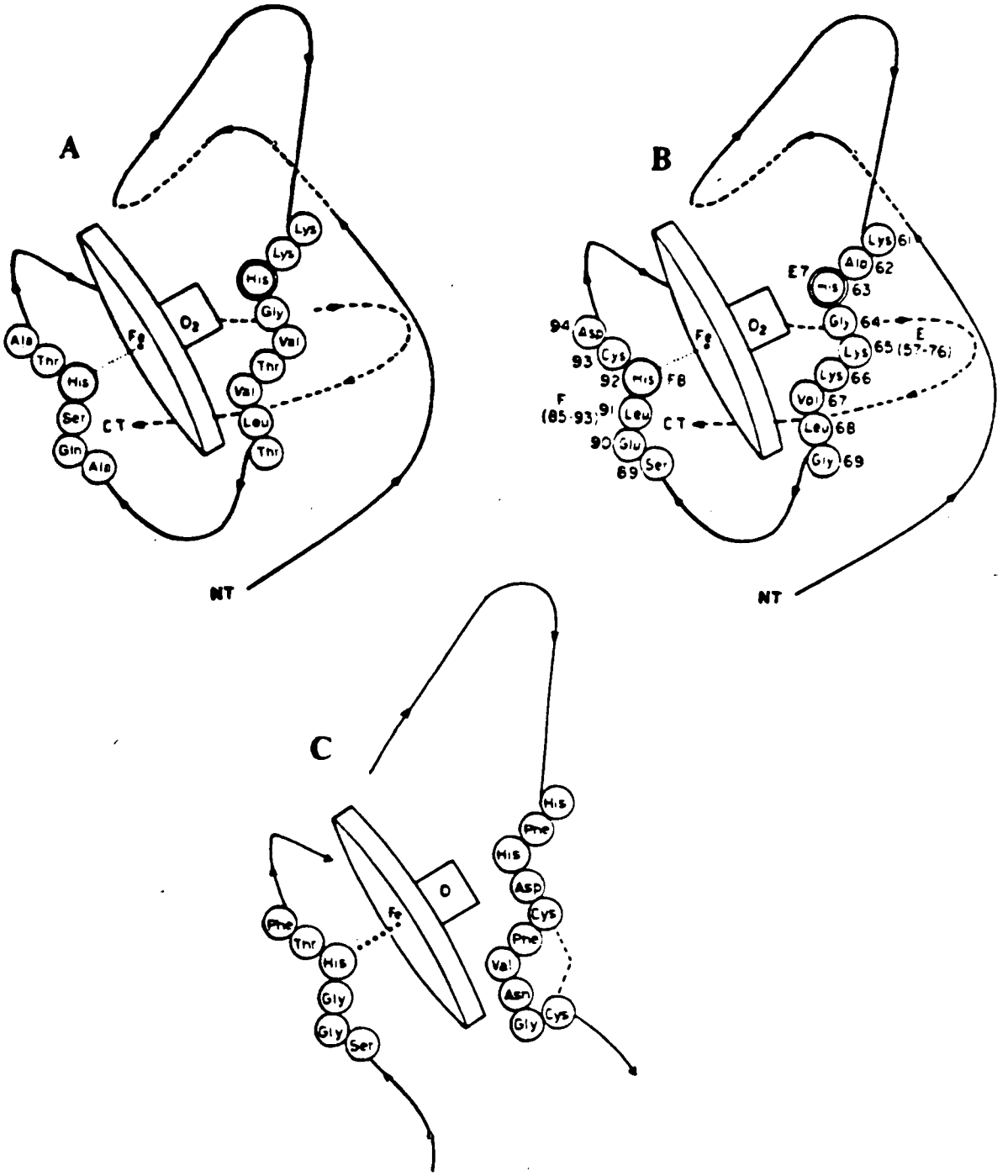


Figure II.9 Heme environments of myoglobin (A), hemoglobin (B), and peroxidase (C). (from ref. 81).

$$Fe(II)+CO \rightleftharpoons Fe(II)-CO, \quad K_{co}=k_{on}/k_{off} \quad (II.2)$$

where Fe(II) is the reduced heme unit, K_{co} is the equilibrium constant, and k_{on} and k_{off} are association and dissociation rate constants, respectively. The ligand association reaction is an over all second order process, while the dissociation reaction is first order.[82,83] The kinetic and equilibrium data for CO binding to P-450 and related systems are given in Tables II.1 and II.2, respectively.

Table II.1

Kinetic Data for CO Binding to Cytochrome P-450 and Related Systems.

System	Method	pH	T°C	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	Ref.
P-450cam	SF	7.4	4	5.1×10^{6}	2.3	77
(cam.-free)	FP	7.4	5	2.7×10^{6}	1.2	82
			15	4.7×10^{6}		
			25	8.4×10^{6}		
P-450cam	SF	7.4	4	3.8×10^{4}		77
(cam.-bound)	SF	7.0	20	3.6×10^{5}	2.8	84
	SF	7.0	24	2.2×10^{5}	1.7	75
	FP	7.0	5	8×10^{4}		82
			15	1.3×10^{5}		
			25	2.3×10^{5}		
	FP	7.3	12	3.5×10^{4}		85
Microsomal	FP	7.4	4	3.4×10^{5}	0.068	13
P-450	SF	7.5	4	4.5×10^{5}	0.63	88
Horse Mb	FP	7.0	20	5×10^{5}	0.02	83
	FP	7.0	25	3.8×10^{5}		86
P-450 Model	FP		23	1.1×10^{5}	18	87

SF=stopped-flow, FP=flash photolysis

Table II.2

Equilibrium Data for CO Binding to Cytochrome P-450 and Related Systems.

System	pH	T°C	K_{CO} (M^{-1})	ΔH° (kcal /mol)	ΔS° (cal /mol.deg)	Ref.
P-450cam	7.4	4	2.2×10^{6}			77
(cam.-free)	7.0	24	1.7×10^{5}			75
P-450cam	7.4	4	2.6×10^{5}			77
(cam.-bound)	7.0	12	2.6×10^{5}	-12	-17	75
	7.0	24	1.3×10^{5}			75
P-450 Model		23	1.1×10^{4}	-16	-35	87
Horse Mb	7.0	20	2.9×10^{7}	-12.6	-7.4	83

The CO and O_2 complexes of P-450, Hb and Mb are diamagnetic.[89] The metal-carbon bond-order derived from the relationship with the C-O stretching frequency indicates that carbon monoxide is involved in both σ and π-type molecular orbitals of the complex.[83] The sp-hybridized lone pair of the carbon atom is involved in $O{\equiv}C \rightarrow Fe$ type bonding, while the two $p\pi^{*}$ orbitals overlap with two $d\pi$ orbitals of iron. The Pauling valence bond structures of the CO complex are given in Figure II.10.

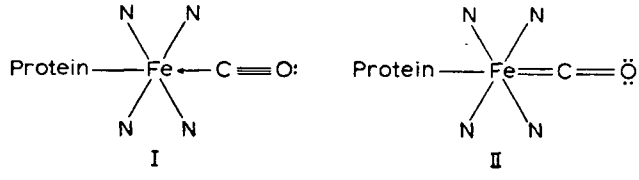


Figure II.10 Pauling valence bond structures of carbon monoxide complex of cytochrome P-450.

Although CO is thought to bind in a linear fashion to transition metals , it can be attached at an angle in a heme protein to minimize the steric hindrance . In the chironomous hemoglobin CO complex the Fe-C-O angle is $145 \pm 15^{\circ}$.[83] This observation provides a basis for the explanation of the lower affinity of substrate-bound P-450 towards CO, compared to the substrate-free system (see Table II.2). The camphor molecule bound in the vicinity of the heme moiety[77] is considered not only to restrict the access of the CO molecule to the heme unit, but also to have a destabilizing effect on the heme-CO complex once it is formed. However, at 24°, the equilibrium constant value for the substrate-free system, reported by Dolphin et al[75],

approaches that for the substrate-bound system. In order to understand better the effect of the substrate on CO binding to P-450, the necessity of more studies has been mentioned.[87,90] More detailed interpretation of the substrate effect on CO binding can be provided by evaluation of the enthalpy and entropy contributions to the binding constant values at different temperatures.

The binding of CO to substrate-bound P-450 is about 200 times weaker than that for Mb (see Table II.2). This difference in affinity towards ligand binding probably reflects the effect of the proximal ligand, imidazole in Mb, and thiolate in P-450. The thiolate anion coordinated to heme may produce an electron-rich iron atom, thereby lowering the affinity for the sixth ligand.[91] Since imidazole is a weaker σ-donor than thiolate, this could account for the difference in binding constants.

The thermodynamic parameters, calculated from the limited data available [75] for the substrate-bound system (Table II.2), indicate that the ΔH^{o} value is comparable in magnitude to that of Mb (-12 kcal/mol for P-450; -12.6 kcal/mol for Mb). Binding of a gas molecule to a metal centre would be unfavourable in entropy terms due to the loss of translational and rotational motion of the gas molecule. But the entropy of binding of CO to P-450 is more unfavourable (ΔS^{o}=-17 cal/mol.deg.) than for Mb; (ΔS^{o}=-7.4 cal/mol.deg.). Such differences in entropy contributions have been rationalized, based on cis- and trans- effects

and steric effects due to the presence of protein as well as the substrate.[75,77] The ΔH^0 term for the protein free model system (-16 kcal/mol) is more favourable, but the entropy contribution is not so (-35 cal/mol.deg.).

The kinetic and equilibrium data available to date for dioxygen binding to cytochrome P-450 are not as extensive as those for CO binding to P-450, or O_2 binding to Mb. The principal reason for this is the instability of the P-450-O_2 complex at ambient temperatures; in the case of substrate-bound enzyme at room temperature and pH 7.0 the autoxidation (Reaction II.3) occurs with a half-life < 1 minute,[92] while the rate of autoxidation of the dioxygen complex of the substrate-free enzyme is at least 100 times faster.[75]

$$\text{P-450Fe(II)-O}_2 + \text{H}^+ \longrightarrow \text{P-450Fe(III)} + \text{HO}_2 \qquad \text{(II.3)}$$

The binding of dioxygen to substrate bound P-450 is at least 6 times weaker than to Mb (Table II.3). This results mainly from a much slower on-rate in the P-450 system; this could result from a geometrically restricted coordination site due to the presence of the substrate,[75] although the electronic effects due to the sixth ligand may also play a role.

Table II.3

Equilibrium and Kinetic Data for Reversible O_2 Binding to Substrate-Bound Cytochrome P-450[75] and Mb.[93]

System	$P_{1/2}$ (mmHg)	Binding Constant (M^{-1})	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
P-450	2.5 (0°C)	2.2×10^{5} (0°C)	7.7×10^{5} (4°C)	3.5 (4°C)
Horse Mb	.52(20°C)	1.3×10^{6} (20°C)	1.4×10^{7}(20°C)	10.(20°C)

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CHAPTER III

EXPERIMENTAL PROCEDURES

III.1 Growth of Bacteria

III.1.1 General Information

The bacteria Pseudomonas putida strain PpG 786 (ATTC 29607) derived from strain PpG 1 (ATTC 17453) by Gunsalus and Wagner[1] was used throughout these studies to produce cytochrome P-450. The parent strain PpG 1 was originally isolated from soil by enrichment on D-(+)-camphor. The strain was kept alive by bi-weekly transfer to minimal agar plates containing D-(+)-camphor as carbon and energy source.

The four-stage growth procedure for the mass production of bacteria as described by Gunsalus and Wagner[1] was used with several minor modifications. This growth procedure is designed to produce maximum amounts of cytochrome P-450 and other monooxygenase components at the expense of camphor and liquid media and has a relatively short period of growth with a generation time of about three hours. The strain PpG 786 has the ability to release much of the hydroxylase proteins by freeze-thaw autolysis to circumvent more tedious cell breakage techniques. A single freeze-thaw cycle typically liberates about one half of the cytochrome P-450 content as soluble enzyme in the cell-free extract, based on the total amount of enzyme in the whole cell suspension as determined from the ferrous carbon monoxide difference spectrum.

The four stage growth procedure,

1. Minimal agar stage

2. L-Broth stage

3. 500 mL Shake flask stage

4. 14 L Fermenter stage

typically yielded about 500 g of bacteria based on the wet weight in about 96 hours of elapsed time.

All inoculations except for 14 L fermenters were carried out in a laminar flow clean-air station to minimize the possibility of contamination. Three agar plates were inoculated each time and the wire loop used to transfer the bacteria between plates was heated until red hot before each use, in the flame of a Bunsen burner. The L-broth and the shake flasks were inoculated and incubated in a platform shaker thermostated to 30°. Three 14 L fermenters (Labroferm; New Brunswick Scientific, N.J, U.S.A) were used in the final stage of growth. The growth medium was made up to 12 L and stirred at 300 rpm with 15 L/min rate of aeration. The temperature was maintained at 30°. The rate of growth of bacteria was determined turbidometrically by measuring the optical density at 660 nm using a Bausch and Lomb spectrometer model Spectronic 20. During the growth of bacteria in 500 mL shake flask and 14 L fermenter stages, the fluctuation of pH was monitored using a Fisher Accumet model 210 pH-meter equipped with a glass pH-electrode. When the optical density of the medium reached a maximum value, the bacteria were collected using a continuous flow centrifuge (Carl Padberg, Schnell-Zentrifuge, Model LE) at 37, 000 rpm. The bacteria were then stored under dry-ice

temperature in a Dewar in 500 g batches.

III.1.2 Media

Media for all four stages were prepared according to Gunsalus and Wagner[1] using reagent grade chemicals and freshly distilled water. All solutions were sterilized at 121° under pressurized steam using an autoclave (American Sterilizer Co. Model AS-DTT616GE). Solutions of volumes less than 200 mL required 20 min autoclaving while 14 L fermenters required 45 min to ensure complete sterilization. The sterilized solutions were used as early as possible to avoid re-contamination.

III.1.2.1 Minimal Agar

In order to store the bacteria alive, minimal agar plates were prepared using the following ingredients.

K_2HPO_4	3.5 g
KH_2PO_4	1.0 g
NaCl	0.06 g
$MgSO_4.7H_2O$	0.05 g
$(NH_4)_2SO_4$	0.5 g
D-(+)-camphor	0.68 g
Bacto agar	7.5 g
Water	540 mL

The salts, camphor and agar were dissolved in water, sterilized, cooled, combined and poured into disposable petri dishes obtained from Canlab. The minimal agar dishes

were stored at 5°, three of which were used every two weeks to inoculate with the bacteria.

III.1.2.2 L-Broth

At the initial stage of large scale growth of bacteria, a very rich medium called L-broth[2] was used to facilitate the rapid multiplication of the organism. The following ingredients were mixed, poured into five 50 mL Erlenmeyer flasks and sterilized.

Bactotriptone	1.0 g
Yeast extract	0.5 g
NaCl	0.5 g
Glucose	0.1 g
Water	100 mL

This solution was brought to pH 7.0 by adding 1M NaOH dropwise before the sterilization.

III.1.2.3. 500 mL Shake Flask

At this stage of growth a phosphate-ammonium (PA) buffer solution was used to maintain the desired pH level. Essential mineral salts were supplied from a hundred-fold strong stock solution (100-X salts) and a carbon and energy source was also added to the medium. In order to maximize the production of cytochrome P-450 content, the 500 mL shake flask stage was performed twice , first with glutamic acid as the carbon and energy source and secondly with camphor as the only carbon and energy source. In each case 1 L of medium was prepared using the following ingredients and divided into five 500 mL Erlenmeyer flasks and

sterilized.

PA buffer ..... 1 L

100-X salts .....16 mL

Glutamate .....2.9 g or

Camphor .....2.5 g

PA buffer and 100-X salts were prepared by mixing following ingredients.

PA buffer:

K_2HPO_4 .....10.7 g

KH_2PO_4 ..... 3.1 g

NH_4Cl ..... 4.0 g

Water ..... 1 L

100-X salts:

$MgSO_4.7H_2O$ .....19.5 g

$MnSO_4.H_2O$ ..... 5.0 g

$FeSO_4.7H_2O$ ..... 5.0 g

$CaCl_2.2H_2O$ ..... 0.3 g

L-Ascorbic acid ..... 1.0 g

Water ..... 1 L

III.1.2.4 14 L Fermenter

The final stage of growth was carried out in a somewhat similar medium to the 500 mL shake flask stage, but it was essential to keep the medium always saturated with camphor during the whole period of growth. The following chemicals were added in each fermenter.

K_2HPO_4	128.2 g
KH_2PO_4	36.3 g
NH_4Cl	46.8 g
100-X salts	120 mL
Bacto yeast extract	3.0 g
Antifoam	1.0 mL
Camphor in DMF (3M)	30 mL
Water	11.6 L

III.1.3 Growth Procedure

Four of the five Erlenmeyer flasks, each containing 20 mL of L-broth medium, were inoculated with the bacteria and shaken at 30° for 10 hours. The fifth flask served as a reference. At the end of the 10 hour period it was noted that the flasks that were inoculated turned turbid, while the reference flask remained clear. On swirling the inoculated flasks, clouds of bacteria could be seen.

The third stage of growth was started by inoculating each of the first set of four 500 mL shake flasks with 5 mL of L-broth culture. The fifth flask served as a reference. Each of these flasks contained 200 mL of basal PA buffer , 3.2 mL of 100-X salts and 10 mM glutamate as the carbon and energy source. All five flasks were shaken at 30° until the optical density at 660 nm (OD_{660}) reached 0.3. At this time the induction of camphor 5-monooxygenase system was initiated by the addition of stock camphor solution (3M in DMF) to a final concentration of 5 mM. The OD_{660} and the pH of one of the inoculated flasks and the reference flask were taken every hour in order to monitor the growth and the metabolism of camphor. Addition of camphor was continued at OD_{660} 0.7, 1.0 and once every three hours thereupon until the growth reaches its late-logrithmic phase (see Figure III.1). At this time the second set of shake flasks with camphor as the only carbon and energy source was inoculated with 10 mL each of this solution. For this inoculation the culture medium was used only from the

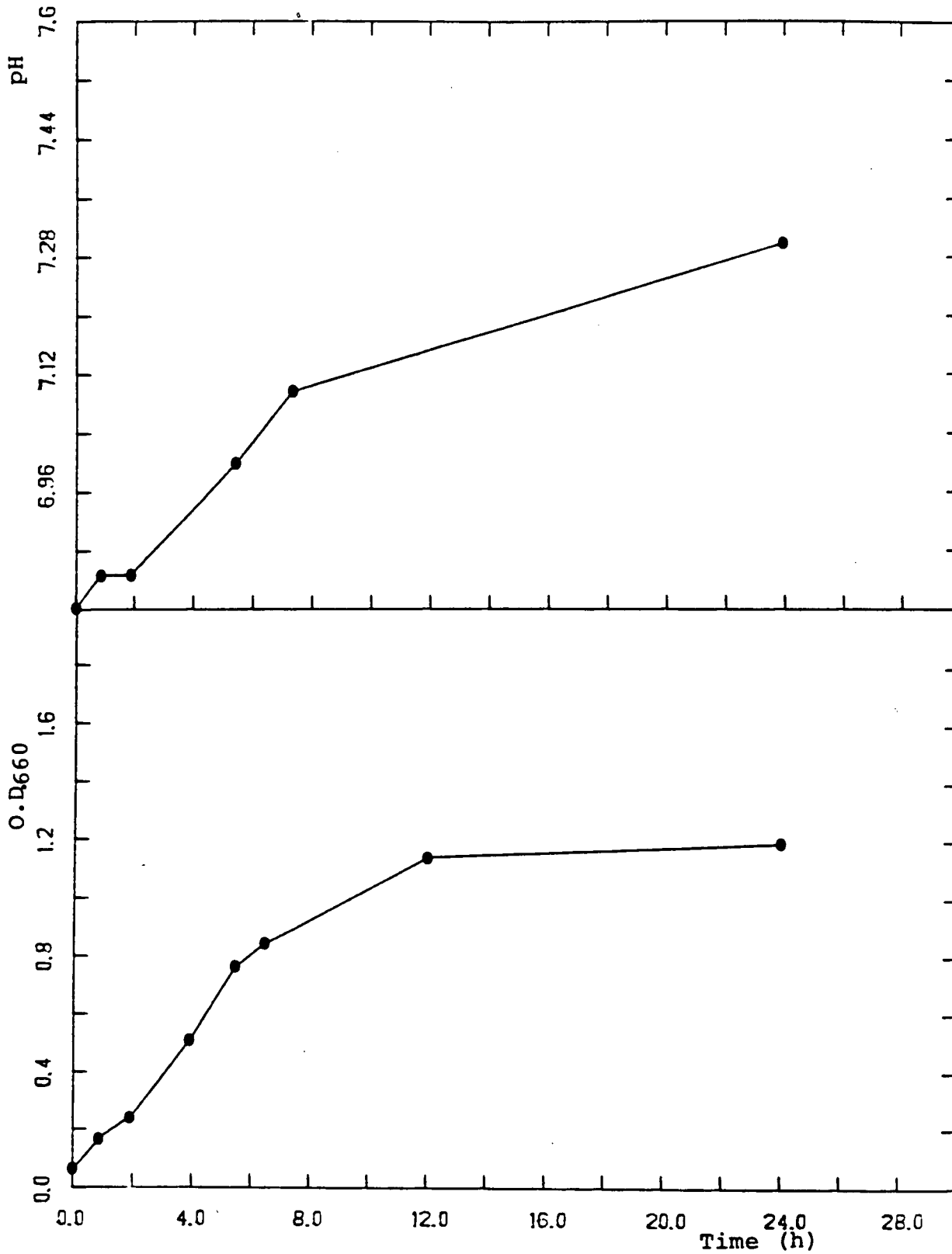

Figure III.1 $O.D_{660}$ and pH profiles for the bacterial growth in the first set of shake-flasks.

three shake flasks that were left undisturbed while shaking at 30°. The flask that was used to withdraw aliquots for pH and optical density measurements was never used as an inoculum, in order to avoid any possibility of contamination. The flasks were heated around the neck before opening, by rotating on the flame of a Bunsen burner whenever they were opened either for addition of camphor or for withdrawing aliquots. The needle of the 1 mL disposable syringe that was used to measure the camphor solution was heated to red hot before use. Pre-sterilized 2 mL Pasture pipettes were used to withdraw aliquots from the flasks.

The initial rate of growth in the presence of camphor as the only carbon and energy source was smaller compared to that in the presence of glutamate (see Figure III.2), but the optical density reached similar values after about 24 hours. When the growth reached its late-logarithmic phase (about 14 hours), the contents of the three undisturbed flasks were used to inoculate 14 L fermenters. The bacteria were allowed to grow under continuous agitation and aeration (see Section III.1.1) until the OD_{660} reached 0.6 (see Figure III.3), at which time the feeding with camphor was started. A solution of camphor in DMF (3M) as well as solid camphor was used at a rate of 15 mL/h or 6.7 g/h, respectively. The use of a solution of camphor was preferred towards late stages of growth since the solid camphor tended to clog the continuous flow centrifuge. However , the use of solid camphor helped to reduce the

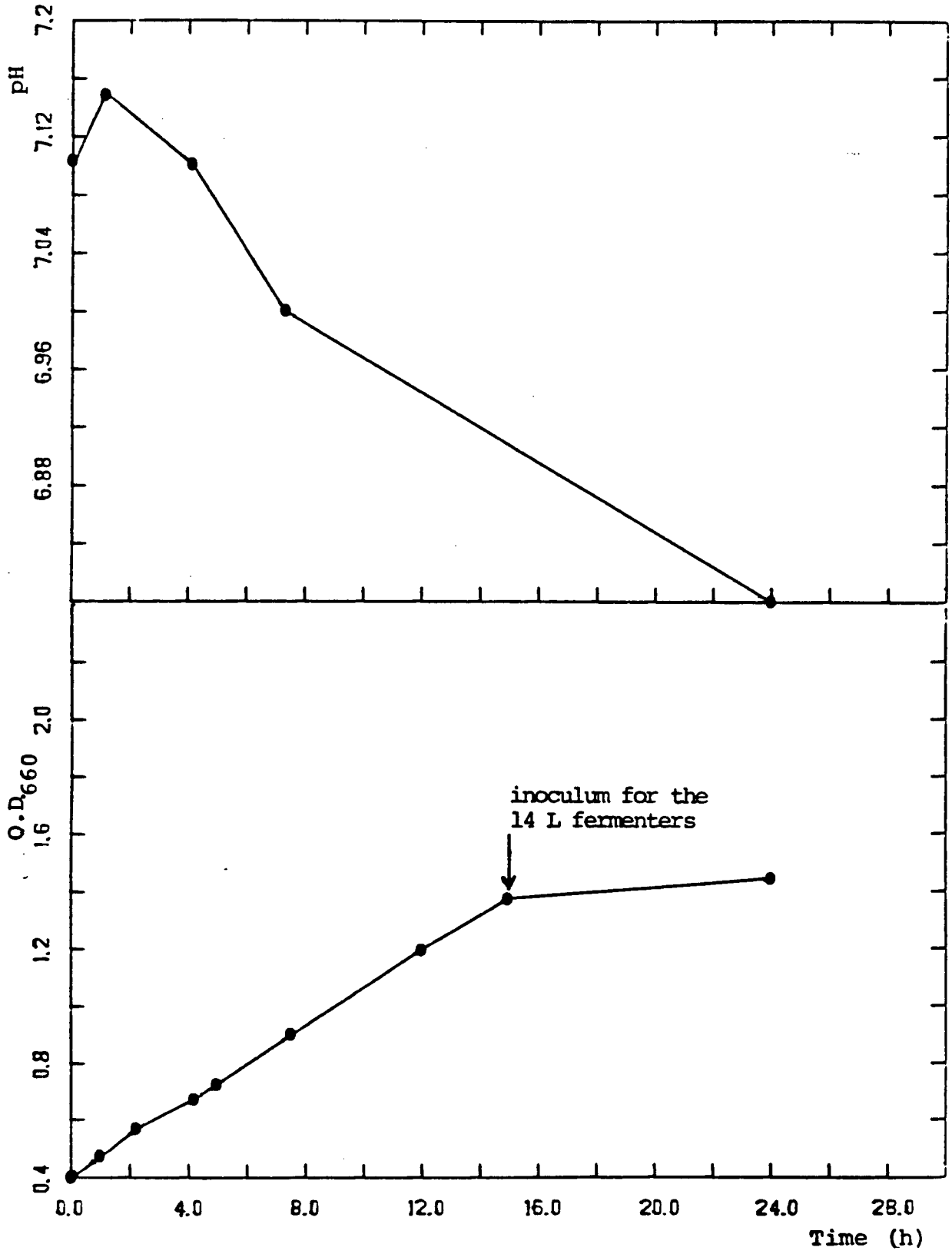

Figure III.2 $O.D_{660}$ and pH profiles for the bacterial growth in the second set of shake-flasks.

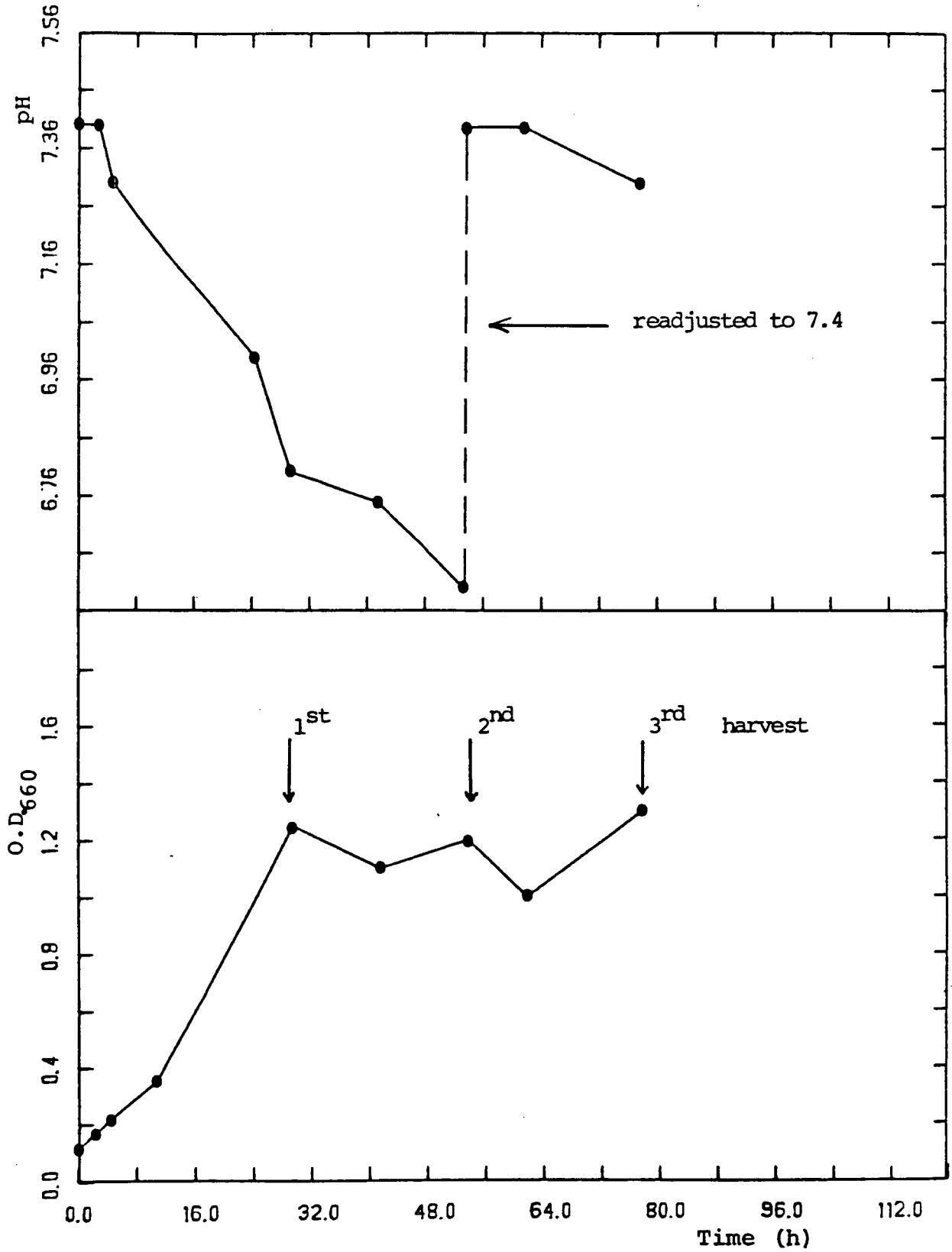

Figure III.3 $O.D_{660}$ and pH profiles for the bacterial growth in the 14 L fermenters.

amount of DMF added to the media. It is likely that an excess of DMF may interfere with the growth. The centrifugation was carried out when the OD_{660} reached about 1.2 (about 24 hours) and about 150 g of bacteria had collected. The growth was continued until the optical density again reached 1.2 (about 24 hours from last harvest), at which time the centrifugation was carried out again. The pH of the medium drops below 6.0 at this time and enough potassium hydrogen phosphate (K_2HPO_4) was added to the medium to bring the pH value to 7.4. Another 120 mL of 100-X salts was added also in order to replenish the medium. The final centrifugation was carried out again when the optical density reached 1.2. The total wet weight of the bacteria collected was 450-500 g. The total growth procedure takes about 5.5 days. The paste of bacteria was stored under dry-ice (-78°) for at least 10 days before being used to isolate cytochrome P-450.

III.2 Isolation and Purification of Cytochrome P-450

III.2.1 General Information

The freeze-thaw autolized cells of bacteria Pseudomonas putida strain 786 (see Section III.1.3) were used to isolate cytochrome P-450. The method of Gunsalus and Wagner[1] was used with some minor modifications. Basically, the isolation procedure involves removal of cell debris by centrifugation of stirred cell suspension in a buffer solution, and separating cytochrome P-450 from other monooxygenase components by a two-step anion exchange column chromatography procedure. The purification of isolated crude cytochrome P-450 was done by a two-step gel filtration column chromatography procedure. Once isolated and purified, P-450 samples can be stored frozen in liquid nitrogen (-196°) or can be kept as a liquid in the refrigerator (5°). The camphor substrate was removed by another two-step gel filtration chromatographic procedure.

III.2.2 Materials

The chemicals and sources were as follows. Diethylaminomethyl cellulose (DE 52) from Whatman Chemical Co.; Sephadex G-10 and G-100 from Pharmacia Fine Chemicals; Enzyme grade ammonium sulphate from Schwarz/Mann Chemical Co.; β-mercaptoethanol (βME), deoxyribonuclease 1 (DNase) isolated from bovine pancrease, ribonuclease-A (RNase) isolated from bovine pancrease, dithiothreitol (DTT) and

tris-(hydroxymethyl)aminomethane (Trizma Base) from Sigma Chemical Co.; potassium chloride from American Scientific Chemical Co..

III.2.3. Buffer Solutions

All buffer solutions were prepared from glass-distilled water, deaerated by repeated aspiration followed by flushing with argon. The argon was passed through a column of copper catalyst pellets (BASF Chemical Co.) heated to 50° to remove trace dioxygen, followed by drying through phosphorus pentoxide and Indicating Drierite (Hammond Drierite Co.). The Tris.Cl buffer solutions were prepared from a 1.0M stock Trizma Base (see Section III.2.2.) solution by diluting to 50 mM with distilled water followed by addition of 6M HCl to obtain a pH of 7.4. Four buffer solutions (4 L of each) were prepared with different ionic strengths by adding 0, 50, 100 and 600 mM KCl to 50 mM Tris.Cl buffer. For simplified nomenclature these buffers are specified as T, T-50, T-100, T-600, respectively. Prior to use, all the buffer solutions were made 10 mM with βME .

The phosphate buffer solutions were prepared by mixing appropriate volumes of 1 M K_2HPO_4 and 1 M KH_2PO_4 stock solutions to obtain a pH value of 7.4, when diluted to a 50 mM solution. The 50 mM phosphate buffer solution (P-buffer) was made 100 mM with KCl to produce buffer P-100. Solid camphor was added to appropriate buffers that were stirred for about 6 hours to obtain a saturated (8 mM)

solution.

The approximate volumes of buffer solutions used in a typical preparation of cytochrome P-450 from about 400 g of bacteria are listed in Table III.1. The quantities of reagents used are listed in Table III.2.

Table III.1

Volumes of Buffer Solutions Required for a Typical Isolation and Purification of Cytochrome P-450.

Step	Buffer	Volume
Cell-Free Extract	T	0.6 L
Anion Exchange Chromatography (DE52)		
(a) Equilibration of column A	T	4.0 L
(b) Equilibration of column B	T-50	4.0 L
(c) Separation of protein components	T-100	4.0 L
	T-600	4.0 L
Gel- Filtration (Sephadex G-100)		
(a) Column A	T-100 (100mM DTT)	1.0 L
(b) Column B	P-100 (8 mM Cam.)	1.0 L

Table III.2

Quantities of Reagents Required for a Typical Isolation and Purification of Cytochrome P-450.

Reagent	Quantity
Trizma base, 1M	800 mL
KH_2PO_4, 1M	20 mL
K_2HPO_4, 1M	35 mL
KCl	250 g
$(NH_4)_2SO_4$	100 g
NH_4OH, 15M	2 mL
βME	10 mL
DTT	0.2 g
DNase	1.0 mg
RNase	1.0 mg

III.2.4 Column Chromatography

Since the success of isolation and purification of cytochrome P-450 very much depend on the precision exercised in the preparation of chromatographic resins and operation of the chromatography columns, the manufacturer's recommendations were followed with special care. The columns for DE-52 anion exchange chromatography were manufactured by Bio-Rad Laboratories (Model Bio-Rex) and the columns for Sephadex gel filtration column chromatography were constructed in the mechanical shop of the Chemistry Department, U.B.C. The ends of the DE-52 columns were fitted with porous polyethylene and 100 mesh nylon inserts. The ends of Sephadex columns contained only 100 mesh nylon inserts. Both DE-52 columns were equilibrated in the descending flow mode using a peristaltic pump (Buchler Instruments) to achieve constant flow rates. The Sephadex columns were equilibrated under gravity feed. The enzyme samples were collected with a LKB Broma, 7000 Ultrarac fraction collector, and the enzyme samples were concentrated using an Amicon ultrafiltration apparatus fitted with a YM-10 Diaflow ultrafiltration membrane (10, 000 molecular weight cut-off).

III.2.5. Isolation of Cytochrome P-450

III.2.5.1. Cell-Free Extract

About 400 g of bacteria, which had been frozen for at least two weeks in dry-ice, were thawed at room temperature and made into a creamy paste with 320 mL of T-buffer. This suspension was then stirred in the cold room (5°) for 8 hours at which time 2 mg each of DNase 1 and RNase A were added, and the stirring continued overnight (ca. 12 h) at 5°. Another 120 mL of T-buffer were then added and the mixture centrifuged at 10,000 rpm for 15 minutes in a Sorvall RC-2 refrigerated centrifuge equipped with a GSA rotor. This removed most of the cell debris from the enzyme extract. The resultant cloudy liquid was again centrifuged on a SS-34 rotor at 15,000 rpm for 30 minutes to obtain a clear pink coloured solution. This cell-free extract was kept on ice at all times under an argon atmosphere.

III.2.5.2. Separation of Cytochrome P-450

Separation of cytochrome P-450 from the other monooxygenase components was carried out at 25°, unless otherwise specified, using a two-step anion exchange column chromatographic procedure. The cell-free extract derived from about 400 g of bacteria was applied in the descending mode at a flow rate of 180 mL/h to a DE-52 anion exchange column (150 g, 5×15 cm) previously equilibrated with buffer T (see Table III.1). This column was then washed with buffer T-100 for 0.5h and the effluent discarded.

Subsequent effluent from this column was then applied in the ascending mode to a second DE-52 anion exchange column (300 g, 5×30 cm) equilibrated with buffer T-50. The elution with buffer T-100 continued until the three enzyme bands were well separated. At this time the yellow putidaredoxin reductase band was running in front of the pink cytochrome P-450 band which was followed by the brown putidaredoxin band. At this point a linear salt gradient from T-100 to T-600 was started in order to further separate yellow and pink bands from the brown band. The first DE-52 column was disconnected when the yellow and pink bands entered the second column, and the elution of second column was continued. The yellow band which eluted first was discarded and the pink cytochrome P-450 band was passed through a 6 m coil of small diameter tubing immersed in ice prior to collection at 5°. The total time required for running the two columns was about 14 hours.

III.2.5.3. Ammonium Sulphate Fractionation

The crude enzyme solution from the second DE-52 column was made 1mM with D-(+)-camphor and fractionated with ammonium sulphate (enzyme grade) by adding 200 g/L (36% saturation) over a period of 30 minutes. The mixture was kept on ice at all times under an argon atmosphere with gentle stirring. To maintain a constant pH value, 1 mL of concentrated NH_4OH was slowly added to the mixture for each 30 g of $(NH_4)_2SO_4$ added. The mixture was stirred for

another 30 minutes and centrifuged at 10, 000 rpm for 10 minutes; the resulting white apo-cytochrome P-450 precipitate was discarded. The supernatant was precipitated by adding 140 g/L of $(NH_4)_2SO_4$ (60% saturation) using the same procedure. The precipitate was collected by centrifugation and the yellow supernatant discarded. The precipitate was resuspended in a minimum volume of buffer P-100 containing 8 mM camphor. This crude enzyme solution was stored under argon at 5^0 in a Schlenk tube until purified by gel filtration chromatography.

III.2.6. Gel-Filtration Chromatography

III.2.6.1. Purification of Cytochrome P-450

The crude enzyme samples were made 50 mM with DTT and incubated for 30 minutes at room temperature prior to purification by two Sephadex G-100 (particle size 40-120 micron) columns equilibrated with T-100 (10 mM DTT) and P-100 (8 mM camphor) buffers, respectively. All enzyme samples were concentrated to 1-2 mL (ca. 0.5 mM) aliquots by ultra-filtration before applying to the columns. The enzyme solution from 400 g of bacteria was divided into two portions and purified separately in order to avoid overloading the columns. Both columns were equilibrated and run at 5° under gravity flow with a head-pressure less than 200 mm. The fractions from the first column were selected by inspecting the colour density so that about 60-70% of the pink enzyme elute was included; after being concentrated to about 2 mL those fractions were then applied to the second column. The middle fractions of this column contained the most pure enzyme samples which were spectroscopically analyzed. The usual yield calculated from a molecular weight of 45,000 Daltons amounted to about 135 mg of protein (ca. 3 mL of 1 mM solution) isolated from 400 g of bacteria.

III.2.6.2. Removal of Camphor Substrate

The camphor substrate was removed by another two-step gel-filtration column chromatographic procedure using Sephadex G-10 at 5°. Pure cytochrome P-450 solutions were

made 50 mM with DTT and incubated at room temperature for 30 minutes. Camphor was then removed by gel-filtration on a Sephadex G-10 column (1.0×20 cm) equilibrated with T-100 buffer containing 10mM DTT. Only about 1.0 mL of enzyme solution was used for each run and the head-pressure was adjusted so that the column ran over a 24 hour period. The effluent enzyme solution was then chromatographed on a second Sephadex G-10 column equilibrated with buffer P-100 in order to effect a buffer exchange and to remove the excess thiol reagent. During the removal of camphor, special care was taken to avoid the glassware and tubing which came in contact with camphor, as this sometimes led to the isolation of partly substrate-bound enzyme. The enzyme samples prepared in this manner demonstrated no apparent residual bound camphor, as indicated by the absence of an optical absorption shoulder at 391 nm in the presence of 50 mM potassium ion (see Section III.2.7.).

III.2.7. Spectral Analysis

The purity of cytochrome P-450 samples can be estimated conveniently by correlating the extinction coefficient of the electronic absorption maximum of the heme protein with the electrophoretic mobility in sodium dodecyl sulphate (SDS) gels.[1] The analytical SDS-tube gel electrophoresis by the integration of densiometric recordings from an automatic gel scanner using the method of Weber and Osborn,[3] combined with the optical absorption spectral data of the heme protein, indicate that crystalline cytochrome P-450 (greater than 99% homogeneous by the SDS-tube gel electrophoresis) is characterized by an optical absorption ratio of the peak at 391 nm to the peak at 280 nm of 1.63.[1] Purified but non crystallized samples greater than 95% homogeneous have an absorption ratio, $A_{391}:A_{280}$ of 1.45.

Electronic absorption spectra were recorded from 250-700 nm in a Cary spectrophotometer model 17D at room temperature, a special cell tonometer (Figure III.4) designed to handle enzyme solutions anaerobically being used. The cell tonometer consisted of a clear rectangular quartz cell (1x1x5 cm)attached to a sidearm reservoir (10 mL) through glass tubing (20 cm) containing a silicon rubber septum fitting. A high- vacuum O-ring joint (Kontes Glass Co.) with a B-14 sidearm socket was utilized for attachment to the vacuum-gas handling line. The total volume of the cell tonometer was 60 mL.

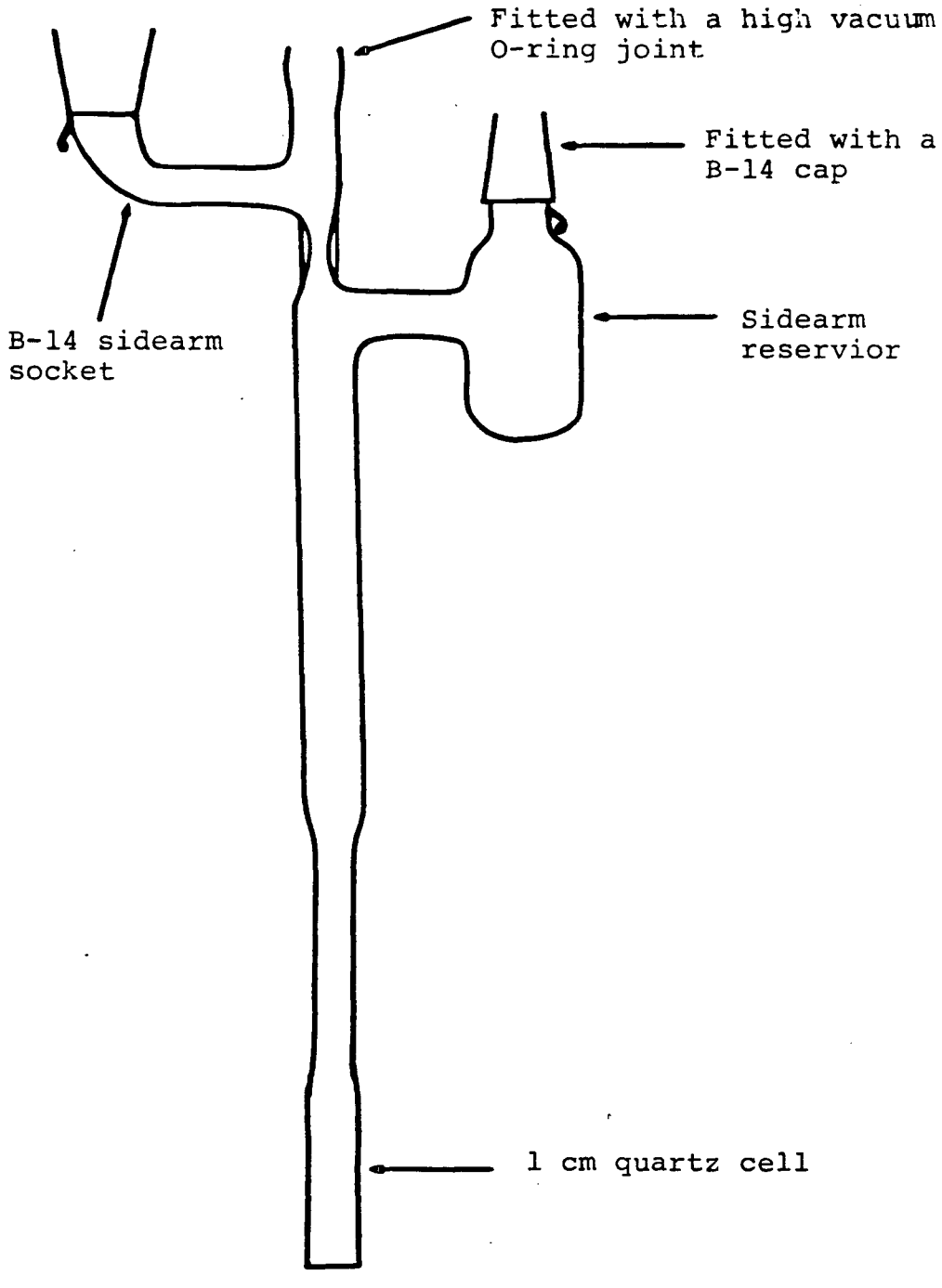

Figure III.4 The cell-tonometer.

Previously degassed P-100 buffer (4.8 mL) was transferred to the sidearm reservoir and repeatedly flushed with argon and evacuated three times. Stock enzyme solution (ca.0.2 mL) was added to the reservoir, mixed by swirling and then transferred to the cell cavity by tilting the tonometer. The optical absorption spectrum of the ferric state of the enzyme was then recorded. Stoichiometric reduction of the enzyme to the ferrous state was carried out by adding small amounts of a sodium dithionite solution (ca. 4 mg/mL in degassed buffer P-100) through the rubber septum using a syringe, until a small absorption peak appeared at 314 nm due to excess dithionite. Any excess sodium dithionite was removed by adding small amounts of dioxygen via a gas-tight syringe; the optical spectrum of reduced enzyme was then recorded. The carbon monoxide complex of the reduced enzyme was conveniently formed by gently bubbling the gas through the solution for 30 seconds at room temperature.

III.3 Determination of the Equilibrium Constant for the Reaction of Carbon Monoxide with Substrate-free Cytochrome P-450 at Different Temperatures

III.3.1 General Information

The equilibrium constant for the reaction of carbon monoxide with reduced substrate-free cytochrome P-450 at a given temperature can be measured conveniently by observing the electronic absorption spectral changes as a function of gas pressure. The procedure for measuring the spectral changes as a function of gas pressure and determining the equilibrium constant via Hill log/log plots has been described previously.[4,5]

In order to allow for the addition of small partial pressures of CO gas (ca.0.8 mm Hg), a vacuum-gas handling apparatus was constructed with an incorporated di-n-butylphthalate (DBT) manometer instead of the usual mercury manometer. Partial pressures of CO in mm DBT were converted to mm Hg for data analysis using the formula, mm DBT 7.73×10^{-2} = mm Hg, derived using density data.

III.3.2 Procedure

All buffer solutions were freeze-thaw degassed and argon-saturated before use. About 4.8 mL of camphor-free P-100 buffer was added to the optical cell tonometer and the system evacuated and refilled with argon three times. The cell was then transferred to the thermostated cell compartment of the Cary 17D and the base-line spectrum

recorded from 250-700 nm. For thermostating , two Haake model FK constant temperature baths were utilized, one connected to the special cell compartment of the spectrophotometer and the other at the CO-gas handling line. Before the begining of each experiment, the constant temperature baths were brought to the required temperature ($\pm 0.2^{o}$) and allowed to equilibrate at the temperature for at least 30 minutes . The cell tonometer was again attached to the argon line and about 0.2 mL of stock substrate-free enzyme solution added anerobically with gentle swirling. At all times, care was taken not to form bubbles in the enzyme solution as this can lead to denaturation of the protein. Then the cell tonometer was again placed in the thermostated cell compartment and the spectrum of oxidized substrate-free enzyme recorded. The stoichiometric reduction of the enzyme was then carried out as described in Section III.2.7 and the spectrum recorded. The cell tonometer was then taken to the the CO-gas handling line and thermostated to the desired temperature by immersion in a Dewar full of water containing a spiral copper coil attached to the water circulation port of the constant temperature bath. Two thermometers were used to measure the temperature ($\pm 0.2^{o}$), one at the CO-line and the other at the spectrophotometer.The cell tonometer was then slowly evacuated to remove the argon atmosphere and the system allowed to equilibrate (>5 minutes) before reading the vapour pressure of the enzyme solution. This procedure was

repeated three times. The value of vapour pressure obtained was converted to mm Hg and checked with published data[6] for accuracy, in order to make sure that there were no leaks in the apparatus. Then a small amount of CO gas was carefully admitted to the system and allowed to equilibrate (3 minutes). The pressure was recorded and the cell tonometer was closed, removed from the gas handling line, slowly swirled and taken to the spectrophotometer and allowed to equilibrate for another 3 minutes prior to the spectrum of partially carbonylated enzyme being recorded. Once the spectrum was recorded, the cell was taken back to the CO-line and the pressure was readjusted using the same procedure as above. At least five different CO-pressures were added and the spectra recorded in each case. The final spectrum was recorded under 1 atmosphere of CO.

This general procedure was followed at four different temperatures in order to investigate the temperature dependence of the equilibrium constant.

III.4 Determination of Equilibrium Constant for the Reaction of Dioxygen with Substrate-bound cytochrome P450cam

III.4.1 General Information

The equilibrium constant for the reaction of dioxygen with substrate-bound P-450 can be estimated by an experimental procedure similar to that used for the determination of CO binding constant, discussed in the previous section. In order to equilibrate the system at sub-zero temperatures a slush bath (Figure III.5) was used.Therefore, the use of constant temperature baths as well as the special cell compartment was not necessary. The buffer solution used was 50% diethyleneglycol in P-buffer.

III.4.2 Procedure

The cell-cavity of the slush bath was filled to 2/3 of its volume (30 mL) with the buffer solution and the base-line spectrum was recorded in the Cary spctrophotometer as described in Section III.3.2. Stock substrate-bound enzyme solution was then added to a final concentration of about 2 10^{-6} M and the spectrum recorded. Then, the stoichiometric reduction was carried out as given in Section III.3.2. The cell was then cooled to the desired temperature (± 1°) using the suitable slush[7] from the list below.

Slush	Temperature
Benzonitrile/liquid N_2	-13°
Carbon tetrachloride/liquid N_2	-23°
o-Xylene/liquid N_2	-29°

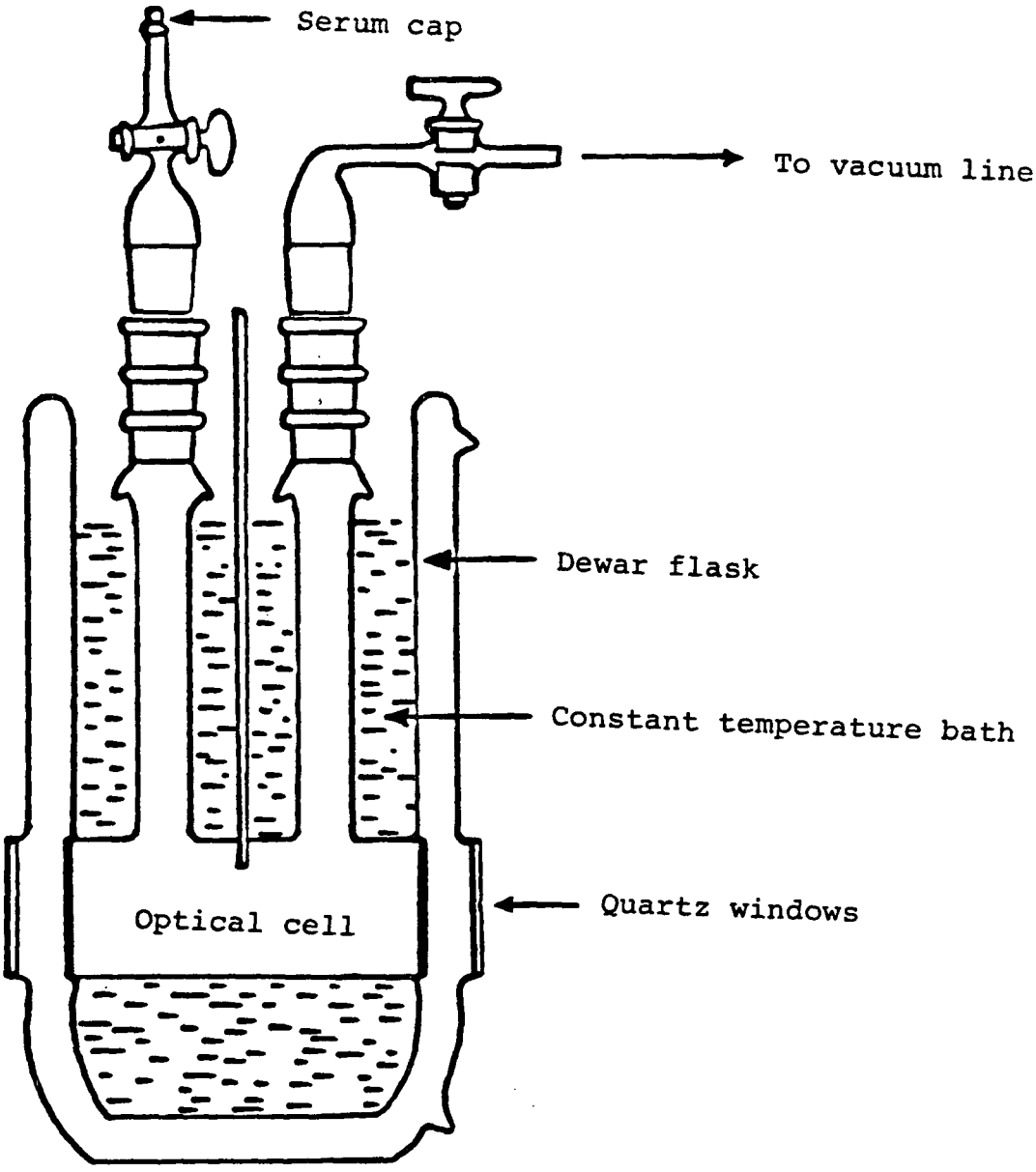

Figure III.5 10 cm path-length optical cell (Slush Bath).

The temperature was maintained constant by the addition of small amounts of liquid N_2 periodically. Small partial pressures of O_2 were then added using a low pressure gas handling line as described in Section III.3.2, and the corresponding spectra were recorded. Attempts were made to record the spectrum of the fully formed oxygenated species under 1 atmosphere of dioxygen (see Section IV.4).

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CHAPTER IV

RESULTS AND DISCUSSION

IV.1 Growth of Pseudomonas putida Strain 786

The monooxygenase enzyme system present in the bacterium Pseudomonas putida has provided a definitive biological model for extensive biochemical and physical studies relevant to the cytochrome P-450 mediated hydroxylation reactions occurring in mammalian systems.[1] In the present study, the Pseudomonas putida strain 786 was used to isolate the camphor hydroxylating monooxygenase component, cytochrome P-450cam. The strain 786 possesses several advantages over the parent strain PpG 1 which was originally isolated from soil by enrichment on D-(+)-camphor.[1] Among these advantages are the shorter generation time, resistance to lysis by bacteriophage, and the ability to release the hydroxylase proteins by freeze-thaw autolysis, thereby providing easy access to the P-450 enzyme.

In order for the bacteria to retain the ability to metabolize camphor, and hence to produce the P-450 enzyme, it was necessary to maintain the strain in minimal agar plates containing camphor as the only carbon source. Failure to do so results in the bacterium losing the ability to multiply in camphor saturated media, with subsequent long lag-phases in the growth curves.[2]

It was observed that the pH trends of the culture media depend on the basic carbon and energy source provided for the growth. In the case of L-broth and 500 mL shake flask stages, where glucose and glutamic acid were used,

respectively, the pH of the media increased, while the growth on camphor resulted in a decrease of pH. The pH decrease during camphor metabolism is known to be due to the accumulation of acidic metabolites,[34] while no clear explanation has been given for the increase in pH during the metabolism of the other organic substrates.

The basic difficulty encountered in the growth of the bacteria is the 'foaming out', despite the use of antifoaming agents, at the later part of the 14 L fermenter stage. The foam caused the exit air filters to clog and the air supply to the culture medium was thus cut off by the build-up of high pressure in the fermenters. This process, which usually happened during overnight growth, resulted in the death of bacterial cells; the use of an external container, connected to the fermenters via rubber tubing (with air filters removed), in order to collect the foam (which contained pink bacterial cells) served as an alternative, but this also resulted in the death of a considerable fraction of the cells due to lack of aeration and agitation.

The duration of the growth procedure was found to be at least twice as long as that given in the literature procedure,[1] although all the instructions were followed closely. As a consequence of the lengthy growth period, the amount of enzyme isolated was generally smaller than that reported.[1]

IV.2 Properties of Cytochrome P-450cam

IV.2.1 Substrate-Bound Cytochrome P-450cam

The absorption spectra of the various states of pure, substrate-bound cytochrome P-450cam (Sections II.2.5 and II.2.6.1) are given in Figure IV.1. The wavelengths of the absorption maxima (λmax), as well as the corresponding millimolar extinction coefficients (ε mM) are presented together with literature data in Table IV.1.

The oxidized, substrate-bound enzyme absorbs at 280 nm (protein peak), 391 nm (Soret peak), and 510, 542, and 643 nm (visible peaks). The calculated εmM values agree reasonably well with the published data, except for the absorption at 280 nm, which is due to aromatic groups in the protein chain. The intensity of the 280 nm peak varied slightly (± 5%), in different enzyme preparations, due to the presence of small amounts of apo-cytochrome P-450.

On reducing the oxidized state with stoichiometric amounts of sodium dithionite (λmax 314 nm), the colour of enzyme solution changes from light brown to light pink with generation of absorption peaks at 408 and 544 nm; the protein absorption remains unchanged as expected. The CO-complex of the reduced enzyme, formed by bubbling CO through the enzyme solution for a few seconds, has a golden yellow colour with absorptions at 365 and 445 nm (near uv and low energy bands of a split Soret) and 550 nm. The discrepancy in values for the 365 nm peak (Table IV.1) is due to the interference of excess dithionite in the

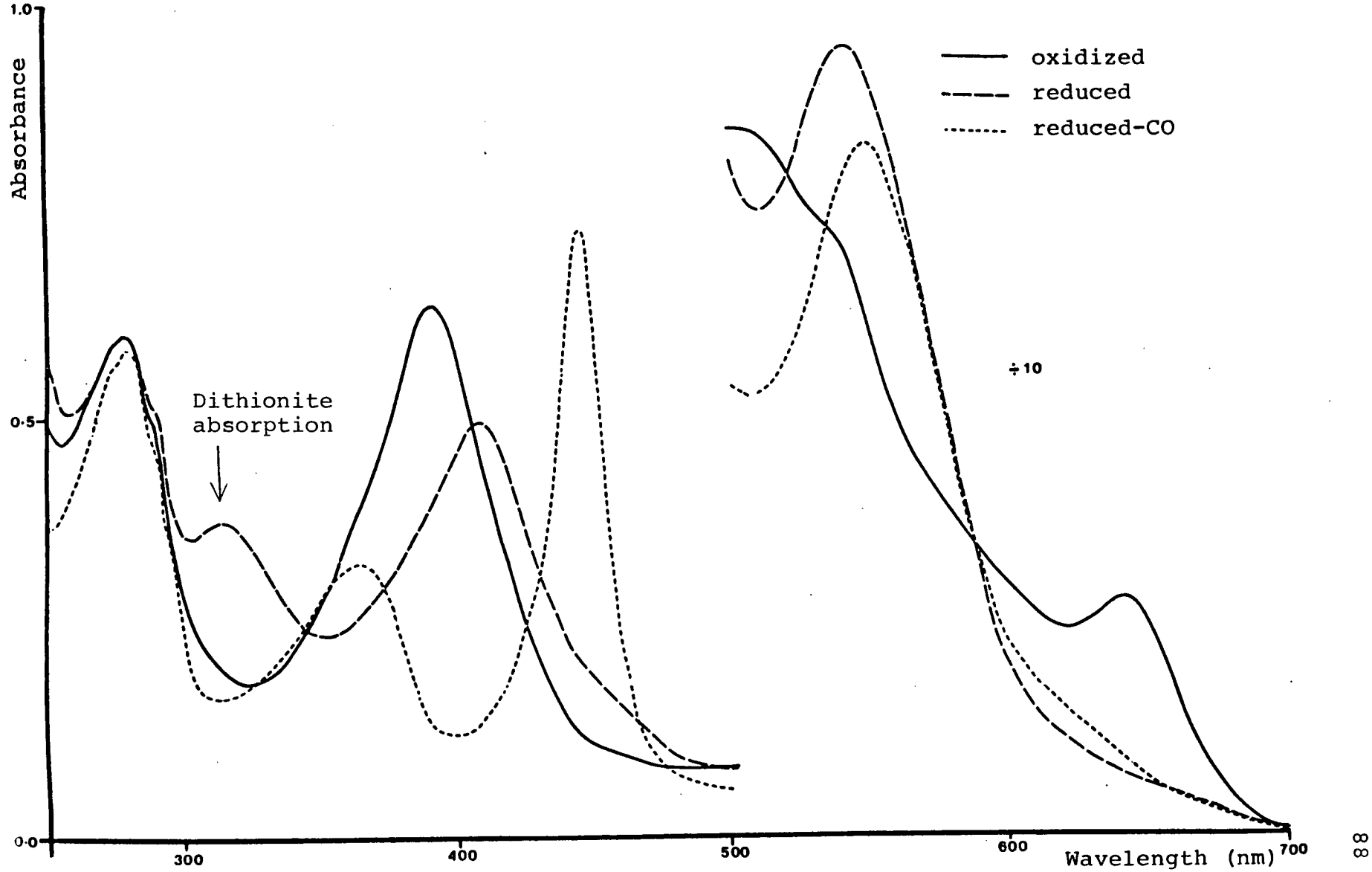

Figure IV.1 Various states of pure, substrate-bound cytochrome P-450cam.

Table IV.1

Absorbance Data for Substrate-Bound Cytochrome P-450cam.

State	(a) Expt. Prep.		(b) Lit. Prep.		(c) Lit. Prep.	
	λ_{max}	ε_{mM}	λ_{max}	ε_{mM}	λ_{max}	ε_{mM}
Oxidized	280	81.2	-	-	280	63.3
	391	102	391	101	391	102
	510	12.2	510	-	510	13.0
	542	10.9	540	-	540	11.2
	643	4.2	645	-	645	5.4
Reduced	408	80.7	408	83	408	86.5
	544	14.5	542	15.2	542	16.0
Reduced-CO	365	51.1	-	-	364	60.8
	445	120	446	119	446	120
	550	13.8	550	13.0	550	14

(a) Determined in P-100 buffer, pH 7.4, 8 mM camphor at 23±2°; ε mM calculated assuming ε mM=120 for reduced-CO species at 445 nm.

(b) From ref. 5

(c) From ref. 1

literature preparation; and the smaller ε value obtained in the present work is considered more accurate.

IV.2.2 Substrate-Free Cytochrome P-450cam

The absorption spectra of the various states of pure, substrate-free cytochrome P-450cam (Section II.2.6.2) are shown in Figure IV.2; the λmax and εmM values for the experimental preparation are given with the literature data in Table IV.2.

Examination of the spectral characteristics of various states of the substrate-bound and substrate-free enzyme (Tables IV.1 and IV.2) reveals that both forms have very similar absorption spectra in the reduced and reduced-CO states. However, the oxidized states of the forms have remarkably different spectral properties (Figure IV.3). The binding of camphor to substrate-free enzyme is strong and occurs rapidly (Section II.4.2.1). Because of the similiarities in spectra of reduced states (and reduced-CO states) of substrate-free and substrate-bound enzymes, it was essential to make sure that no trace camphor was present in the buffers or glassware used in the studies of substrate-free enzyme.

IV.2.3 Purity and Stability of the Enzyme Preperations

The isolated and purified (Sections II.2.5 and II.2.6.1) substrate-bound enzyme samples were at least 80% homogeneous,[1] having an absorption ratio $A_{391}:A_{280}$= 1.14. The absorption ratio for some of the literature preparations was 1.00 [5], 1.13 [2], 1.37 [6], 1.45 (crystalline sample)[1].

All substrate-free enzyme samples were of the same

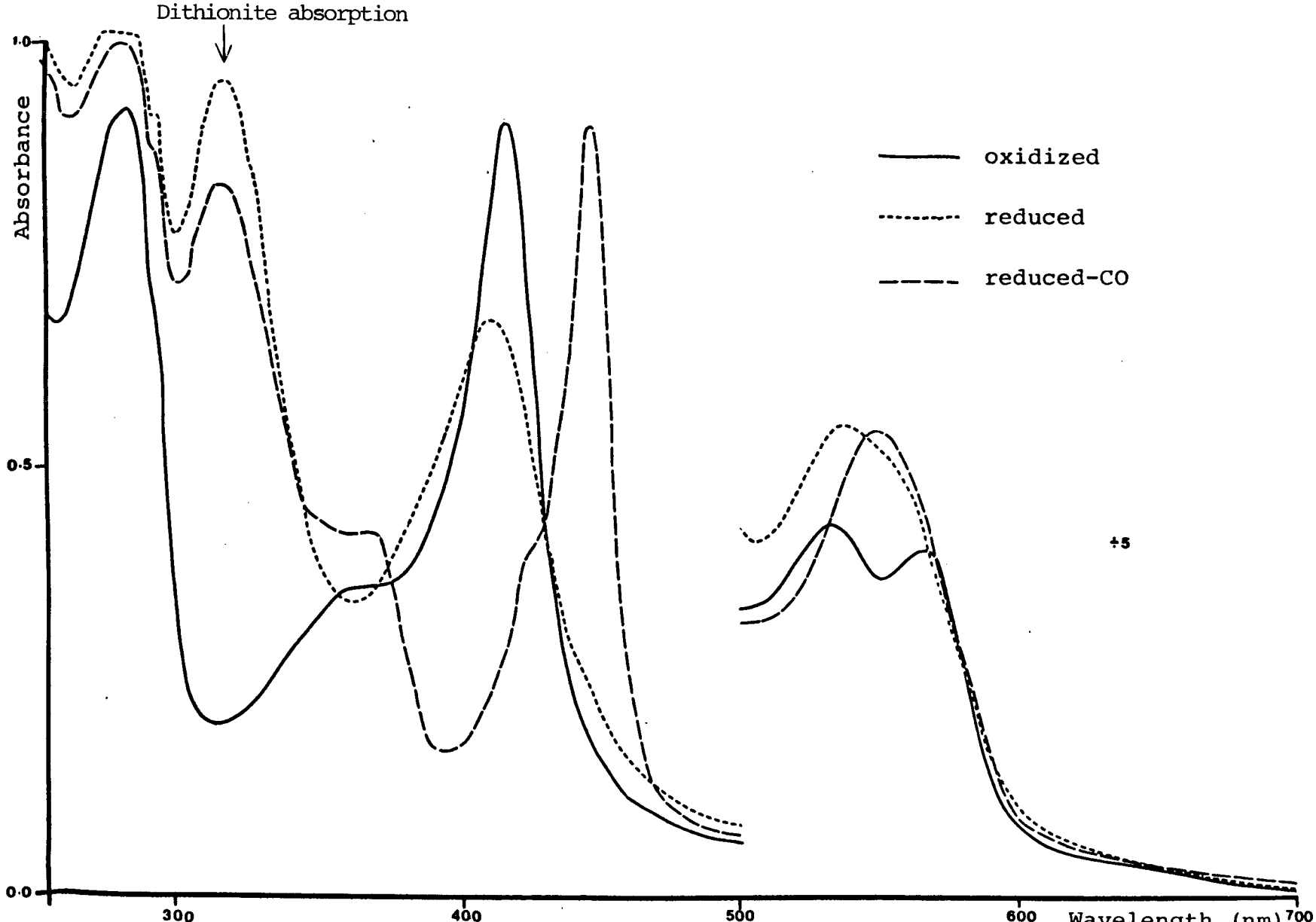

Figure IV.2 Various states of pure, substrate-free cytochrome P-450cam.

Table IV.2

Absorbance Data for Substrate-Free Cytochrome P-450cam.

State	(a) Expt. Prep.		(b) Lit. Prep.		(c) Lit. Prep.	
	λ_{max}	ϵ_{mM}	λ_{max}	ϵ_{mM}	λ_{max}	ϵ_{mM}
Oxidized	278	107	-	-	280	68.3
	360	41.0	-	-	360	36.7
	416	117.9	418	104	417	115
	535	9.7	535	10.3	535	11.6
	568	9.3	570	10.4	569	11.9
Reduced	408	78.9	408	69.0	408	76.7
	540	12.5	540	13.5	540	15.1
Reduced-CO	365					
	445	120	447	104	447	120
	550	12.5	550	12.1	550	14.3

(a) Determined in buffer P-100, pH 7.4, no camphor added, at 23±2°; ϵ mM calculated assuming ϵ_{mM}=120 for reduced-CO species at 445 nm.

(b) From ref. 5

(c) From ref. 1

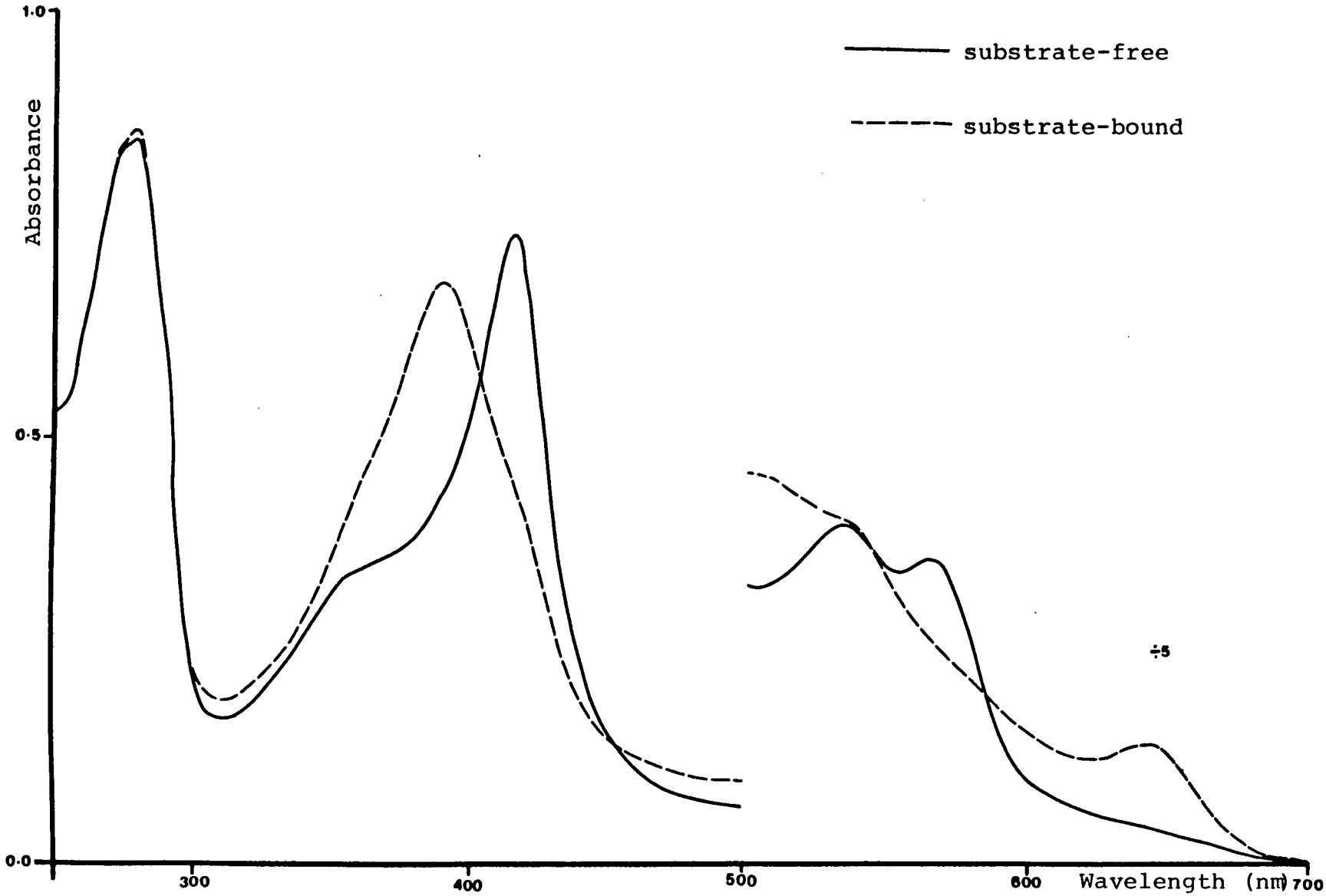

Figure IV.3 Oxidized states of substrate-free and substrate-bound cytochrome P-450cam.

order of purity as the substrate-bound enzyme, from which they were prepared. But, storage at 5° under argon for more than 48 hours caused the formation of detectable amounts of P-420 species (denatured P-450) as seen in the absorption spectrum (Figure IV.4). Therefore, substrate-free enzyme samples for the equilibrium ligand binding studies were prepared on a daily basis. The substrate-bound enzyme, once purified, could be stored at 5° for over 6 months without the formation of any P-420 species. The substrate-bound enzyme could be stored indefinitely at liquid nitrogen temperature without detectable loss of purity.

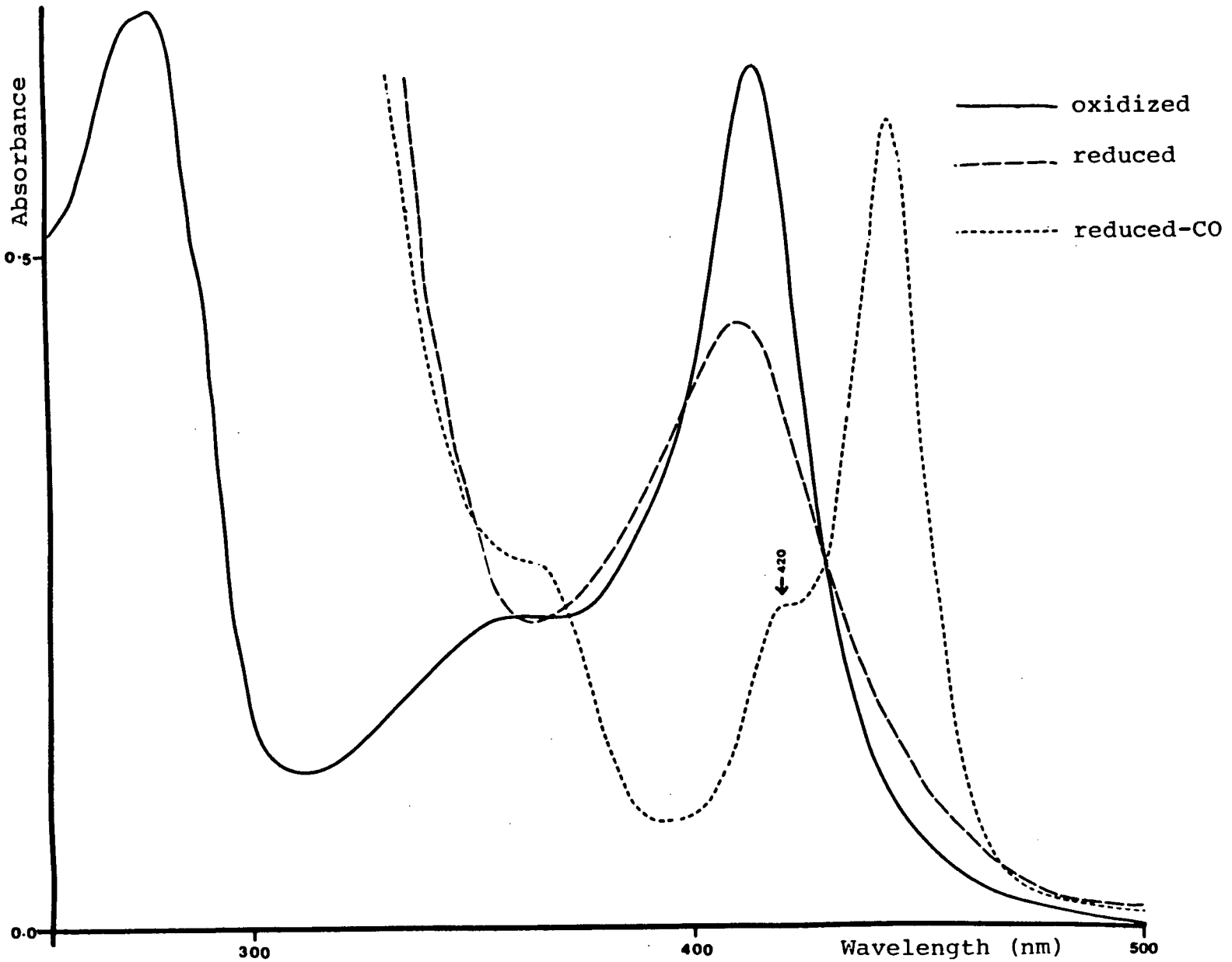

Figure IV.4 P-420 species formed in substrate-free cytochrome P-450cam solution.

IV.3 Determination of the Equilibrium Constant at Different Temperatures for the Binding of Carbon Monoxide by Substrate-Free Cytochrome P-450cam

IV.3.1 Acquisition and Treatment of Data

Equilibrium constant values for the binding of CO with substrate-free cytochrome P-450 were estimated from the experimental data obtained as given in Section III.3.

At each temperature, at least three different determinations were carried out to give a mean value for the equilibrium constants. The spectral changes observed for experiment no.5 (Table IV.3) are given in Figure IV.5. At a fixed enzyme concentration (7-8 10^{-6} M), the CO pressure was varied from 0.4-760 mm Hg, resulting in a decrease of the 408 nm peak (reduced) and an increase in 445 nm peak (CO-complexed). Isosbestic points were observed at 373, 430, 466 and 583 nm. Raw data from all the experiments are given in Appendix (i), along with the processed data.

The degree of formation of the CO-complex of the substrate-free cytochrome P-450cam (Reaction II.2) can be determined using the familiar Hill log/log plot which yeilds the $P_{1/2}$ and hence K_{CO} value.

The ratio of the Fe(II)-CO to the decarbonylated complex present at a given CO pressure (P_{CO}), can be expressed as $(A-A_0)/(A_\infty-A)$, where,

A = absorbance of Fe(II)-CO at a known P_{CO}

A_0 = absorbance of Fe(II) enzyme when $P_{CO}=0$

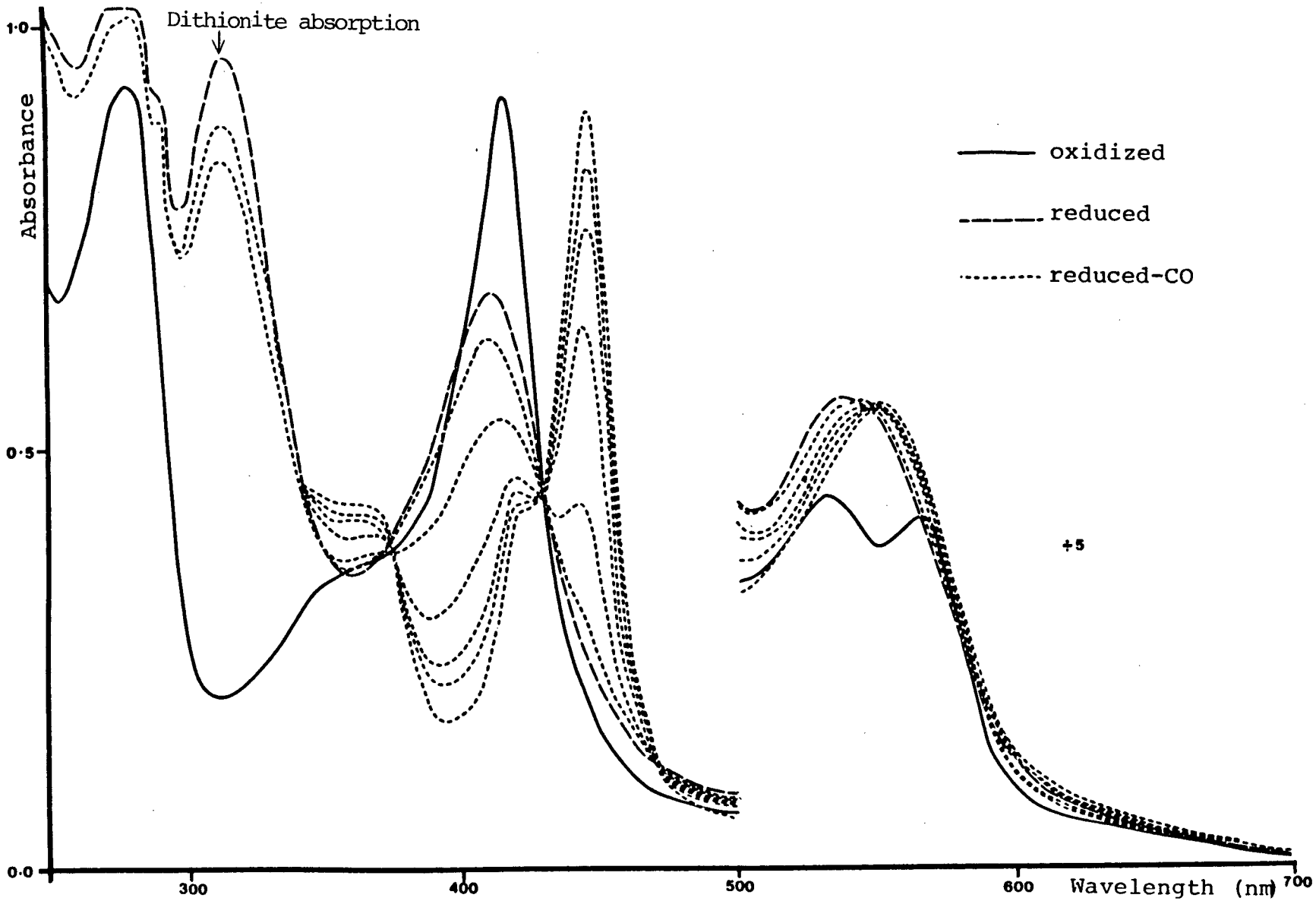

Figure IV.5 Spectral changes observed for the $P_{1/2}$ experiment no. 5.

A_∞= absorbance of fully formed Fe(II)-CO

Clearly, $A-A_0 \propto$ [Fe(II)-CO] , and

$A_\infty-A \propto$[Fe(II)].

Since, K_{CO} =[Fe(II)-CO]/[Fe(II)][CO], the following relationships are derived.

$$\text{Log } K_{CO}=\text{Log } (A-A_0)/(A_\infty-A) - \text{Log } [CO]$$

$$\text{Log } (A-A_0)/(A_\infty-A)=\text{Log } P_{CO}+ \text{Log } K'$$

where, $K'= x.K$ and

x= the solubility of CO in water expressed in M/(mm Hg) at the reaction temperature.

Therefore, the plot of log $(A-A_0)/(A_\infty-A)$ vs. log P_{CO} should give a straight line with a theoretical gradient equal to unity for the simple equilibrium; and when $A-A_0=A_\infty-A$ or, log $(A-A_0)/(A_\infty-A)=0$, the corresponding P_{CO} value would be the $P_{1/2}$. The $K_{CO}(M^{-1})$ values can be calculated using the equation,

$$K_{CO} = 1/x.P_{1/2}$$

The log $(A-A_0)/(A_\infty-A)$ values were calculated from the corresponding absorbance values at 445 nm. P_{CO} values were converted from mm DBT to mm Hg as described in Section III.3.1. The data were plotted as in Figure IV.6 using the computer program "ace:graph" available from the computer facility at U.B.C. The gradients of all the plots were in the range 0.90-1.15; in certain instances , removal of an experimental data point (considered to contain a large error), applying the condition that at least four other data points remained, resulted in a much more satisfactory

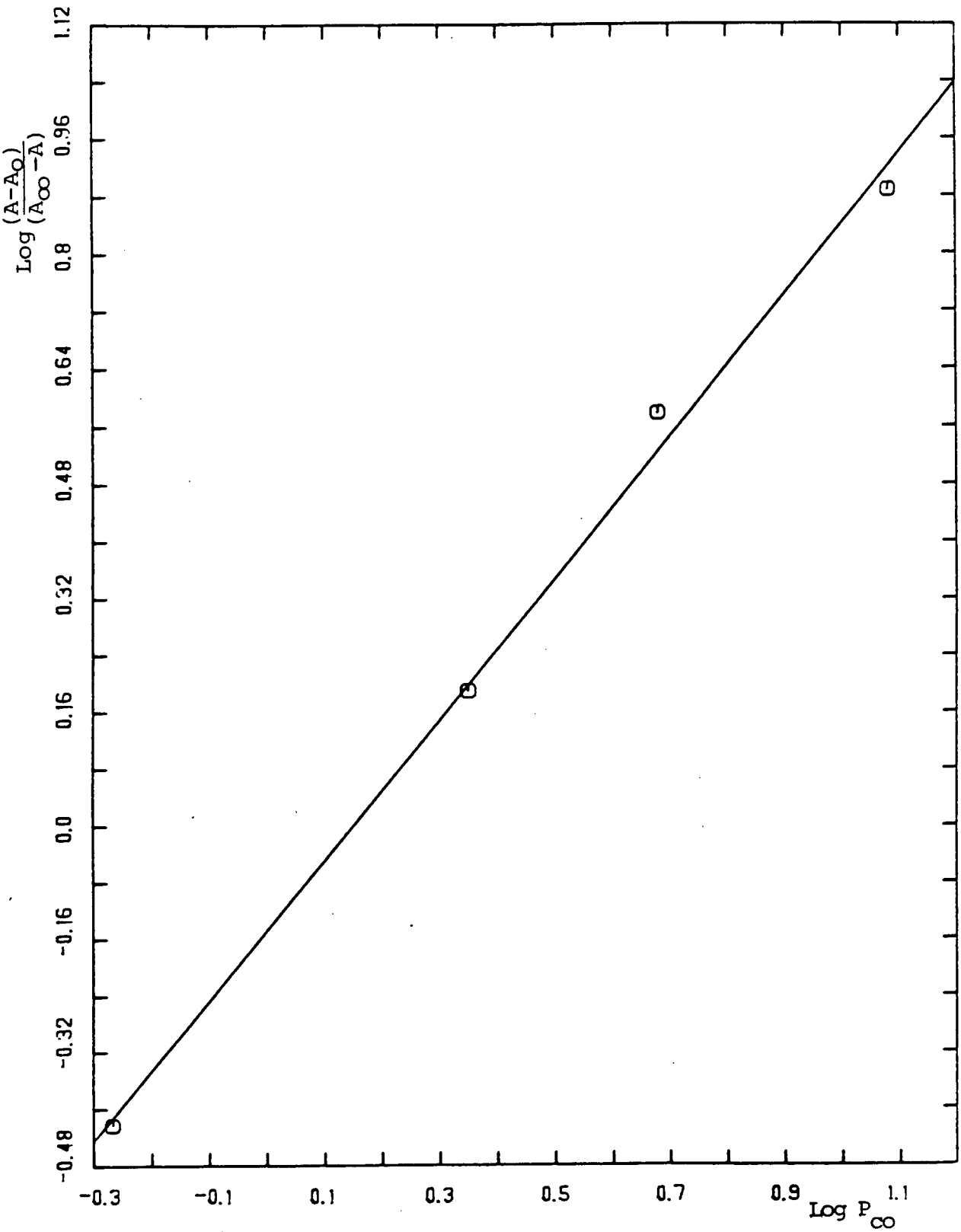

Figure IV.6 A typical Hill log/log plot.(Expt. no.5).

correlation of data, according to least-squares linear regression analysis. The plots obtained for all $P_{1/2}$ experiments are given in Appendix (ii). Each $P_{1/2}$ value calculated from these plots w recorded and the mean value calculated (Table IV.3). The $P_{1/2}$ values were converted to equilibrium constant (K_{CO}, M^{-1}) values using published data[7] for the solubility of CO in water at various temperatures.

An important aspect of the study of reactions of hemoproteins with ligands is the determination of the thermodynamic parameters ΔH^0 and ΔS^0. In the case of biological systems, the determination of such parameters has usually been via van't Hoff plots,[8] although highly sensitive microcalorimetric techniques are also available.[9]

The van't Hoff plot, (log K_{CO} vs. 1/T ; Figure IV.7) was obtained using the computer program mentioned above, and the thermodynamic parameters were calculated. The thermodynamic relationship,

$$\text{Log } K = -(\Delta H^0/2.303R).1/T + \Delta S^0/ 2.303R ,$$

allows for a plot of log K vs. 1/T to give ΔH^0 from the gradient and ΔS^0 from the y-intercept. Good linearity is observed in these plots owing to the fact that the measurements are done in a narrow temperature range[8] (usually 0^0-40^0) because of the instability of proteins at higher temperatures.

The ΔH^0 value for the CO binding to reduced substrate-free cytochrome P-450cam was estimated to be -18±1 kcal/mol

Table IV.3

The Equilibrium Constant Values for CO Binding to Substrate-Free Cytochrome P-450cam.

T(°C)	#	$P_{1/2}$(mm Hg)	Mean $P_{1/2}$	K_{co} (M^{-1})	Mean K_{co}	Log K_{co}	$1/T \times 10^{-3}$
4.0	1	0.372		1.55×10^6			
	2	0.423	0.398	1.37×10^6	1.46×10^6	6.1644	3.608
	3	0.398		1.45×10^6			
12.0	4	1.38		5.07×10^5			
	5	1.32	1.40	5.30×10^5	5.00×10^5	5.6990	3.507
	6	1.57		4.63×10^5			
18.0	7	2.63		2.95×10^5			
	8	2.69	2.78	2.88×10^5	2.80×10^5	5.4472	3.434
	9	3.02		2.57×10^5			
24.0	10	4.68		1.57×10^5			
	11	4.47	4.59	1.65×10^5	1.60×10^5	5.2041	3.365
	12	4.62		1.59×10^5			

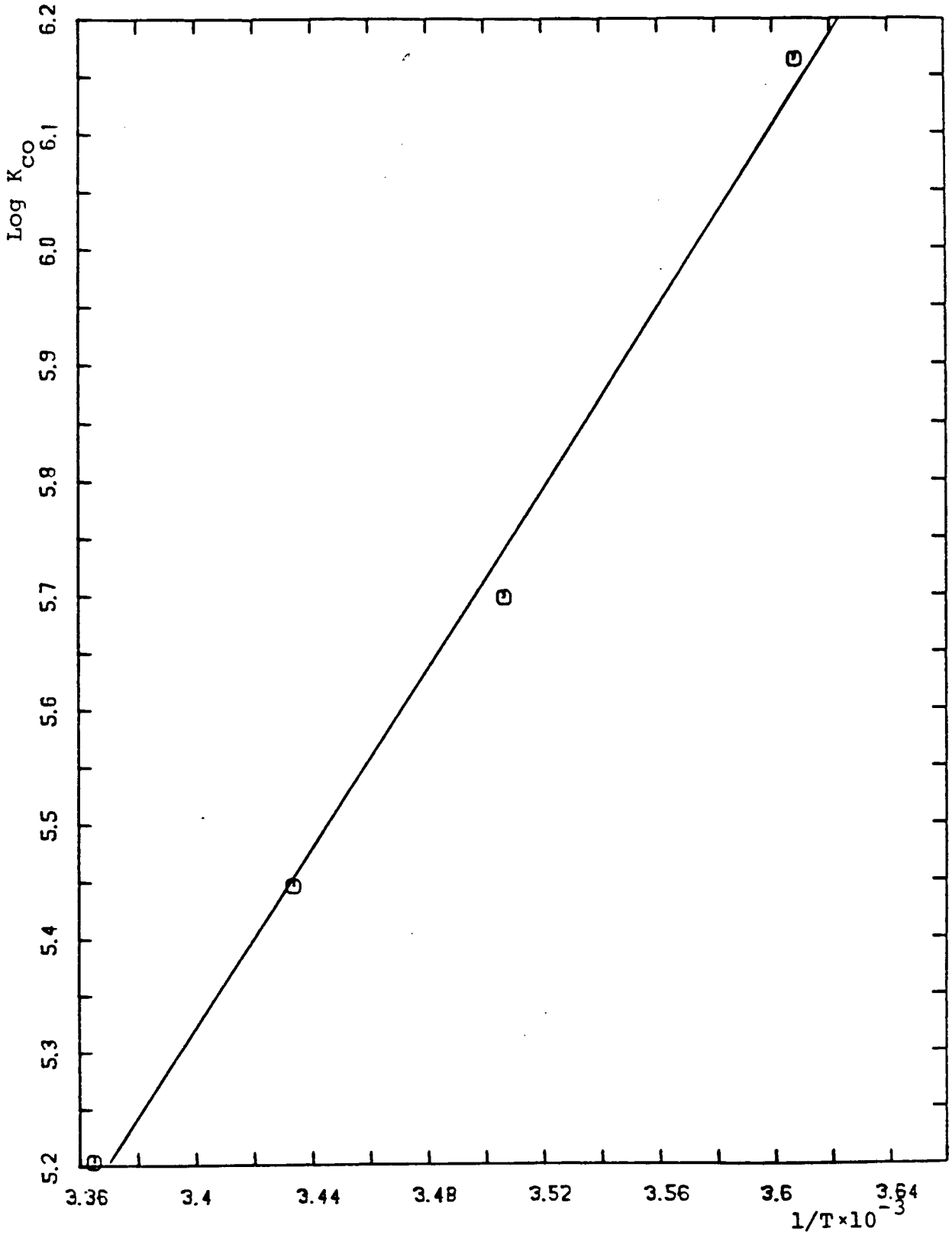

Figure IV.7 The van't Hoff plot.

The ΔS° value was -37±4 cal/mol.deg. (standard state of 1 M). Table IV.4 summarizes published thermodynamic data for the CO binding to substrate-bound cytochrome P-450cam, horse Mb, sheep Hb (per mole of heme), and to some model compounds. Given also are the values determined in this study for substrate-free cytochrome P450cam.

Table IV.4

K_{CO}, ΔH° and ΔS° Values for CO Binding to Some Hemoproteins and Models of Cytochrome P-450.

System	$K_{CO}, 25^{\circ}$ (M^{-1})	ΔH° (kcal/mol)	ΔS° (cal/mol.deg.)	Ref.
P-450cam (sub.-bound)	1.3×10^{5}	-12	-17	11
Horse Mb	2.5×10^{7}	-12.6	-7.4	8
Sheep Hb	-	-16	-	8
Human Hb	-	-17.7	-	9
Model* (a)	1.3×10^{4}	-16	-35	10
(b)	2.8×10^{3}	-19	-48	10
P-450cam (sub.-free)	1.3×10^{5}	-18±1	-37±4	this work

* Fe(II)Pp(IX)DME-BuS complex

(a) in a polar medium (N,N-dimethylacetamide)

(b) in a non-polar medium (toluene)

IV.4 Comparison of Measured ΔH^0 and ΔS^0 Values to those of Other Hemoproteins and Model Systems

Structural analyses of carbonylated hemoproteins reveal that the CO unit is bent and/or tilted from the perpendicular to the porphyrin plane, due to interactions with the distal residues.[12] However, in the protein-free model systems, as expected, the Fe-C-O unit is linear and normal to porphyrin plane.[13] It has been proposed [14-17] that in hemoproteins, the CO affinities are lowered significantly due to this 'distal-side steric effect' which causes the Fe-C-O unit to be bent.

Results from the present study show that the binding of CO to substrate-free cytochrome P-450cam is at least 5 kcal/mol more favourable in terms of ΔH^0 compared to the published value[11] for the substrate-bound system (Table IV.4). The reverse trend observed in terms of ΔS^0 is considered to be due mainly to differences in conformational changes in the protein chain.[18]

As proposed by Lumry and Rajender,[18] there exist a specific linear relationship between the entropy change and enthalpy change within a variety of protein reactions termed 'enthalpy-entropy compensation phenomena'. It has been demonstrated that the 'compensation effect' produces parallel enthalpy and entropy changes so that the net change in the free-energy of the system under investigation is relatively minor. Although this principle is not rigorously est lished, the feeling is that expansions and

contractions of the protein result in changes in the 'free volume' of water, which contributes to the entropy change of the system. For example, the effect of varying ethanol concentrations on the reversible thermal unfolding transition of ribonuclease A at various temperatures is to produce parallel ΔH^0 and ΔS^0 changes that give rise to the same $\Delta(\Delta H^0)/\Delta(\Delta S^0)$ value at each temperature. Also similar compensation effects have been noted in reactions of hemoglobin and myoglobin with ligands such as F^-, N_3^-, CN^-, SCN^-.[18]

The compensation hypothesis appears to apply to the results of the present study; the large negative entropy contribution observed in the case of CO binding to substrate-free P-450cam compensates for the large enthalpy change observed, the overall process resulting in no change in the ΔG^0 of the reaction, compared to the substrate-bound system.

Qualitatively, the observed less-favourable ΔH^0 for the binding of CO to substrate-bound enzyme, compared to the substrate-free system, may be due mainly to the presence of the substrate molecule sitting close to the active-site, perhaps causing the CO molecule to bind in a more angular fashion. In fact, such a hypothesis indirectly supports the observations by Peterson et al.[19] in their inhibition experiments where they conclude that the bonding site of the substrate must lie in the immediate vicinity of the heme iron.

The relatively larger CO binding constant to myoglobin (Table IV.4) may result from the weaker σ-donor property of the fifth ligand, imidazole, compared to the thiolate ligand in P-450. The ΔH^0 term for the CO binding to sheep Hb is more favourable than that for substrate-bound P-450cam or Mb, and indeed it is similar to the ΔH^0 determined for the substrate-free P-450cam. However, the entropy term for the sheep Hb system is not available for comparison. The relatively small entropy change in the Mb system may reflect that only minor conformational changes occur in the protein chain upon CO binding compared to the substrate-bound P-450 system; this results in a much higher binding constant. The binding of CO to the model system (Table IV.4) is equally favourable in terms of ΔH^0 as the binding of CO to substrate-free P-450cam. Therefore, it is conceivable that the Fe-C-O unit is linear (or almost so) in the substrate-free P-450-CO complex.

There are no thermodynamic data available on O_2 binding to P-450 system. In the present work, a large number of attempts were made to determine the $P_{1/2}$ values, at temperatures ranging from 0° to -30°, for the substrate-bound system using the procedure given in Section III.4. None of these experiments were successful because of several difficulties that were not resolved. At temperatures near 0°, the enzyme-O_2 complex was formed, but autoxidized (according to reaction II.3, to give the Fe(III) enzyme) during the time of the experiment. The only

reported $P_{1/2}$ value for O_2 binding to P-450 enzyme at 0° was determined using the same conventional cell tonometer method used in the present study to determine the CO binding constants. It should be noted that the same report gives the half life of autoxidation at 0° as 75 minutes.[11] However, the published [11,20,21,22] rates of autoxidation of the substrate-bound P-450cam-dioxygen complex (Table IV.5) are rather inconsistent; the rates determined in the present work were higher than most of the reported values [Table IV.5; Raw Data in Appendix (iii)].

At lower temperatures (-10° to -30°) the autoxidation reaction was indeed prevented, but the co-solvent system used (ethyleneglycol/water) became more viscous, thus causing small bubbles to form when the slush-bath was shaken to mix the added gas. These bubbles floated to the surface over a long period of time (ca. 1 h), and a single bubble rising to the surface during the recording of a spectrum altered absorption considerably such that the isosbestic points were not observed. After many unsuccessful attempts it was decided that the current approach was not suitable for determination of the $P_{1/2}$ value (and ΔH°, ΔS° values) for the binding of dioxygen to substrate-bound cytochrome P-450cam.

Table IV.5

Half-lives of Autoxidation Reaction of Dioxygen Complex of Substrate-Bound Cytochrome P-450cam.

pH	T (°C)	Half-life (min.)	Ref.
7.0	0.0	75	11
7.4	4.0	45	20
7.4	4.0	115	21
7.4	2.0	21	22
7.4	4.0	18	22
7.4	10.0	9.2	22
7.4	0.0	20	this work
7.4	10.0	6.7	this work

Unfortunately, no equilibrium or kinetic data are available on P-450 model systems that do not use a vast excess of a 'non-innocent' ligand.[23] Mercaptan-tail porphyrins, prepared recently by Collman et al.[23] by covalently attaching a thiol group to the porphyrin so that the thiol can serve as a built-in axial ligand, appear to be good candidates for studies on CO vs. O_2 binding for comparison with P-450 systems in the context of steric considerations.

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APPENDICES

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: 4 ± 0.2° C Buffer: P-100

Vapour Pressure of Water: 79 mm DBT pH: 7.4

Experiment No. 1

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	-	76	-	0.177
2	5	80	4	0.327
3	10	89	13	0.416
4	20	97	21	0.443
5	40	111	35	0.467
6	100	183	107	0.492

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.309	0.150	0.180	0.833	-0.07918	-0.5098
1.01	0.239	0.091	2.626	0.4194	-0.002120
1.62	0.266	0.064	4.156	0.6187	0.2104
2.71	0.290	0.040	7.250	0.8603	0.4323
8.27	0.315	0.015	21.00	1.322	0.9176

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: 4±0.2°C

Buffer: P-100

Vapour Pressure of Water: 79

pH: 7.4

Experiment No. 2

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	-	76	-	0.205
2	5	80	4	0.385
3	10	85	9	0.475
4	15	91	15	0.490
5	25	98	22	0.548
6		-	-	0.063

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.309	0.189	0.245	0.735	-0.1339	-.5100
0.696	0.270	0.155	1.742	0.2410	-0.1600
1.16	0.285	-.140	2.036	0.3087	0.0643 (?)
1.70	0.343	0.082	4.183	0.6215	0.2306

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: $4 \pm 0.2^{o}C$ Buffer: P-100

Vapour Pressure of Water: 79 mm DBT pH: 7.4

Experiment No. 3

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	-	74	-	0.150
2	5	79	5	0.350
3	20	95	21	0.485
4	40	119	45	0.520
5		-	-	0.560

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	$\text{Log}\ \frac{A-A_o}{A_\infty-A}$	$\text{Log}\ P_{co}$
0.387	0.200	0.210	0.9524	-0.0212	-0.4129
1.62	0.335	0.0750	4.466	0.6499	0.2104
3.48	0.370	0.0400	9.250	0.9661	0.5414

Appendix (i) cont'd.

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: 12±0.2°C Buffer: P-100

Vapour Pressure of Water: 135 mm DBT pH: 7.4

Experiment No. 4

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	-	135	-	0.195
2	5	140	5	0.285
3	10	164	29	0.480
4	30	215	80	0.565
5	50	405	270	0.610
6	∞	-	-	0.640

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.387	0.090	0.355	0.254	-0.5960	-0.4129
2.24	0.285	0.160	1.78	0.2507	0.3506
6.18	0.370	0.075	4.93	0.6931	0.7913
20.9	0.415	0.030	13.8	1.141	1.320

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: 12+ 0.2°C Buffer: P-100

Vapour Pressure of Water: 135 mmDBT pH: 7.4

Experiment No. 5

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	-	134	-	0.222
2	5	138	4	0.260 ?
3	10	141	7	0.397
4	20	163	29	0.610
5	50	196	62	0.727
6	150	290	156	0.787
7	∞	-	-	0.860

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.310	0.038	0.600	0.0633	-1.198	-0.5100?
0.541	0.175	0.463	0.378	-0.4225	-0.2668
2.24	0.388	0.250	1.15	0.1909	0.3506
4.79	0.505	0.133	3.79	0.5794	0.6806
12.1	0.565	0.073	7.74	0.8887	1.081

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: Buffer:

Vapour Pressure of Water: pH:

Experiment No. 6

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	-	135	-	0.190
2	10	153	18	0.372
3	20	163	28	0.430
4	40	188	53	0.478
5	60	246	111	0.530
6	∞	-	-	0.582

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
1.39	0.182	0.210	0.867	-0.0621	0.1435
2.16	0.240	0.152	1.58	0.1984	0.3353
4.10	0.288	0.104	2.77	0.4424	0.6125
8.58	0.340	0.052	6.54	0.8155	0.9335

Appendix (1) (cont'd)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: 18± 0.2°C Buffer: P-100

Vapour Pressure of Water: 200 mm DBT pH: 7.4

Experiment No. 7

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	0	192	-	0.220
2	10	200	8	0.325
3	20	219	27	0.470
4	40	268	76	0.595
5	80	313	121	0.635
6	120	371	179	0.685
7	∞	-	-	0.770

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	$\text{Log}\ \frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.618	0.105	0.445	0.236	-0.6270	-0.2087
2.09	0.250	0.300	0.833	-0.0792	0.3195
5.88	0.375	0.175	2.14	0.3310	0.7690
9.35	0.415	0.135	3.07	0.4877	0.9710
13.8	0.465	0.085	5.47	0.7380	1.141

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: Buffer:

Vapour Pressure of Water: pH:

Experiment No. 8

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	0	203	-	0.165
2	10	212	9	0.252
3	20	313	110	0.482
4	40	403	200	0.528
5	80	429	226	0.565 ?
6	∞	-	-	0.585

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.696	0.0870	0.333	0.261	-0.5829	-0.1576
8.50	0.317	0.103	3.08	0.4882	0.9295
15.5	0.363	0.057	6.37	0.8040	1.189
17.5	0.400	0.020	20.0	1.301	1.242

Appendix (1)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: Buffer:

Vapour Pressure of Water: pH:

Experiment No. 9

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	0	205	-	0.225
2	10	216	11	0.310
3	20	221	16	0.348
4	40	239	34	0.400
5	∞	-	-	0.605

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.850	0.085	0.295	0.288	-0.5404	-0.07040
1.24	0.123	0.257	0.479	-0.3200	0.09230
2.63	0.175	0.205	0.854	-0.06872	0.4197

Appendix (i) (cont'd)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: 24±0.2° C Buffer: P-100

Vapour Pressure of Water: 290 mm DBT pH: 7.4

Experiment No. 10

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	0	250	-	0.290
2	15	266	16	0.326
3	25	275	25	0.390
4	40	286	36	0.490
5	65	318	68	0.600
6	100	360	110	0.652
7		-	-	0.826

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
1.23	0.036	0.500	0.0720	-1.1427	-0.1118
1.91	0.100	0.436	0.229	-0.6395	0.2810
2.82	0.200	0.336	0.595	-0.2253	0.4502
5.25	0.310	0.226	1.37	0.1373	0.7201
8.51	0.362	0.174	2.08	0.3182	0.9299

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: Buffer:

Vapour Pressure of Water: pH:

Experiment No. 12

Raw Data

Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	0	263	-	0.190
2	10	365	102	0.415
3	30	454	191	0.455
4	80	533	270	0.480
5		-	-	0.540

Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
7.88	0.225	0.125	1.80	0.2553	0.8968
14.81	0.265	0.085	3.12	0.4938	1.170
20.9	0.290	0.060	4.83	0.4842	1.320

Appendix (i)

Equilibrium Data for CO Binding to Substrate-free Cytochrome P-450cam.

Temperature: Buffer:

Vapour Pressure of Water: pH:

Experiment No. 11

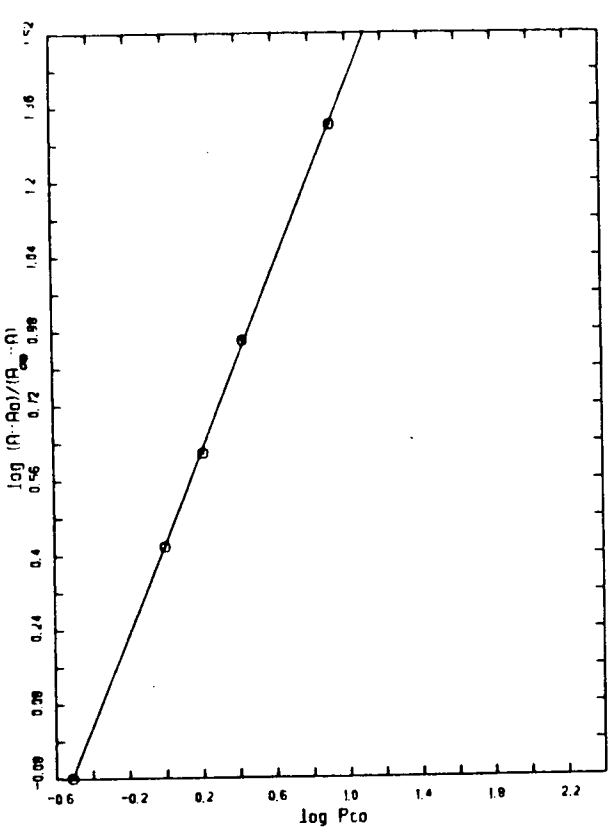
Raw Data

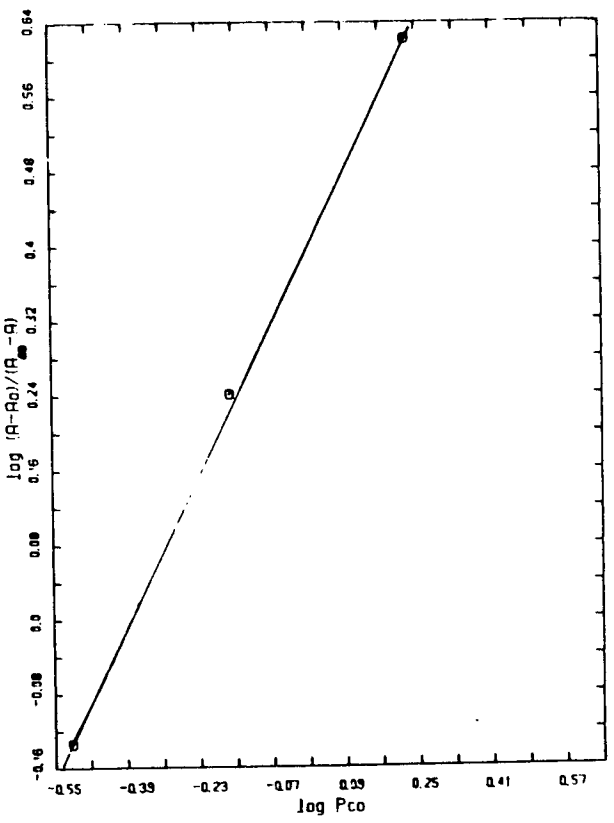
Species	P_{co} wanted (mm DBT)	P_{total} (mm DBT)	P_{co} (mm DBT)	A_{445nm}
1	0	272	-	0.220
2	5	277	5	0.260
3	10	281	9	0.293
4	20	290	18	0.340
5	50	356	84	0.543
6	80	387	115	0.590
7		-	-	0.770

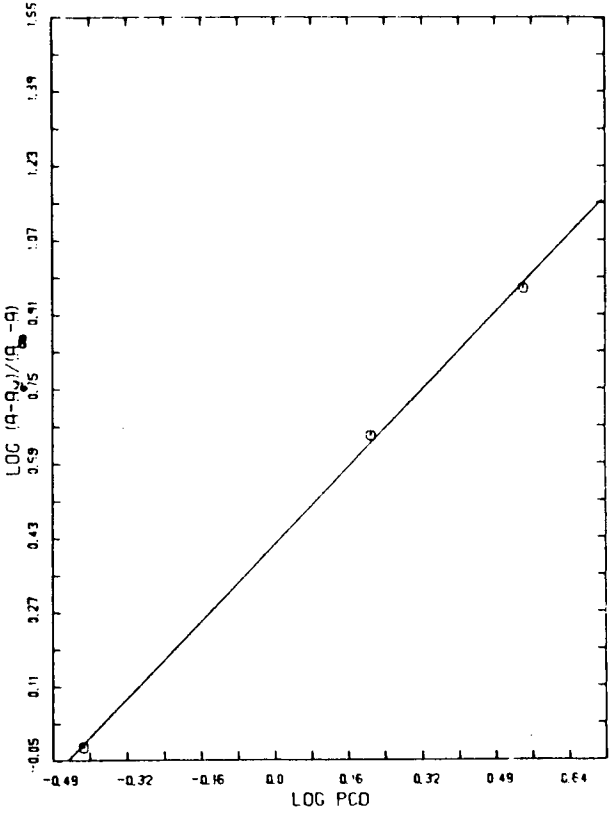
Processed Data

P_{co} (mm Hg)	$A-A_o$	$A_\infty-A$	$\frac{A-A_o}{A_\infty-A}$	Log $\frac{A-A_o}{A_\infty-A}$	Log P_{co}
0.387	0.040	0.510	0.0784	-1.1055	-0.4129
0.696	0.073	0.477	0.153	-0.8152	-0.1577
1.39	0.120	0.430	0.279	-0.5543	0.1435
6.49	0.323	0.227	1.42	0.1532	0.8125
8.89	0.370	0.180	2.06	0.3129	0.9489

Appendix (ii)



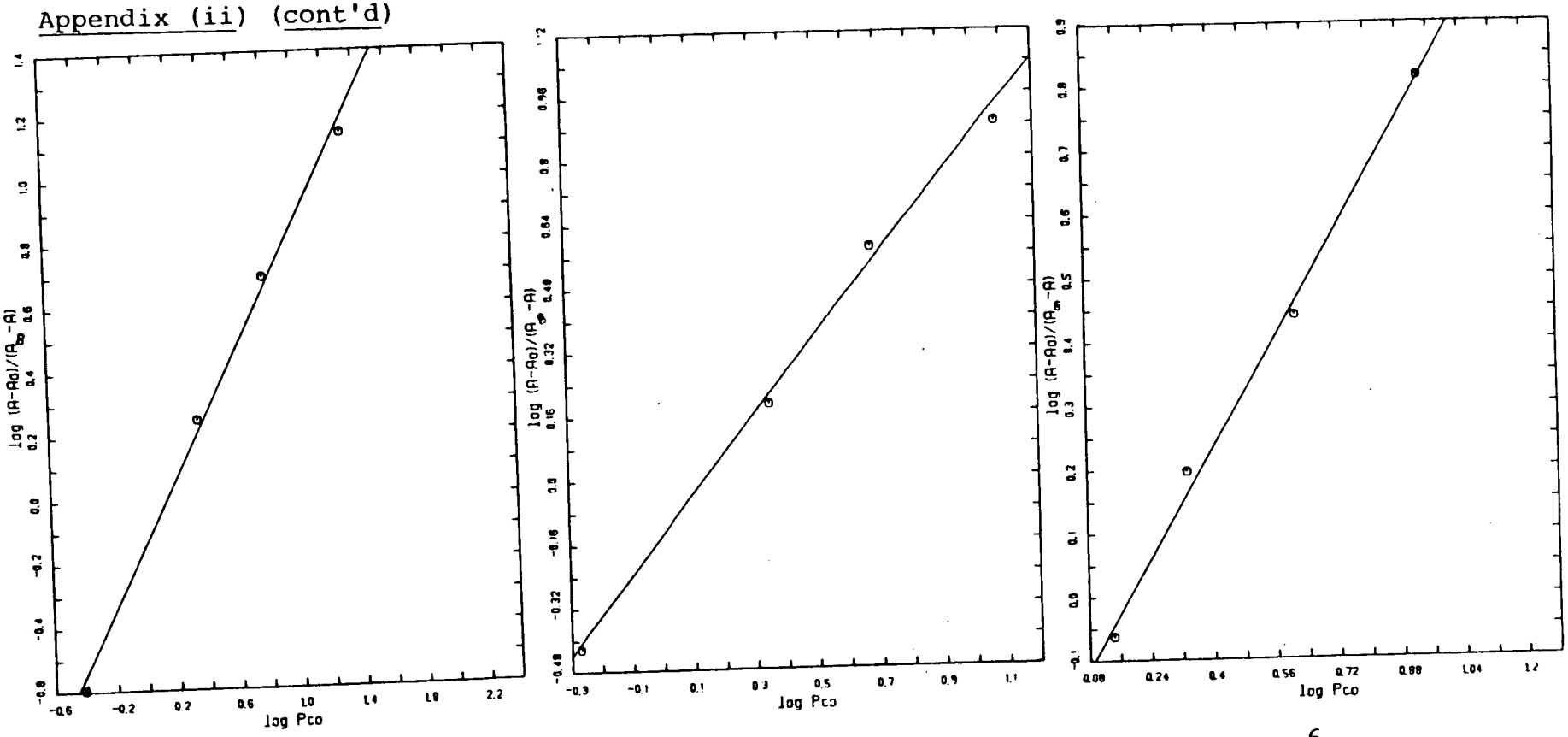




Experiment No. 1 2 3

The Hill log/log plots obtained for the CO-binding to substrate-free cytochrome P-450cam at 4°C.

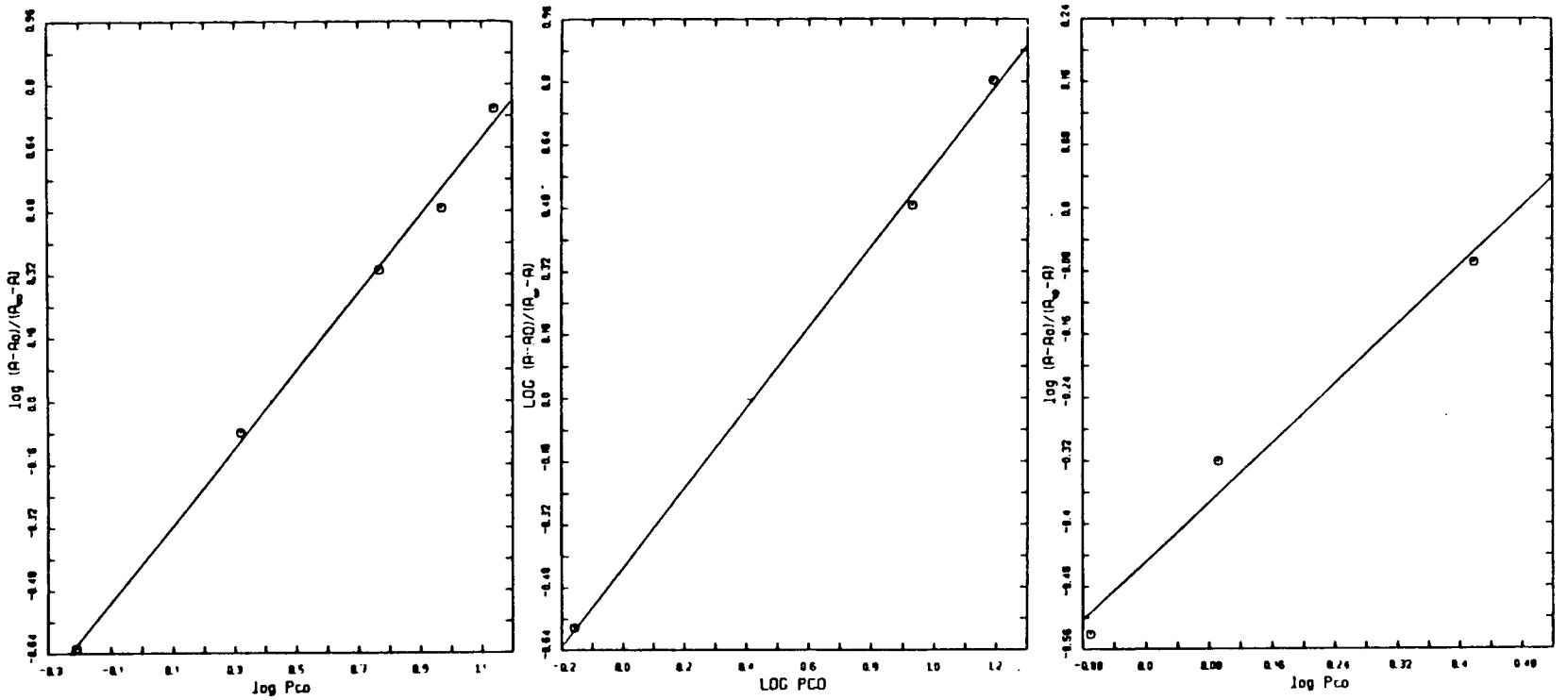
Appendix (ii) (cont'd)



Experiment No. 4 5 6

The Hill log/log plots obtained for the CO-binding to substrate-free cytochrome P-450cam at 12°C.

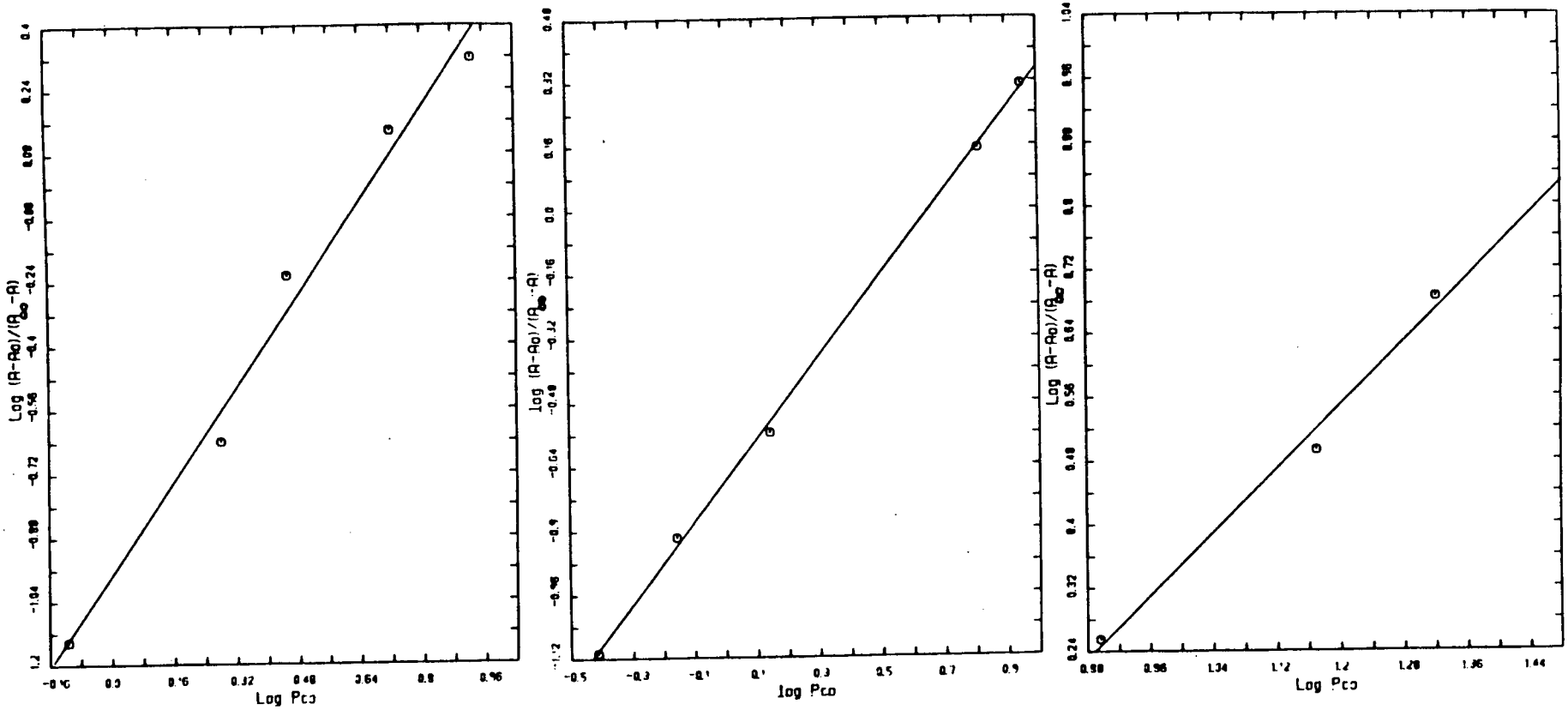
Appendix (ii) (cont'd)



Experiment No. 7 8 9

The Hill log/log plots obtained for the CO-binding to substrate-free cytochrome P-450cam at 18°C.

Appendix (ii) (cont'd)



Experiment No. 10 11 12

The Hill log/log plots obtained for the CO-binding to substrate-free cytochrome P-450cam at 24°C.

Appendix (iii)

Determination of rate of autoxidation of the dioxygen complex of reduced, substrate-bound cytochrome P-450cam.

Raw Data

Expt. No. : 1
Temperature : 0 ± 0.2 °C
Buffer : P-100 A_{∞}=1.68

A	A_{∞}-A	-ln(A_{∞}-A)	Time (min.)
1.28	0.396	0.9263	1.0
1.45	0.230	1.4697	15
1.50	0.176	1.7323	20
1.55	0.130	2.0402	30
1.58	0.100	2.3025	36
1.62	0.0560	2.8824	55

Expt. No. : 2
Temperature : 0 ± 0.2°C
Buffer : P-100 A_{∞}=1.09

A	A_{∞}-A	-ln(A_{∞}-A)	Time (Min)
0.790	0.306	1.1841	1.0
0.856	0.240	1.4271	7.0
0.920	0.176	1.7372	16
0.996	0.100	2.30 6	30
1.05	0.0460	3.0790	54

Expt. NO. : 3
Temperature : 10 ± 0.2°C
Buffer : P-100 A_{∞}=1.63

A	A_{∞}-A	-ln(A_{∞}-A)	Time (Min.)
1.38	0.254	1.3704	1.0
1.48	0.146	1.9241	5.0
1.53	0.100	2.3026	9.0
1.57	0.0600	2.8134	14
1.61	0.0200	3.9120	24