ENZYME DESIGN FOR THE STEADY-STATE OF METABOLISM

BY

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ABSTRACT

The steady-state of metabolism has been investigated. The equilibrium thermodynamic properties of metabolic enzymes have been related to their role in the steady-state of metabolism. Simplified free energy profiles that include the reverse reaction have been constructed for the enzyme fumarase under a variety of conditions using the thermodynamic interpretation of the Haldane relationship. The difference in the Gibbs free energy change of activation forward compared to that of the reverse direction (ΔG_R) has been found not to equal the Gibbs free energy change reactants to products. The binding of substrate may be perturbed independently of the catalytic event. It is suggested that this is important in the determination of ΔG_R. ΔG_R may be perturbed to a considerable extent in fumarase. The importance of ΔG_R is suggested to lie in the steady-state of metabolism. Many steady-states, giving different affinities are theoretically possible. Biological systems have selected a continuous steady-state where ΔG_R is numerically equal to affinity over a range of velocities. In order to achieve this steady-state two important conditions must be imposed on the design of the two enzymes sharing a given substrate. The first is that the Km for the shared substrate must be the same in both enzymes. The second is that enzyme concentration must be adjusted. When these two constraints are imposed on the design of the enzymes the magnitude of the steady-state affinity equals the size of ΔG_R.

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CHAPTER 1

GENERAL INTRODUCTION

The steady-state of metabolism is one of the simplest forms in which metabolism may be discussed. There are many examples of studies of steady-state transitions (Roberts,1977) but few studies of the basic properties of the steady-state itself. In the case of metabolism the steady-state is that state wherein the concentration of metabolites and their rates of interconversion are constant. One basic question stemming from this definition is how this homeostasis is achieved. How are enzyme concentrations of two sequential enzymes in a pathway related? Why are some enzymes, notably the regulatory enzymes, present in lower activity than non-regulatory enzymes? Similarly, it is not known what kinetic parameters are crucial to the achievement of the metabolic steady-state. Mass action effects, cofactor availability and feedback inhibition have been variously invoked to explain aspects of metabolic regulation (Atkinson,1977). The steady-state of metabolism presents an example of the principle of least action upon which much of our understanding of the laws of physics rest. As such, the steady-state may simply be a design feature of the non-regulatory enzymes in a pathway. If so it may not be necessary to postulate other effectors to explain the steady-state. The purpose of this thesis is to describe some of the properties of the steady-state of metabolism and what features of enzyme design are required to produce the steady-state of metabolism. The nature of the steady-state itself needs further definition. Is it stable or

unstable? When a transition from one steady-state to another occurs is it along a predictable and stable path?

Chemical systems are affected by the same forces which affect most physical systems. Of these, temperature and pressure are the most frequently investigated. Chemical systems are also subject to another force which has been called the affinity (affinity=ΔG = RT ln(mass action ratio/Keq). De Donder and van Rysselberghe (1936) have adequately shown that this force has all the attributes of a state function . Most of the previous work on enzyme thermodynamics has concentrated on the effects of temperature and pressure on enzyme design and function. While temperature and pressure do affect biological systems it is the affinity which provides the major driving force for living systems. To date I am aware of no work on the role of the affinity of a chemical reaction on the design of the enzyme catalyzing it. Rottenberg (1973) has investigated the relationship between the rate of an enzyme reaction and its driving force or affinity. He has shown the rate of reaction to be a linear function of the affinity over a range of more than 2 kcal/mol. It is important therefore, to investigate the relationship between the affinity of a chemical reaction in vivo and the enzymes that catalyze it.This must be done within the constraints imposed by the concept of the steady-state.

It is widely assumed that enzymes behave essentially as other catalysts. All of the known catalysts function to speed the approach to equilibrium. All enzymes of course, do this when outside of the organism (exoenzymes or others in vitro) but there is a large class of enzymes which are designed to function

inside of the cell ; they are designed not to simply speed the approach to equilibrium but to control the rate of flux and maintain the concentrations of all metabolically useful compounds constant. Although metabolic enzymes are clearly capable of function at different steady-states, most previous work on metabolic regulation has emphasized instead the transient state (Atkinson,1977). From this has arisen the idea that metabolic enzymes fall into two general categories which are termed regulatory and non-regulatory; the former being subject to activation or inhibition by various metabolic 'signals' (hormones, cations, metabolites etc.) are considered to play a much more important role in metabolic regulation than do non-regulatory enzymes. In particular any given steady-state, it is proposed (Newsholme and Start, 1973; Atkinson, 1977) is maintained by a balance between substrate availability, modulator concentration, and other such factors. As I shall show in greater detail later (Chapter 5), this role for regulatory enzymes is critical but is insufficient to explain how non-regulatory enzymes pace each other and pace regulatory ones. For this reason, and because this study represents an initial exploration of the problem, I will mainly concentrate on the functional properties of non-regulatory enzymes requisite for the development and maintenance of any given steady-state.

Implicit in an understanding of the steady-state is that it is a flux situation. At every point in a pathway catalyzed by an enzyme the net flux through that point is described by the difference between the flux produced by the forward reaction and the flux produced by the reverse reaction. This simplest concept

has been almost universally ignored in the study of enzyme design. To understand the steady-state of metabolism it is therefore necessary to understand the functional design of the enzymes which govern it. Both forward and reverse reactions must be taken into account and related.

These relationships have been analyzed using computer simulations of model systems. Using this approach some of the design features of metabolic enzymes have been determined. In this general context, then, I set out to answer the following questions.

1) What relationships exist between the thermodynamic properties of sequential non-regulatory enzymes in a metabolic pathway allowing function at different steady-states?

2) What relationships exist between the kinetic properties of sequential non-regulatory enzymes in a metabolic pathway allowing function at different steady-states?

3) How are the concentrations of sequential enzymes related to allow function at different steady-states?

4) Does the affinity for any given reaction change at different steady-states?

5) Is the steady-state perturbed by fluctuations in concentration of pathway intermediates or is it a stable state?

6) Why do the maximum activities of enzymes in any given pathway vary by up to four orders of magnitude (Guppy et al.,1979) when in the steady-state they all must function at the same rates?

I found remarkably simple answers to all these questions by approaching them with the use of model systems. In the first place, I used fumarase as a 'model' non-regulatory enzyme. A

thermodynamic approach was employed for this part of the thesis since it provides a way of comparing different types of processes in terms of the same units (calories). In this part of study I found that the difference in the Gibbs free energy of activation in the forward direction compared to the reverse direction (ΔG_R) is not necessarily equal to ΔG°. That this observation had metabolic impact I established by comparing the value of ΔG_R with in vivo affinity for a series of twelve metabolic enzymes. This part of the study showed that ΔG_R is numerically equal to the in vivo affinity for the reaction.

To explore how this sort of steady-state may be achieved in vivo , I used a computer modeling scheme for two sequential non-regulatory enzymes and found that two constraints must be imposed on such a sequence of enzymes to allow steady-state function wherein ΔG_R is equal numerically to affinity. The first is that the Km for the shared substrate must be the same; the second is that the concentrations of the two enzymes must be appropriately adjusted with respect to each other.

CHAPTER 2

EQUILIBRIUM THERMODYNAMICS OF ENZYMES

INTRODUCTION

For the simplest fully reversible enzyme catalyzed reaction is:

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EB \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + B \qquad (2\text{-}1)$$

Where $Vmaxf = EAk_2$, $Vmaxr = EBk_{-2}$, $Ka = k_1/k_{-1}$ and $Kb = k_3/k_{-3}$. The net flux of A to B is given by:

$$v = \frac{Vmaxf([A]/Ka) - Vmaxr([B]/Kb)}{1 + ([A]/Ka) + ([B]/Kb)} \qquad (2\text{-}2)$$

Equation (2-2) describes the rate of reaction in the presence of both reactants and product. When one or the other is totally absent equation (2-2) reduces to the Michaelis-Menten equation. At equilibrium the flux is zero. If the left hand side of equation (2-2) is set equal to zero and rearranged the following equation is obtained:

$$[B]/[A] = Keq = \frac{VmaxfKb}{VmaxrKa} \qquad (2\text{-}3)$$

Equation (2-3) is the Haldane relationship (Haldane, 1930) for this type of reaction. Haldane relationships exist for all types of enzyme catalyzed reactions. The Haldane relationship has previously only been used to verify the validity of kinetic

determinations of its constituent constants or to determine the Keq of reactions in which it is otherwise difficult (Purich et al., 1973).

Since equation (2-3) represents the equilibrium state, it may be simply expressed in thermodynamic terms:

$$RT \ln Keq = RT \ln \frac{(VmaxfKb)}{(VmaxrKa)} \qquad (2\text{-}4)$$

If equation (2-4) is expanded and the terms separated

$$RT \ln Keq = RT \ln Vmaxf + RT \ln Kb - RT \ln Vmaxr - RT \ln Ka) \qquad (2\text{-}5)$$

rearranging (2-5) gives,

$$RT \ln Keq = RT \ln (Vmaxf/Ka) - RT \ln (Vmaxf/Kb) \qquad (2\text{-}6)$$

Fersht (1977) described the relationship between the free energy of binding (ΔG^b) and the free energy of activation ($\Delta G^{\neq}$) in terms of kcat and Km. Since,

$$Vmax = kcat/(\text{enzyme concentration}). \qquad (2\text{-}7)$$

$$RT \ln (kcatf/Ka) = RT \ln (kT/h) - (\Delta G_f^{\neq} + \Delta G_a^b) \qquad (2\text{-}8)$$

The left hand side of equation (2-8) closely resembles either part of the right hand side of equation (2-9) separated by the minus sign.

Since Fersht worked with irreversible proteases this type of equation adequately describes those enzymes. Most metabolic enzymes however, are reversible and the reverse reaction may also be described by a Fershtian equation with opposite sign:

$$-RT \ln (kcatr/Kb) = -RT \ln (kT/h) + (\Delta G_r^{\neq} + \Delta G_b^b) \qquad (2\text{-}9)$$

Substituting (2-6), (2-8) and (2-9) in (2-6) gives:

$$RT \ln Keq = RT \ln (kT/h) - (\Delta G_f^{\neq} + \Delta G_a^b) - RT \ln (kT/h) \qquad (2\text{-}10)$$

$$+ \left(\Delta G_r^{\neq} + \Delta G_b^{b} \right)$$

Simplifying (2-10)

$$RT \ln Keq = \Delta G_r^{\neq} + \Delta G_b^{b} - \left(\Delta G_f^{\neq} + \Delta G_a^{b} \right) \qquad (2\text{-}11)$$

The terms kT/h represents the rate of decay of the activated complex and is the same for all chemical reactions (Johnson, Eyring and Stover, 1974). This term disappears in equation (2-11) since it is identical but of opposite sign for the reverse reaction. Since,

$$-RT \ln Keq = \Delta G^{\circ} \qquad (2\text{-}12)$$

equation (2-11) may be rearranged and written,

$$\Delta G_f^{\neq} + \Delta G_a^{b} = \Delta G_r^{\neq} + \Delta G_b^{b} + \Delta G^{\circ} \qquad (2\text{-}13)$$

Equation (2-13) describes the free energy profile for an enzyme catalyzed reaction as depicted in Figure 3-1.

The Gibbs free energy of binding of both substrate and product are negative as is ΔG°. The free energies of activation in both the forward and reverse directions and positive.

In a similar way the Gibbs free energy profiles may be derived from the Haldane relationships of more complex reactions. Table 2-1 presents the thermodynamic equations describing these reactions at equilibrium. The terminology uni-uni, bi-bi, etc. is that of Cleland (1970) and refers to the number of kinetically important reactants in the forward and reverse directions (ie. One reactant in the forward and one in the reverse is designated a uni-uni mechanism; two reactants in the forward and one in the reverse is a bi-uni mechanism and so on). In the case of bi-reactions the order of binding of reactants may be <u>random</u> or <u>ordered</u> . The <u>Theorrell-Chance</u>

mechanism is similar to the ordered bi-bi type except that the concentration of the ternary complex is vanishingly low. If all substrates bind before any products are released the reaction is termed sequential and if some product is released before all substrates have bound the mechanism is termed ping-pong. The Michaelis constants for all substrates and products are assumed to equal the dissociation constants. The occurence of inhibitory ternary complexes in these mechanisms results in the coefficients 1/2 before the binding free energies of that ligand. The strength of binding of the substrate to its "proper" site is not the same as binding in the unproductive ternary complex. The effective binding of the particular ligand is therefore the average of the proper binding and the inhibitory binding, thus the sum of the free energies of binding with coefficients of 1/2.

The Haldane relationship therefore provides a useful approach to understanding the equilibrium charactersitics of enzymes. A study of the properties of enzymes at equilibrium is essential to an understanding of their function in situations displaced from equilibrium and will ultimately lead to an understanding of their function in the steady-state of metabolism.

Table 2-1 Haldane relationships and thermodynamic interpretations of several enzyme reaction mechanisms

Mechanism	Haldane *	Thermodynamic interpretation
Theorell-Chance Non-sequential Ping-pong Random bi-bi	$Keq = \frac{V1KpKipKqKiq}{V2KaKiaKbKib}$	$\Delta G^\circ = \Delta G_f^{\ddagger} + 1/2\Delta G_a^b + 1/2\Delta G_a^{ib} + 1/2\ G_b^b + 1/2\ G_b^{ib} - (\Delta G_r^{\ddagger} + 1/2\Delta G_p^b + 1/2\Delta G_p^{ib} + 1/2\Delta G_q^b + 1/2\Delta G_q^{ib})$
Ordered uni-bi	$Keq = \frac{V1KpKipKqKiq}{V2KaKia}$	$\Delta G^\circ = \Delta G_f^{\ddagger} + 1/2\Delta G_a^b + 1/2\Delta G_a^{ib} - (\Delta G_r^{\ddagger} + 1/2\Delta G_p^b + 1/2\Delta G_p^{ib} + 1/2\Delta G_q^b + 1/2\Delta G_q^{ib})$
Bi-uni	$Keq = \frac{V1KpKip}{V2KaKiaKbKib}$	$\Delta G^\circ = \Delta G_f^{\ddagger} + 1/2\Delta G_a^b + 1/2\Delta G_a^{ib} + 1/2\Delta G_b^b \cdot + 1/2\Delta G_b^{ib} - (\Delta G_r^{\ddagger} + 1/2\Delta G_p^b + 1/2\Delta G_p^{ib})$

* see list of abbreviations at end of thesis for descriptions of parameters.

CHAPTER 3

THE RELATIONSHIP BETWEEN BINDING AND CATALYSIS

Introduction

An important characteristic of a catalyst is its ability to lower the Gibbs free energy of activation. Haldane (1930) stated that enzymes (and other catalysts) cannot alter the true equilibrium position of a reaction. He also stated that enzymes accelerate the reverse reaction to the same extent as the forward giving several examples in which this was shown. Since enzymes bind substrates and products with differing affinities it would seem likely that independent perturbation of binding of substrate and product could occur.

Surprisingly, the relationships between the two enzyme properties, termed Km and kcat respectively (the binding and catalytic constants for the enzymes displaying Michaelis kinetics) have not been clarified for both the forward and the reverse reactions. For enzymes whose function is to turn over large quantities of substrate quickly, Fersht (1977) and Crowley (1975) envisaged that the ratio of kcat/Km is high in order to maximize reaction rates. Their conclusion, implying a direct relationship between kcat and Km, was based on catalysis by proteases in only one direction; the consequences to the reverse reaction of maximizing rates in the forward direction were not considered. Other workers (Cornish-Bowden, 1975) have concluded that maximizing kcat/Km is not necessarily advantageous and, on theoretical grounds, have shown that Km and kcat can be treated

as independent variables. However, the possible relationships between Km and kcat were not investigated experimentally.

As a first approach to improving our understanding of the relationships between Km and kcat, a relatively simple metabolic enzyme, fumarase (E.C. 4.2.1.2) was selected which catalyzes the reversible hydration of fumarate.

$$\text{fumarate} + H_2O \underset{\text{reverse}}{\overset{\text{forward}}{\rightleftharpoons}} \text{malate}$$

A variety of conditions were used to perturb either Km or kcat, or both, in order to explore the relationship between them.

MATERIALS AND METHODS

Enzyme assays. Purified pig heart fumarase (E.C.4.2.1.2) was obtained from the Sigma Chemical Co., St.Louis, Mo. It was assayed in a 50 mM potassium phosphate buffer at pH 7.3 unless otherwise stated. The change in absorbance at 240 nm in the malate direction using the millimolar extinction coefficient 2.44 and at 280 nm in the fumarate direction with the millimolar extinction coefficient 0.26 was followed with a Unicam SP 1800 recording spectrophotometer. Cuvette temperature was kept constant ($\pm$0.1 ° C) with a Lauda constant temperature bath and circulator.

Kinetic and thermodynamic calculations . Km and Vmax were determined in triplicate at five temperatures from 20° C to 40° C using the modified Cornish-Bowden/Eisenthal plot (Cornish-

Bowden and Eisenthal, 1978). An example is presented in Figure 3-1. Substrate concentrations ranged from one tenth of the Km to two or three times the Km. $\Delta G°$ was determined from the Keq using the relationship $\Delta G° = -\{RT \ln(\gamma_m/\gamma_f) + RT \ln Keq\}$. Since the pKa of malate and fumarate are similar (Sober, 1970) the activity coefficients γ_m and γ_f were assumed to be equal. The relationship then simplifies to $\Delta G° = - RT \ln Keq$. Keq for the reaction was determined in both the forward and reverse directions. The Gibbs free energy of binding was determined from the Km values using the relationship $\Delta G^b = - RT \ln (1/Km)$. The Km is expressed in molar units.

Fumarase obeys Michaelis-Menten kinetics at substrate concentrations below five times the Km (Hill and Teipel, 1971) and equilibrium dialysis binding studies (Teipel and Hill, 1968) indicate that the Km is equal to the dissociation constant. The kcat was determined from Vmax using kcat/(enzyme concentration). The Gibbs free energy of activation was determined using the method of Low et al. (1973) where:

$$\Delta H = Ea - RT$$

Ea was determined from Arrhenius plots of log kcat versus $1/T$ using the least squares regression analysis.

$$\Delta S = 4.576 (\log kcat - 10.753 - \log T + Ea / 4.756 T)$$

where kcat (in 1/sec.) = Vmax/mg enzyme x molecular weight of the enzyme (194,000 for fumarase) x 10 mmol/ mol. X 1min./60 sec. ΔS and ΔH were substituted into the following equation to obtain ΔG .

$$\Delta G = \Delta H - T\Delta S$$

RESULTS AND DISCUSSION

Perturbation of ΔG^b and $\Delta G^{\ddagger}$. By perturbing the reaction catalyzed by fumarase with salt, pH or temperature it is possible to alter ΔG^b and $\Delta G^{\ddagger}$ .If the relationship between ΔG^b and $\Delta G^{\ddagger}$ is not fixed by chemical necessity then the two can change independently and four primary relationships are possible:

1. Increasing $\Delta G^{\ddagger}$ with decreasing ΔG^b
2. No change in $\Delta G^{\ddagger}$ with increasing ΔG^b
3. Increasing $\Delta G^{\ddagger}$ with no change in ΔG^b
4. Increasing $\Delta G^{\ddagger}$ with increasing ΔG^b

The five other possiblities are simply the opposites of the above plus the case where no change in either parameter occurs. Since no hysteresis has been observed under any of these conditions, it is assumed that all nine are possible but only four are conceptually important.

For fumarase, it was possible to obtain all four patterns (Table 3-1). The first and second relationships occur in the reverse reaction with salt and temperature transitions respectively; the third and fourth relationships hold in the forward reaction during the same transitions.

The relationship between ΔG^b and $\Delta G^{\ddagger}$. The fact that all possible combinations of changes in ΔG^b and $\Delta G^{\ddagger}$ were obtained (Table3-1) can best be explained by assuming that the active site contains two functional domains, the first concerned with substrate binding and the second with catalysis. This concept agrees with other studies (Hill and Teipel, 1971) which consider the fumarase active site to be specialized into binding regions

(interacting with the carboxyl groups of the substrate) and catalytic regions (involved in hydration or dehydration of fumarate or malate respectively). In such event, the possibility of changes in ΔG^{b} independent of changes in $\Delta G^{\neq}$ can easily be envisaged.

Although the model is useful in understanding the specific relationships observed between ΔG^{b} and $\Delta G^{\neq}$ for fumarase, it is widely applicable since a characteristic of many, if not all enzymes is the spatial and functional separation of binding and catalysis (Holbrook et al., 1976). Such a separation may be an important design constraint on the active site of enzymes for without it, perturbation of ΔG^{b} should always lead to similar changes in $\Delta G^{\neq}$ and vice versa, a situation that would not allow for the independent modulation of binding and activation. Independent binding and catalysis should allow greater flexibility in the design of enzymes suited to function in different environments, since adjustments may be made in either parameter without necessarily affecting the other. In addition it may facilitate the design of metabolic enzymes in which strong binding interactions are important without sacrificing catalytic efficiency.

Free energy profiles Simplified free energy profiles for the reaction catalyzed by fumarase (Figure 3-2) can be constructed using the equation:

$$\Delta G^{\neq}_{fum} + \Delta G^{b}_{fum} = \Delta G^{\neq}_{mal} + \Delta G^{b}_{mal} + \Delta G^{\circ} \qquad (3\text{-}1)$$

Because ΔG^{b} and $\Delta G^{\neq}$ were determined for both the forward and the reverse reactions, it is important to point out that the ΔG°

calculated from equation (3-1) is in good agreement with the value determined experimentally, independently of either ΔG^{b} and $\Delta G^{\neq}$. This equivalence supplies an independent check of the validity of ΔG^{b} and $\Delta G^{\neq}$ estimates and of the free enrgy profile itself (Table 3-2). An analysis of the free energy profiles for fumarase under a variety of conditions (Table 3-2) reveals several points of interest. The binding of substrates proceeds with negative Gibbs free energy. The Gibbs free energy of activation in both the forward and reverse direction is positive. The difference in the Gibbs free energies of activation of the forward compared to the reverse directions (ΔG_R) does not equal the free energy drop of reactants to products ($\Delta G°$), a result of the binding of ligands in the reaction. Chapter 4 will deal with the mechanism by which this is achieved. This non-equivalence of ΔG_R and $\Delta G°$ in enzyme catalyzed reactions appears to have escaped general notice since most kinetic data are not expressed in thermodynamic terms, where the relationship is most obvious.

The independent modulation of binding and catalysis is a crucial prerequisite for design of reversible enzymes which must function in a steady-state.Other features required for complete freedom of design will be dealt with in the next chapter.

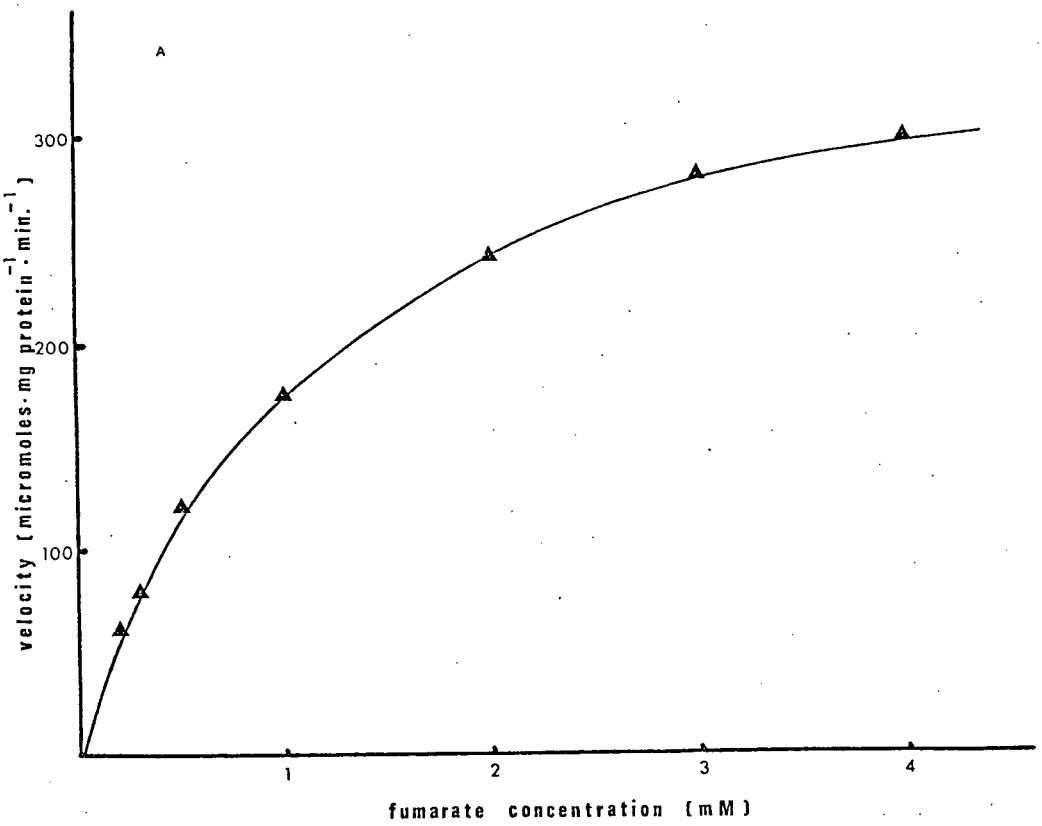
Table 3-1. The effects of perturbing conditions on $\Delta\Delta G^{\neq}$ and $\Delta\Delta G^{b}$ for the fumarase reaction. Two kinds of step-wise transitions with and without 0.25 M salt, and low vs. high temperature were utilized. $\Delta\Delta G^{\neq}$ and $\Delta\Delta G^{b}$ were obtained from the difference in thermodynamic parameters obtained in the intial state and under the perturbing conditions. All assay conditions and calculations are given in the text. Values are subtracted means for which the maximal standard error is ± 2.7%.

Transition conditions	$\Delta\Delta G^{\neq}$ (cal/mol)	$\Delta\Delta G^{b}$ (cal/mol)
malate, pH 7.3, 30°C 0-0.25 M KCl	945	-645
malate, pH 8.0 20-40°C	5	220
fumarate, pH 7.3, 30°C 0-0.25 M KCl	210	-10
fumarate, pH 8.0 20-40°C	270	220

Table 3-2. Gibbs free energy changes of binding and activation under various conditions. Assay conditions and calculations are given in the text. The calculated values for ΔG° were obtained using equation (3-1) Values given are means ± standard error.

Conditions	ΔG_f^b	$\Delta G_f^{\ddagger}$	ΔG_m^b	$\Delta G_m^{\ddagger}$	ΔG° calculated	ΔG° observed	ΔG_R
	cal/mol	cal/mol	cal/mol	cal/mol	cal/mol	cal/mol	cal/mol
pH 7.3,							
20°C	-4010 ± 42	13100 ± 76	-3235 ± 151	13425 ± 95	-1100 ± 81	-930 ± 10	-325 ±110
30°C	-3980 ± 36	13245 ± 51	-3375 ± 72	13510 ± 74	-865 ± 55	-790 ± 16	-265 ± 46
40°C	-3915 ± 35	13420 ± 51	-3275 ± 57	13560 ± 61	-780 ± 52	-680 ± 20	-140 ± 34
pH 7.3,+ 0.25 M KCl							
20°C	-4025 ± 78	13420 ± 132	-4150 ± 44	14545 ± 76	-1000 ± 85	-975 ± 20	-1125 ± 159
30°C	-3985 ± 54	13455 ± 93	-4020 ± 91	14455 ± 101	-965 ± 99	-835 ± 23	-1000 ± 139
40°C	-3565 ± 212	13465 ± 115	-3980 ± 39	14410 ± 122	-525 ± 330	-725 ± 21	-945 ± 219
pH 8.0,							
20°C	-4135 ± 61	13405 ± 56	-3050 ± 22	13165 ± 23	-845 ± 29	-875 ± 18	240 ± 34
30°C	-4075 ± 53	13600 ± 14	-2835 ± 90	13150 ± 38	-795 ± 77	-785 ± 24	450 ± 24
40°C	-3865 ± 73	13625 ± 115	-2775 ± 74	13170 ± 111	-640 ± 23	-700 ± 25	455 ± 54

Figure 3-1 Example of velocity versus substrate concentration a) and modified Cornish-Bowden/Eisenthal plots b) for Km and Vmax determinations with fumarase.


A
300
200
100
velocity [micromoles·mg protein^{-1}·min.$^{-1}$]
1
2
3
4
fumarate concentration (mM)

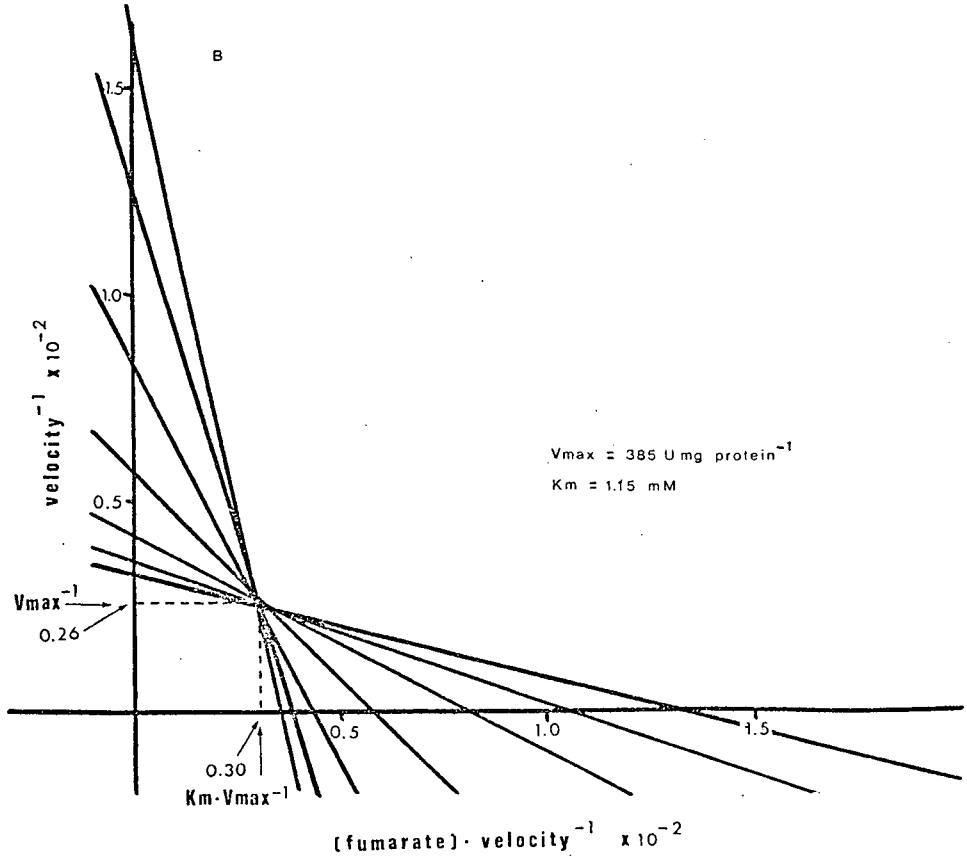
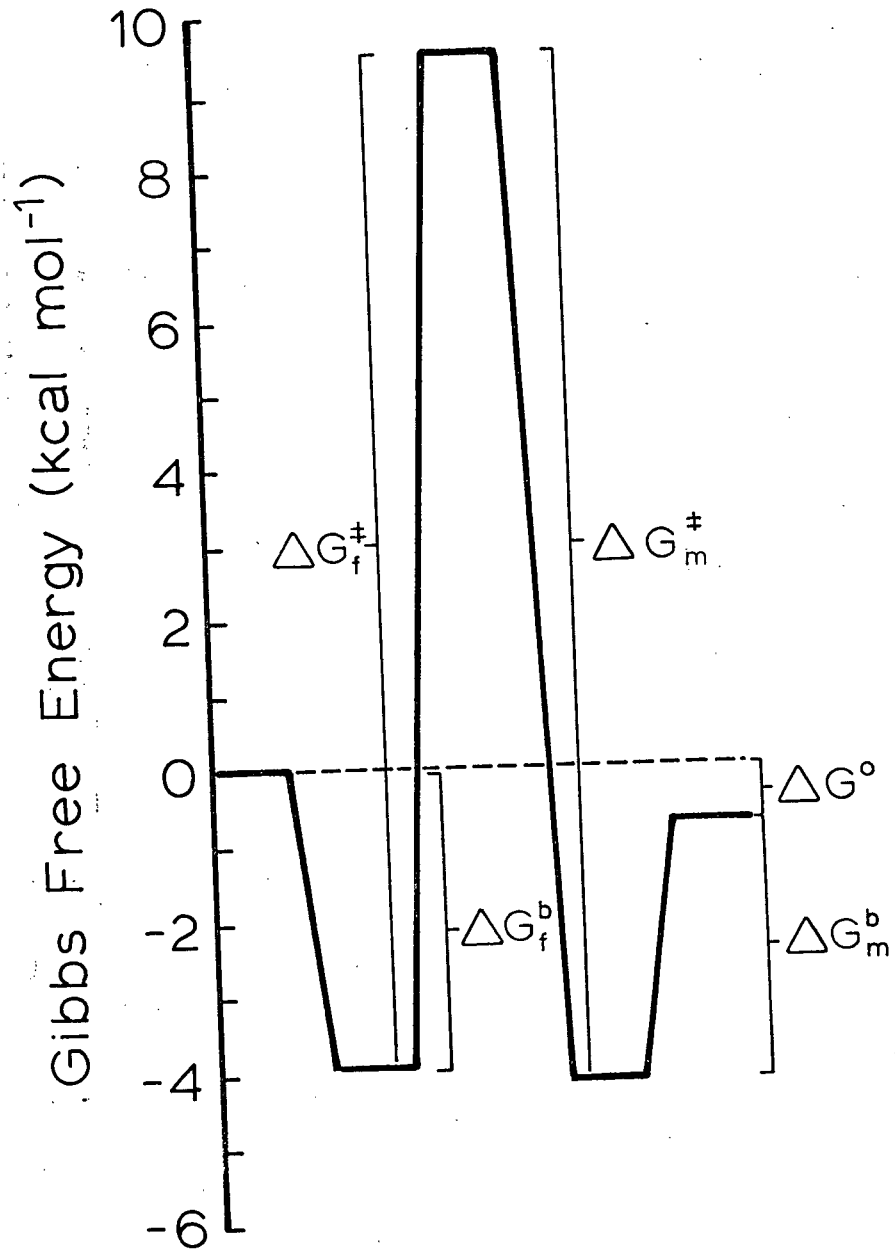

B
1.5
1.0
0.5
velocity^{-1} x 10^{-2}
Vmax = 385 U mg protein^{-1}
Km = 1.15 mM
Vmax^{-1}
0.26
0.5
1.0
1.5
0.30
Km·Vmax^{-1}
[fumarate]·velocity^{-1} x 10^{-2}

Figure 3-2. Simplified free energy profile for the reaction catalyzed by fumarase at 40°C, pH 7.3, and 50 mM KPO4. Symbols are explained in the text.


Gibbs Free Energy (kcal mol^{-1})
10
8
6
4
2
0
-2
-4
-6
$\Delta G_f^{\ddagger}$
$\Delta G_m^{\ddagger}$
ΔG°
ΔG_f^{b}
ΔG_m^{b}

CHAPTER 4

INDEPENDENT MODULATION OF FORWARD AND REVERSE BINDING

Introduction

The observation made in the last chapter that $\Delta G_R \neq \Delta G^\circ$ is worthy of further consideration. Firstly, it is a widespread phenomenon of metabolic enzymes as Table 4-1 demonstrates. This implies that such a design feature must have important functions in metabolism.

The theorem that:

$$k1/k\text{-}1=Keq \qquad (4\text{-}1)$$

has been widely assumed to hold not only for chemical reactions but also for enzyme catalyzed reactions. Early workers (Oesper and Meyerhof, 1950) even used this method to calculate the Keq for an enzyme catalyzed reaction using Vmax values for the forward and reverse reactions as the rate constants k1 and k-1 respectively. Gadsby et al. (1946) dealing with heterogenous catalysis and later Manes, Hofer and Weller (1950) and Horiuti (1957) suggested that equation (4-1) is a special case of the more general equation

$$(k1/k\text{-}1)^z = Keq \qquad (4\text{-}2)$$

In the case of enzyme catalyzed reactions the factor z may be expressed as

$$Z = \frac{\ln(Kb)(k\text{-}1)}{(Ka)(k1)} + 1 \qquad (4\text{-}3)$$

Table 4-1. Comparison of ΔG_R* and $\Delta G°$ for several enzyme catalyzed reactions.

Enzyme	ΔG_R (cal/mol)	Ref.	$\Delta G°$ (cal/mol)	Ref.
phosphoglucomutase	-618	1	-1746	1
pyruvate kinase	-2410	2	-5263	3
phosphoglycerate kinase	-613	4	-4767	4
lactate dehydrogenase	-850	5	-4955	5

* ΔG_R was determined from published Vmaxf/Vmaxr using the equation ΔG_R = RT ln (Vmaxf/Vmaxr), where Vmaxf and Vmaxr are determined at the same concentrations of enzyme.

1. Lowry,O.H. and J.V.Passonneau.1969. Phosphoglucomutase with the phosphates of fructose, glucose, mannose, ribose, and galactose. J.Biol.Chem. 244:910-916.

2. Dyson,R.D.,J.M.Cardenas, and R.J.Barsotti.1975. The reversibility of skeletal muscle pyruvate kinase and as assessment of its capacity to support glyconeogenesis. J.Biol.Chem. 250:3316-3321.

3. McQuate,J.T. and M.F.Utter. 1959. Equilibrium and kinetic studies of the pyruvate kinase reaction. J.Biol.Chem.234:2151-2157.

4. Krietsch,W.K.G. and T.Bucher. 1970. 3-phosphoglycerate kinase from rabbit skeletal muscle and yeast. Eur.J.Biochem. 17:568-580

5. Ballantyne,J.S. unpublished data.

When equation (4-2) is substituted into equation (4-1) and expressed in thermodynamic terms the thermodynamic expression of the Haldane relationship results. Equation (4-2) permits the utilization of methods worked out for chemical systems in enzyme systems.

Gadsby et al. (1946) and Horiuti (1957) both hinted at the possibility of catalysis in heterogenous situations where the factor Z was not equal to one. Little further work seems to have been done in this area. This chapter probes ΔG_R, using a simple enzyme catalyzed reaction.

MATERIALS AND METHODS

The same methodology was employed as in Chapter 3 with the additional use of various concentrations of KCl in the reaction medium.

RESULTS AND DISCUSSION

Table 4-2 shows that it is possible to select conditions for fumarase wherein it is possible to independently modulate binding of substrate and product. Presumably if we can select "artificial" conditions to perturb the ratio of Ks to Kp, adaptational expediency may alter the amino acid sequence to effect the same response.

Since, as we have seen in Chapter 3, ΔG^b may be perturbed with respect to $\Delta G^{\neq}$ in any way and since the binding of

substrate and product may be independently perturbed we may next ask whether $\Delta G_f^{\ddagger}$ can be perturbed in any way with respect to $\Delta G_r^{\ddagger}$. Table 4-3 shows again that this is possible to achieve by selecting the appropriate conditions.

The investigations of van't Hoff in the later part of the last century and the beginning of the present on the nature of the effect of temperature on the equilibrium ratios of substrate and products are now fundamental to our understanding of biochemical catalysis. By plotting the ln Keq versus the inverse of the absolute temperature he was able to determine the heat change of a given chemical reaction. This change (enthalpy) is a characteristic of a chemical reaction under a given set of conditions. Arrhenius used a similar graphical technique to determine the energy required for the rate limiting reaction in a given direction.It was also possible to calculate the enthalpy and entropy of the activation event from these plots. Although neither van't Hoff nor Arrhenius studied this it is assumed that at least for chemical reactions the enthalpy differences of the activation event in the forward and reverse reactions would equal the enthalpy change of the overall reaction as determined from van't Hoff plots. In the case of biological catalysis one may expect a different result. Figure 4-1 demonstrates this difference. The slope of the ln(Vmaxf/Vmaxr) versus the inverse of absolute temperature does not equal the slope of ln Keq versus 1/T. Additionally, the position on the ordinate is changed. This result suggests that ΔG_R is determined by both enthalpic and entropic effects. Figure 4-2 shows the enthalpic and entropic differences between ΔG_R and ΔG^0. Overall there is a

trend towards compensating an enthalpy change with a corresponding change of $T\Delta S$. As Table 4-3 has already demonstrated it is possible to independently perturb $\Delta G_f^{\neq}$ with respect to $\Delta G_r^{\neq}$. This means in effect that ΔG_R may assume any value, positive,negative small or large. As will become apparent later this is an important design feature of enzymes and directly affects the nature of the steady-state in the reaction the enzyme catalyzes in its specific metabolic pathway.

The next chapter will demonstrate how the independent modulation of binding and catalysis and the freedom of design required for biological catalysis are necessary to permit the steady-state and also provide for stability during steady-state transitions.

Table 4-2 Independent modulation of binding of substrate and product in the fumarase reaction.
Values are subtracted means for which the maximal standard error is 5.9%.

Transition conditions	$\Delta\Delta G_f^b$ (cal/mol)	$\Delta\Delta G_m^b$ (cal/mol)
20°C,pH 7.3-pH 8.0	-125	185
pH 7.3, 0.25M KCl,30-40°C	420	40
30°C, pH 7.3, 0-0.25M KCl	-5	-645
pH 8.0, 20-40°C	270	275

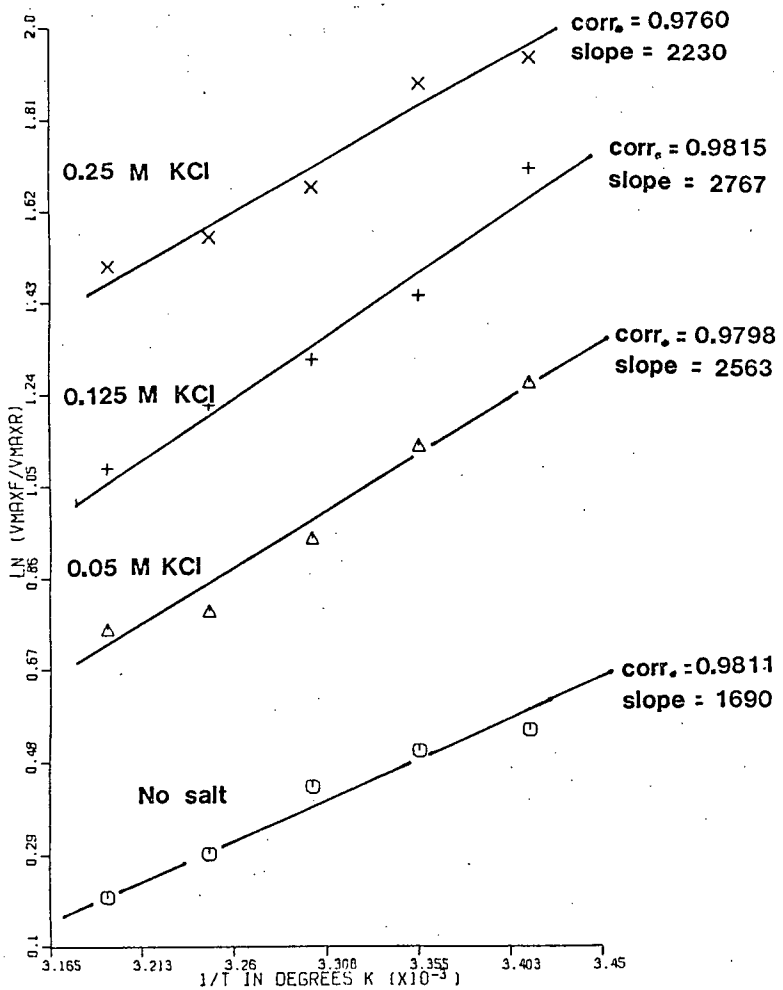
Table 4-3 Independent perturbation of $\Delta G_f^{\ddagger}$ with respect to $\Delta G_r^{\ddagger}$ for the fumarase reaction.

Values are subtracted means for which the maximal standard error is 0.85%.

Transition conditions	$\Delta\Delta G_f^{\ddagger}$ (cal/mol)	$\Delta\Delta G_r^{\ddagger}$ (cal/mol)
pH 7.3, 20-40°C	320	135
pH 7.3, 40°C, 0-0.25M KCl	45	850
20°C, pH 7.3-pH 8.0	305	-260
pH 8.0, 20-40°C	220	5

Figure 4-1a Effect of temperature on ln(Vmaxf/Vmaxr) for fumarase under various conditions.

4-1b van't Hoff plot of fumarase reaction under various conditions


corr = 0.9760
slope = 2230
0.25 M KCl
corr = 0.9815
slope = 2767
0.125 M KCl
corr = 0.9798
slope = 2563
0.05 M KCl
corr = 0.9811
slope = 1690
No salt
LN (VMAXF/VMAXR)
1/T IN DEGREES K (X10⁻³)

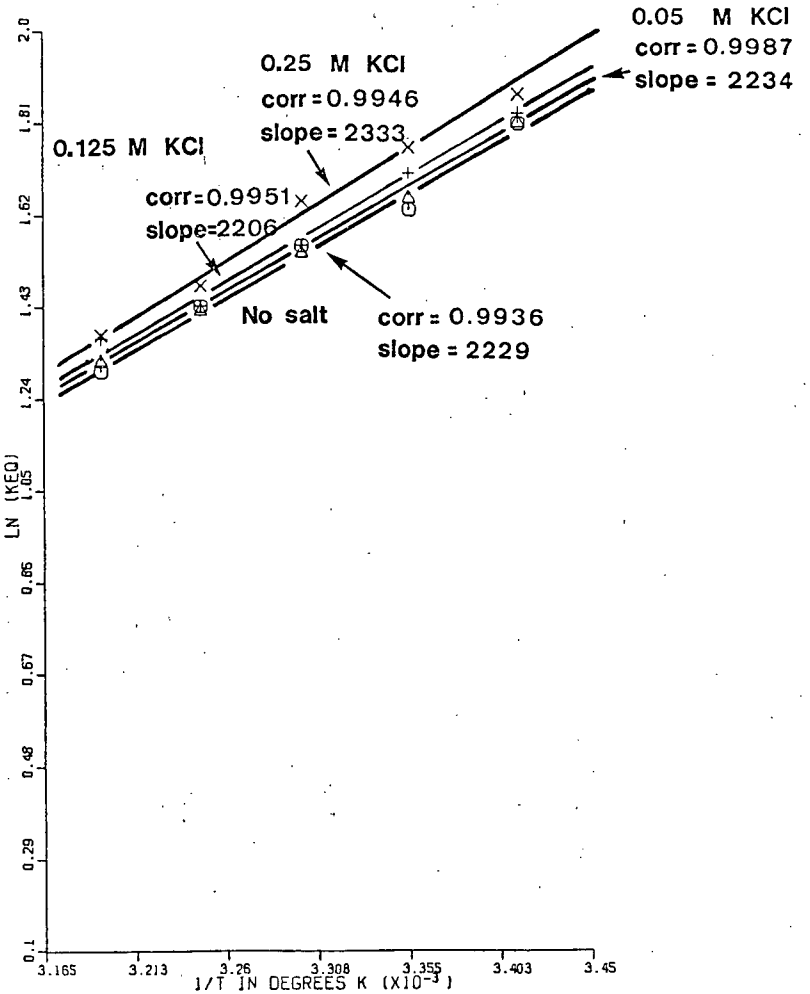
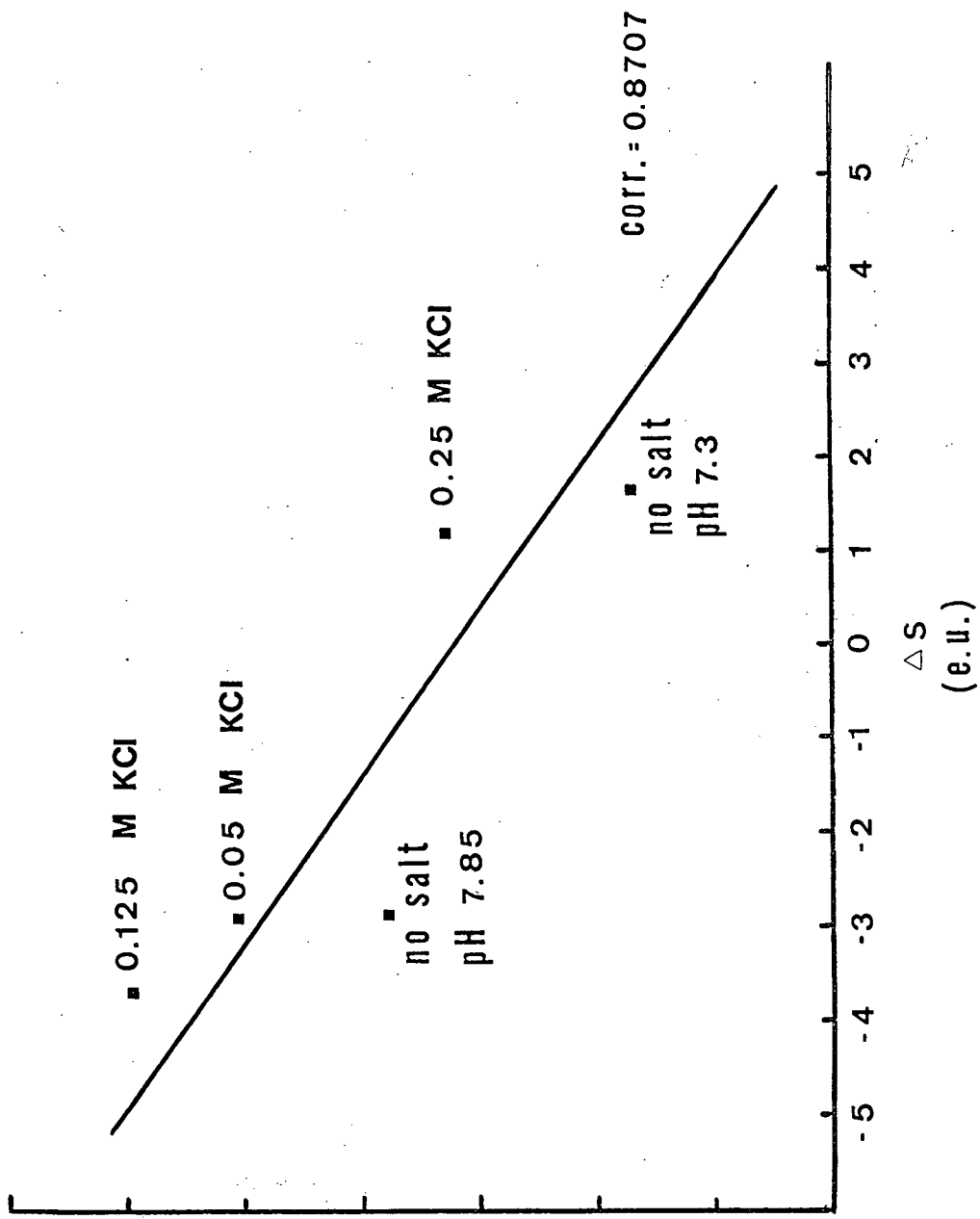

0.05 M KCl
corr = 0.9987
slope = 2234
0.25 M KCl
corr = 0.9946
slope = 2333
0.125 M KCl
corr = 0.9951
slope = 2206
No salt
corr = 0.9936
slope = 2229
LN (KEQ)
1/T IN DEGREES K (X10⁻³)

Figure 4-2 The role of enthalpy and entropy in perturbations of ΔG_R. A plot of enthalpy change (ΔH) versus entropy change (ΔS) under various conditions. $\Delta H = \Delta H_R - \Delta H°$ and $\Delta S = \Delta S_R - \Delta S°$.


0.125 M KCl
0.05 M KCl
no salt
pH 7.85
0.25 M KCl
corr. = 0.8707
no salt
pH 7.3
1000
500
0
-500
-1000
-1500
ΔH
(cal/mole)
-5
-4
-3
-2
-1
0
1
2
3
4
5
ΔS
(e.u.)

CHAPTER 5

ENZYME DESIGN AND METABOLISM

Introduction

The steady-state of a metabolic pathway must encompass the following:

1). Flux of metabolic compounds at any point (i.e. at any enzyme catalyzed reaction) in the pathway must be the same as that at any other point in the same pathway (branch points provide exceptions).

2). The concentrations of all intermediates must not change at any given velocity. Changes may occur during steady-state transitions but they must again be constant at the new velocity. Ideally strict control should be maintained during the transition.

In order to understand the steady-state of metabolism it is first necessary to look at a model system to see what range of steady-states can exist. Once this general steady-state has been examined the biological constraints can be imposed upon the system and any design features (those features which two sequential enzymes in a pathway must have to permit a steady-state within the biological constraints) can be determined.

MATERIALS AND METHODS

The fluxes for the two uni-uni mechanisms catalyzing:

$$A \underset{}{\overset{E1}{\rightleftharpoons}} B \overset{E2}{\rightleftharpoons} C$$

were calculated using the following equations:

$$v = \frac{Va([A]/Ka) - Vb1([B]/Kb1)}{1 + ([A]/Ka) + ([B]/Kb1)} \qquad (5\text{-}1a)$$

$$v = \frac{Vb1([A]/Kb2) - Vc([B]/Kb2)}{1 + ([A]/Kb2) + ([B]/Kc)} \qquad (5\text{-}1b)$$

for E1 and E2 respectively where Va and Vb1 refer to the maximal velocity for the forward and reverse reactions respectively for E1 and Vb2 and Vc refer to the maximal velocity for the forward and reverse reactions respectively of E2. Ka and Kc refer to the Michaelis constants for substrates A and C respectively, while Kb1 and Kb2 refer to the Michaelis constants for the shared substrate B for E1 and E2 respectively. Arbitrary values for the kinetic parameters were used except where specific relationships were being tested. A surface visualization routine available in the public files of the UBC Computer Centre was used to produce a three dimensional representation from equations 5-1a,b varying product concentration at constant substrate concentration over a range of substrate concentrations.

RESULTS AND DISCUSSION

Three dimensional representation of an enzyme catalyzed reaction

Figure 5-1 shows the surfaces produced by a representative enzyme. On the X axis the concentration of A is represented. The Y-axis represents the concentration of B. The region of positive Z (above the X-Y plane represents positive flux in the direction

of B production. Regions of the negative Z-axis represent flux in the direction of A production. The straight line produced by the intersection of the X-Y plane (Z=0) and the enzyme reaction surface represents the equilibrium situation (net flux is zero). The equation of this line is the equilibrium constant (Keq) for the reaction. In a closed or isolated system the reaction would procede to equilibrium along the surface in a path described by the law of conservation of mass.

The General Steady-State

For the sequence:

$$\xrightarrow{} A \underset{}{\overset{E1}{\rightleftharpoons}} B \underset{}{\overset{E2}{\rightleftharpoons}} C \xrightarrow{}$$

if the concentrations of A and C are held constant the steady-state will exist whenever the concentration of B (the shared substrate) is constant and the velocity v for E1 equals the velocity for E2. Using arbitrary values for the kinetic parameters of E1 and E2 the values of B which permit steady-states may be calculated. Figure 5-2 demonstrates such a series of calculations. One line of each pair refers to the relationship between A and B as v increases and the other the relationship between B and C. Changing the kinetic parameters of E1 and E2 generates another such pair of lines, where this new pair of lines defines another equally valid steady-state. There are a large number of such relationships each depending on the kinetic parameters assigned to E1 and E2. However, as outlined in the next section biochemical systems cannot be so loosely designed. Important constaints are placed on biological systems

that severely limit the kind of steady-state solutions that are actually observed in metabolic pathways. I shall consider two apparently most critical constraints below.

The role of affinity

Since the overall affinity of a pathway determines the direction of flux, perhaps tha most obvious constraints imposed on a metabolic pathway, limiting the number of permitted steady-states relate to affinity control. The affinity of the pathway is the sum of all the affinities in the pathway, some of which may be positive and others negative (Prigogine, 1955). To most effectively control the affinity and therefore the flux of a pathway a small number of enzymes are invested with the largest affinities in all the pathway. These "regulatory" enzymes, that determine the direction of flux, must be replaced with other enzymes with an opposite affinity to reverse the pathway. These latter enzymes constitute the so-called by pass reaction pathways and like the enzyme steps they by-pass are usually closely regulated (Atkinson,1977). With control invested in regulatory enzymes, the affinities of the other reactions are typically small (close to equilibrium values). The reactions with steady-states close to equilibrium will require higher enzyme activities for a given equilibrium constant in order to maintain the same flux as those with steady-states far from the equilibrium value. This qualitative observation is evident in the oft cited difference between the regulatory enzymes which catalyze reactions with steady-states far from equilibrium and are found in low activity (as measured by Vmax) and the non-regulatory enzymes that catalyze reactions close to equilibrium

and are found in greater activity (as measured by Vmax). This is most easily understood with reference to an enzyme response surface (eg. Figure 5-1) Close to the equilibrium value the flux is farther from its Vmax value than the far from equilibrium case where the flux is much closer to its Vmax value. Therefore, when measuring enzyme activities by the Vmax method it will be observed that regulatory enzymes will have a lower Vmax values than the non-regulatory enzyme (with similar equilibrium constant), even though in vivo at steady-state both function at the same rate. This difference in Vmax levels of regulatory and non-regulatory enzymes indeed is always observed (Newsholme and Start, 1979). An important consequence of this organization (of varying affinities along the pathway) is that the Vmaxf for one enzyme may be very large with respect to the Vmaxf for the next enzyme in the pathway, in which case the range of possible steady-states narrows (discussed futher below). Thus one biological constraint on conditions for the steady-state requires enzyme function at relatively specific affinities. That is why it is important to explore the determinants of affinity for any given enzyme reaction in vivo. This exploration, it turns out identifies one of the most important constraints on in vivo conditions for the steady-state.

As pointed out in the previous chapters, a characteristic of metabolic enzymes which seems to distinguish them from most if not all other kinds of catalysts is the parameter ΔG_R. Since ΔG_R refers to the difference in the Gibbs free energy of activation of the forward compared to the reverse reaction, one may expect it to be correlated in some way with substrate and

product concentrations in vivo. In fact, this is observed. If the substrate and product concentration ratios for various reactions in vivo are expressed in thermodynamic terms (i.e. As affinity or distance from equilibrium), a correlation may be seen to exist with ΔG_R (Figure 5-3). This is shown for a series of 12 enzymes for which in vivo data on substrate levels could be obtained. These twelve enzymes vary in metabolic function as well as the affinities at which catalysis occurs in vivo, yet there is a reasonably good relationship (correlation coefficient = 0.9371) between affinity in vivo and ΔG_R (Figure 5-3). Thus for the maintenance of steady-state, a second important constraint is this relationship between the in vivo substrate and product concentrations and the design of the enzymes catalyzing the reaction, allowing in vivo equivalence between ΔG_R and affinity.

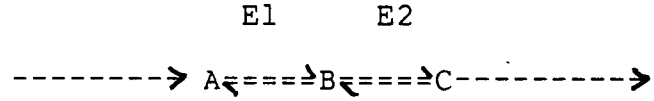
Using these two constraints:

1. Control over affinity
2. Equivalence of ΔG_R with affinity

the general steady-state may be reexamined to determine the natire of the metabolic steady-state and the design features required of the enzymes to allow a biologically meaningful steady- state to be developed and maintained.

The Steady-state of Metabolism

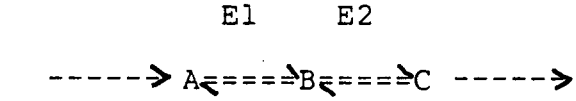
To investigate the enzyme requirements for steady-state in this more restricted context, the following scheme was used to simulate a sequence of two enzymes in metabolism:



where E1 represents the first enzyme in the sequence being considered which catalyzes the conversion of A to B. Similarly E2 catalyzes the conversion of B to C. This treatment differs from the previous treatment of the general steady-state in two important ways. In the first place, A and C concentrations are not held constant. In the second case, ΔG_R is is assumed equivalent to the affinity. By imposing the ΔG_R affinity relationship on E1, it is possible to calculate what the kinetic parameters must be for E2 such that the affinity of E2 is also equivalent to ΔG_R for E2. This was accomplished by imposing, in addition to the steady-state constraints used in the case ofthe determination of the general steady-state, the additional constraints listed above . Iterative changes in the Michaelis constants and Vmax's were made until biologically acceptable relationships between C and B were obtained. Through this procedure the Km vales and the concentrations of both enzymes were always found to be critically important and apparently <u>in vivo</u> closely adjusted, to steady-state function. Each of these is considered separately below.

<u>1.The Km for the shared substrate for both enzymes must be the same.</u>

One important outcome of this analysis is that for the sequence at steady-state,



the Km for B as product for enzyme 1 equals the Km for B as substrate for enzyme 2. In biological terms this relationship

may need only to be approximated to achieve the steady-state. Figure 5-4a shows the effect on the product/substrate ratio of increasing the Km of B for Enzyme 2 by 1, 2, 3, 4, and 5 fold. In this procedure tThe ratio of B/A is fixed at a ratio equal to the ratio Kb1/Ka and the velocity calculated by increasing substrate and product concentrations. The value of C is then calculated for E2 which give the same velocity as E1 at the same concentration of the shared substrate. The more the Km for the shared substrate deviates from the ideal ratio (Kb1=Kb2) the less linear the steady-state becomes and eventually the intersection passes onto the region of negative substrate concentration. In other words the steady-state is not continuous from zero to large substrate concentrations when Kb1 is less than Kb2. Moreover, the steady-state no longer extends over the metabolically important part of the curve (low to intermediate substrate concentrations). Figure 5-4b shows the effect of lowering the Km for B of E2. As the Km drops the region close to the origin curves but becomes linear as concentration increases. The slopes of these lines are similar. Figure 5-4 demonstrates that increasing Kb2 from the ideal situation Kb2/Kb1 = 1 results in a discontinuous intersection. Lowering the ratio Kb2/Kb1 by changing Kb2 produces acceptable intersection of similar slope. If however, Kb1 is changed in the same way as was Kb2, increasing Kb1 results in discontinuous intsections but this time the ratio Kb2/Kb1 has been lowered. From this analysis I conclude that the ideal relationship between Kb1 and Kb2 is Kb1=Kb2. At present it is not possible to determine what the tolerances to Km non-equivalence are <u>in vivo</u>. Km equivalence is

an ideal situation which evolution presumably would tend to produce. However, other factors may prevent this ideal from actually being achieved in vivo.

2.The enzyme concentration of one or both enzymes must be adjusted to the continuous steady-state.

That the concentration of enzyme one in the sequence is related to that of enzyme two is another important outcome of this analysis. The equation describing this relationship is for two enzymes is (when Kb2 = Kb1):

$$E1V1-E2V1 = E1V2-E2V2 \qquad (5\text{-}2)$$

The designation E1V1 refers to the Vmax activity of enzyme one in the forward direction and E1V2 is the maximal activity of enzyme one in the reverse direction. Figure (5-5) shows the effect of changing the amount of one enzyme away from the ideal situation (equation 5-2). As the amount of E2 increases the intersection describing the steady-state becomes less linear and again passes into the region of negative substrate concentration implying a discontinuous steady-state. Increasing the concentration of E2 results in continuous intersections with decreasing slopes. Again, if the same experiment is done to E1 we will find that increasing concentration of E1 results in a discontinuous intersection. Thus, perturbing equation 5-2 results in discontinuous intersections which are biologically unacceptable. While equation (5-2) may be limited to this system of two uni-uni mechanisms presumably other similar relationships exist for other combinations of enzymes. An equation like (5-2) permits an explanation of the observed constant relationship

between enzyme activities in a pathway (the constant-proportion groups of Pette, (1965)).

Once the above two design features are imposed on the two sequential enzymes the relationship between ΔG_R and <u>in vivo</u> is obtained. This result which is a satisfactory explanation of the empirical data in figure 5-3, may be proven by substituting values of:

$$[A] = [B] \times aff1$$

and

$$[B] = [C] \times aff2$$

Where aff1 and aff2 are the product/substrate ratios of the reactions set equal to the Km ratio Kb1/Ka and Kc/Kb2 for E1 and E2 respectively.(This is an equivalent statement of the expression ΔG_R = affinity due to the Haldane relationship).
The equations describing the steady-state between E1 and E2 are:

$$\text{flux} = \frac{Va(B \times aff1)/Ka - Vb1(B/Kb1)}{1+(B \times aff1)/Ka + (B/Kb1)} \quad (5\text{-}4)$$

$$= \frac{Vb1(B/Kb2) - Vc(B/aff2)/Kc}{1+(B/Kb2) + (B/aff2)/Kc}$$

At every point along the line of intersection the rate of synthesis of B by E1 equals exactly the rate of its utilization by E2.

<u>Constancy of affinity during steady-state transitions</u>

It may be seen from Figures 5-4 and 5-5 that the only set of kinetic constraints satisfying the observed <u>in vivo</u> relationships are Km equivalence and the Vmax relationships

contained in equation 5-2. All other solutions which give steady-states are either parallel to the ideal solution (Figure 5-4) or are straight lines radiating from the origin (Figure 5-5) which will never cross the line which desribes the ΔG_R affinity equivalence observed in vivo. It follows that that when this simple relationship is imposed on two sequential enzymes a constancy of affinity is maintained over broad ranges of concentrations of substrate and product.

Accordingly, two three dimensional surfaces of enzymes designed for the steady-state of metabolism are superimposed in Figure 5-6a. In order to achieve this the shared substrate is placed on the same axis . The scales on the other axis are adjusted so the intersection describes the metabolic steady-state. The steady-state can be seen to be linear from the origin to infinite concentrations as velocity increases. The intersection is also continuous from the side when the three dimensional figure is cut along the intersection and viewed from the side (Figure 5-6b). As a transition from one rate of flux to another occurs, the affinity remains the same even though the concentrations of substrate and product change. This relationship explains and confirms the observations of several authors (Lowry and Passonneau, 1964) that many steps in glycolysis are maintained close to equilibrium even during increased flux when the concentration of intermediates in the pathway increase substantially (Lowry,O.H. Personal communication, 1980, Hintz,C.S.,Lowry,C.V., Ing,J.,Fell, R.,Chi,M.M.-Y. And Lowry,O.H., 1980). This most important result has an analogy in the equilibrium situation of most chemical

systems where k_1/k_{-1} = Keq.

Stability of the steady-state of metabolism

A steady-state of any sort may be unstable in which case small perturbations of the variables determining it will result in a new state being reached. Alternatively, it may be stable in which case small perturbations will lead back to the steady-state (Boyce and DiPrima, 1977). The steady-state of metabolism has the property of being stable with respect to perturbations of substrate concentration. This conclusion may be reached by adding the surfaces generated by E1 and E2 (Figure 5-5). The steady-state now appears as the intersection of the surface with the X-Y plane. Above the X-Y plane flux is positive and towards the steady-state. Below the X-Y plane the flux is negative and toward the steady-state. A perturbation of the shared substrate will result in a new flux velocity which will return to the steady-state regardless of the direction of the perturbation. Since the steady-state is stable the flux velocity will be arrested at the steady-state concentrations . The steady-state is therefore self-stabilizing. It resembles thermodynamic equilibrium in this respect.

A theoretical contruct is useful if it explains previously perplexing observations, and this indeed seems to be the case for the above treatment of the steady-state. In the first instance, it predicts a constancy of affinity under different states of activity for this is a function of the steady-state and is a design feature of metabolic enzymes. In fact, such constancy has been frequently reported but never adequately explained (Lowry and Passoneau, 1964; Guppy et al., 1979)

Whereas feedback mechanisms undoubtedly can influence the magnitude of the flux (eg. stable oscillations in glycoltyic flux and substrate concentration are best explained by invoking feedback control at the phosphofructokinase step, (Sel'kov, 1968)), it is evident from this treatment that they are not sufficient to account for the properties of steady-state flux and steady-state substrate concentration.

These early workers grouped enzymes into two broad classes: equilibrium enzymes that catalyze reactions with an affinity close to zero, and non-equilibrium enzymes whose affinity in the steady-state is large. The term "equilibrium" in this sense is inaccurate and completely misleading. Since the system is an open one, the state of least entropy production (steady-state flux) replaces equilibrium as a stable state (Prigogine,1955).

Finally this treatment helps understand why the Km values of shared substrates are often the same (eg. The Km for malate of fumarase and mitochondrial malate dehydrogenase (Ballantyne 1980, unpublished data and numerous examples in glycolysis).

CONCLUSIONS

The six major conclusions of this chapter are:

1. The magnitude of ΔG_R is equivalent to the in vivo affinity.
2. For two enzymes sharing a substrate the Km's for that substrate are the same.
3. The affinity of a reaction in metabolism remains constant during changes in flux (a constraint that may apply only to non-regulatory enzymes).
4. Enzymes catalyzing reactions with affinities close to

the equilibrium value should have higher activities when measured by the Vmax method than those catalyzing reactions with affinities far from equilibrium.

5. Enzyme concentrations of two consecutive uni-uni reactions are related by E1V1-E2V1 = E1V2-E2V2.
6. The steady-state of metabolism is stable with respect to substrate fluctuations.

Of these, the first four have some supporting evidence from the literature (vide supra). More conclusive proof of (3) and (5) may only be obtained by measuring Km's and Vmax's under conditions that mimic the intracellular milieu as closely as possible. The proof of (6) will require a method of perturbing substrate concentration while monitoring flux and substrate concentrations in vivo.

SUMMARY

Metabolic enzymes are designed for the steady-state. The magnitude of the difference in the Gibbs free energy change of activation forward compared to reverse is equal to the affinity of that reaction in the steady-state and is a linear function from zero to infinity. This enzyme parameter is essential to the attainment, stability and displacement from equilibrium of the steady-state. While this has been proven for a simple three enzyme sequence in vivo measurements suggest it applies to many if not all metabolic sequences. The steady-state is not an inevitable consequence of substrate sharing. Several enzyme design parameters, notably the Km for the shared substrate and the enzyme concentration must be modulated to permit control over the affinity of the reaction. The Km for the shared

substrate must be the same. The enzyme concentration must also be adjusted presumably by the interaction of an accumulated substrate (due to non-steady-state behaviour) on the machinery of protein synthesis or by metabolically equivalent signals. With the characteristic of metabolic enzymes that ΔG_R = affinity as a prerequisite for metabolism as we know it, the stable steady-state is achieved. The control of metabolism via the relationship between ΔG_R and affinity depends upon the "plasticity" of enzyme design outlined in previous chapters.

Figure 5-1 Enzyme reaction surface for simple uni-uni mechanism.

Figure 5-2 The general steady-state.

Five pairs of enzymes 1-5E1 and 1-5E2 were simulated. The kinetic parameters for E1 were

Va = 20,30,40,50,60 for sets 1-5 respectively

Vb1 = 10

Ka = 30

Kb1 = 50

The kinetic parameters for E2 were for all sets,

Vb = 10

Vc = 90

Kb2 = 40

Kc = 30

The concentrations of substrates A for E1 was incremented by 1,2,3,4 and 5 and the concentration ratio C/A maintained constant for each pair. The C/A ratio was 0.5,0.6,0.7,0.8, and 0.9 for sets 1-5 respectively. The velocity of the reaction catalyzed by E1 and E2 was incremented by 1,2,3,4 and 5 for sets 1-5 respectively. The concentration of B for E1 and E2 was calculated using the equation

$$B = \frac{Kb1A\left\{\frac{(Va - v)}{Ka} + \frac{(Vc + v)C}{Kc\ A}\right\}}{\left\{(Vb1 + v) + \frac{(Vb2 - v)}{Kb2/Kb1}\right\}}$$

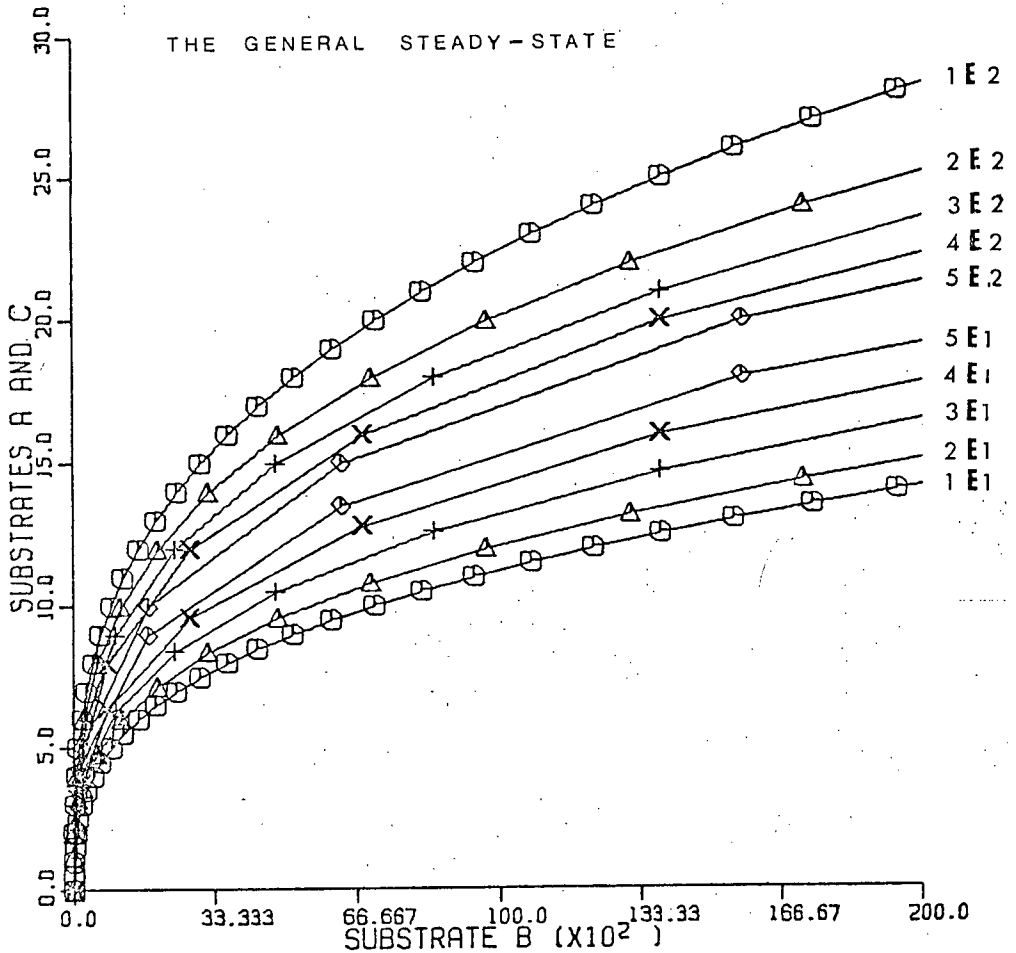

THE GENERAL STEADY-STATE
SUBSTRATES A AND C
SUBSTRATE B (X10²)
0.0
5.0
10.0
15.0
20.0
25.0
30.0
0.0
33.333
66.667
100.0
133.33
166.67
200.0
1 E 2
2 E 2
3 E 2
4 E 2
5 E 2
5 E 1
4 E 1
3 E 1
2 E 1
1 E 1

Figure 5-3 ΔG_R versus ΔG for enzymes and metabolites from several tissues.Data sources are appended. ΔG_R was determined from Vmaxf/Vmaxr in the same way as for Table 4-1.
Unless otherwise designated all data are for muscle enzymes and metabolites.
PGI= phosphoglucose isomerase
PGM= phosphoglucomutase
AK= adenylate kinase
ald= aldolase
FDPase= fructose diphosphatase
CPK= creatine phosphokinase
LDH= lactate dehydrogenase
G3PDH= glyceraldehyde-3-phosphate dehydrogenase
TPI= triosephosphate isomerase
PK= pyruvate kinase
HK= hexokinase

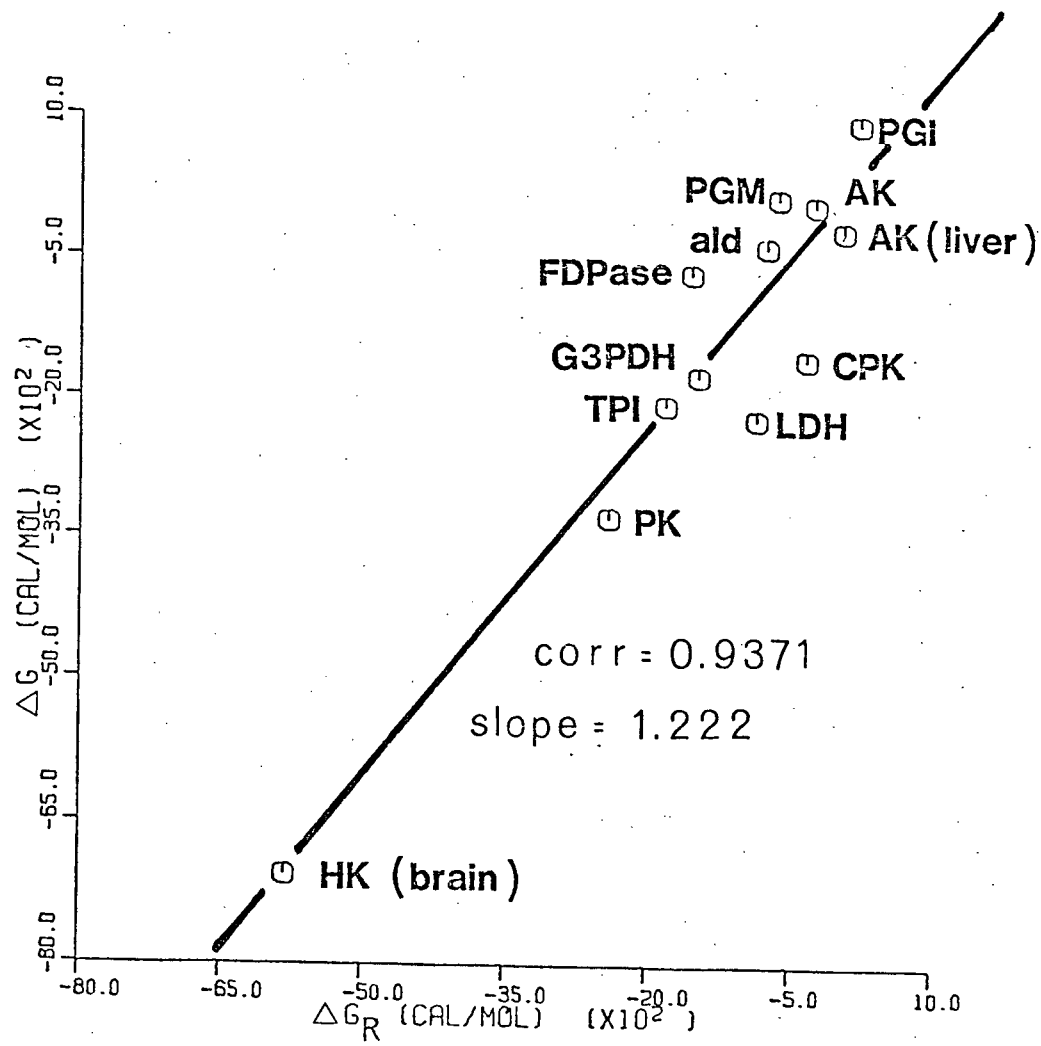

PGI
PGM
AK
ald
AK (liver)
FDPase
G3PDH
CPK
TPI
LDH
PK
HK (brain)
corr = 0.9371
slope = 1.222
10.0
-5.0
-20.0
-35.0
-50.0
-65.0
-80.0
ΔG (CAL/MOL) (X10²)
-80.0
-65.0
-50.0
-35.0
-20.0
-5.0
10.0
ΔG_R (CAL/MOL) (X10²)

Figure 5-4 The effect of changing the Km for the shared substrate on the steady-state.

Equation 5-1b was used to compute the same velocity of E2 as for E1 at the same concentration of B with the kinetic parameters below.

The velocity was computed for E1 using a constant ratio of B to A equal to Kb1/Ka.

The value of Kb2 is indicated beside each line.

Va = 200 Vb2 = 200

Vb1 = 100 Vc = 100

Ka = 1000 Kb2 = varied

Kb1 = 100 Kc = varied to satisfy Haldane relationshi

The solid lines indicate the region where the concentration of substrates is positive.

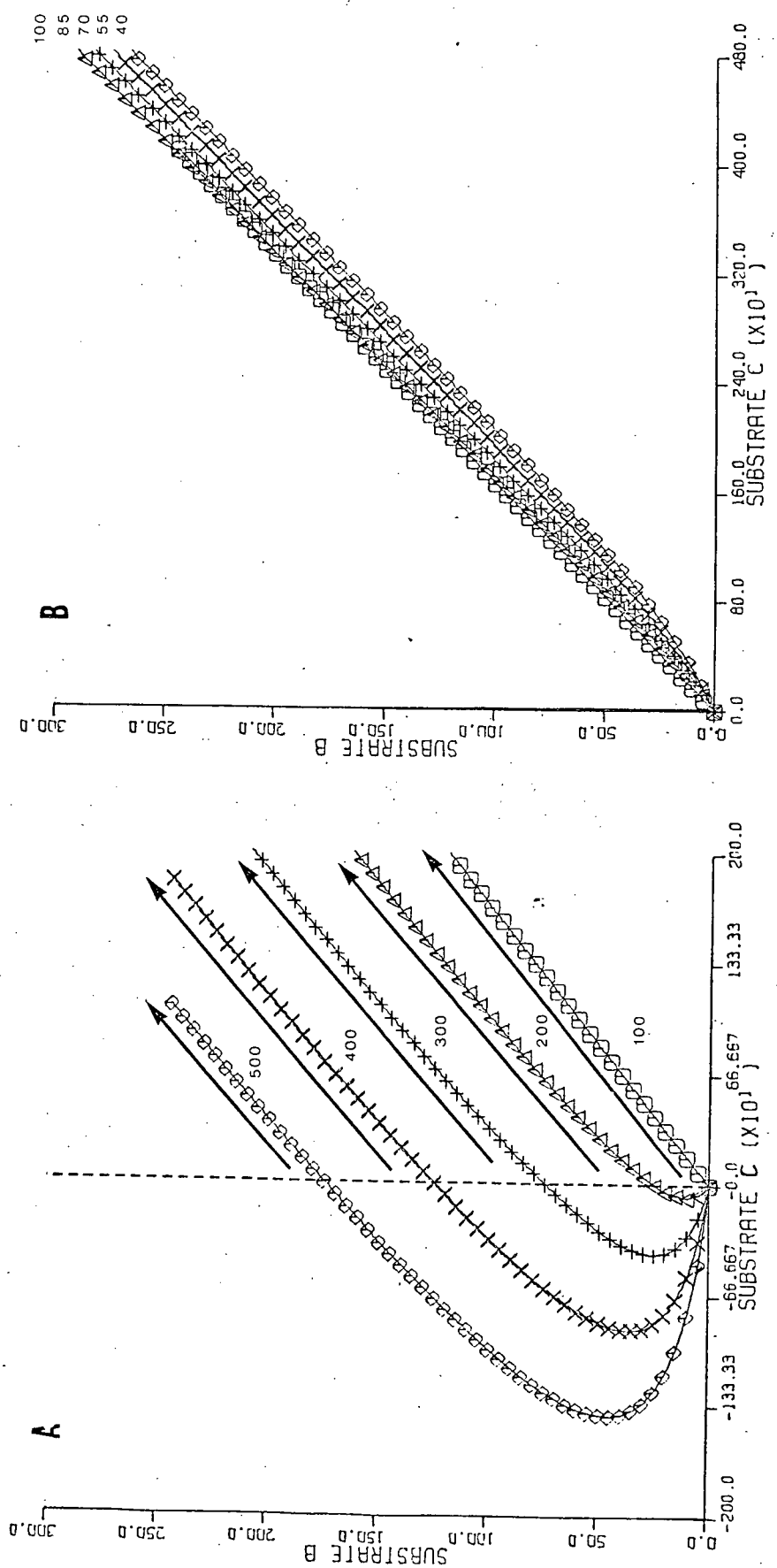

A
SUBSTRATE B
SUBSTRATE C (X10¹)
500
400
300
200
100
B
SUBSTRATE B
SUBSTRATE C (X10¹)
100
85
70
55
40

Figure 5-5 The effect of enzyme concentration on the achievement of the continuous steady-state. Equation 5-1b was used to compute the same velocity of E2 as for E1 at the same concentration of B with the kinetic parameters below. The velocity was computed for E1 using a constant ratio of B to A equal to Kb1/Ka. The ratio Vb2/Vc is indicated beside each line.

Va=300 Vb2=varied

Vb1=225 Vc=varied

Ka=400 Kb2=100

Kb1=100 Kc=200

The solid lines indicate where the concentration of substrates is positive.

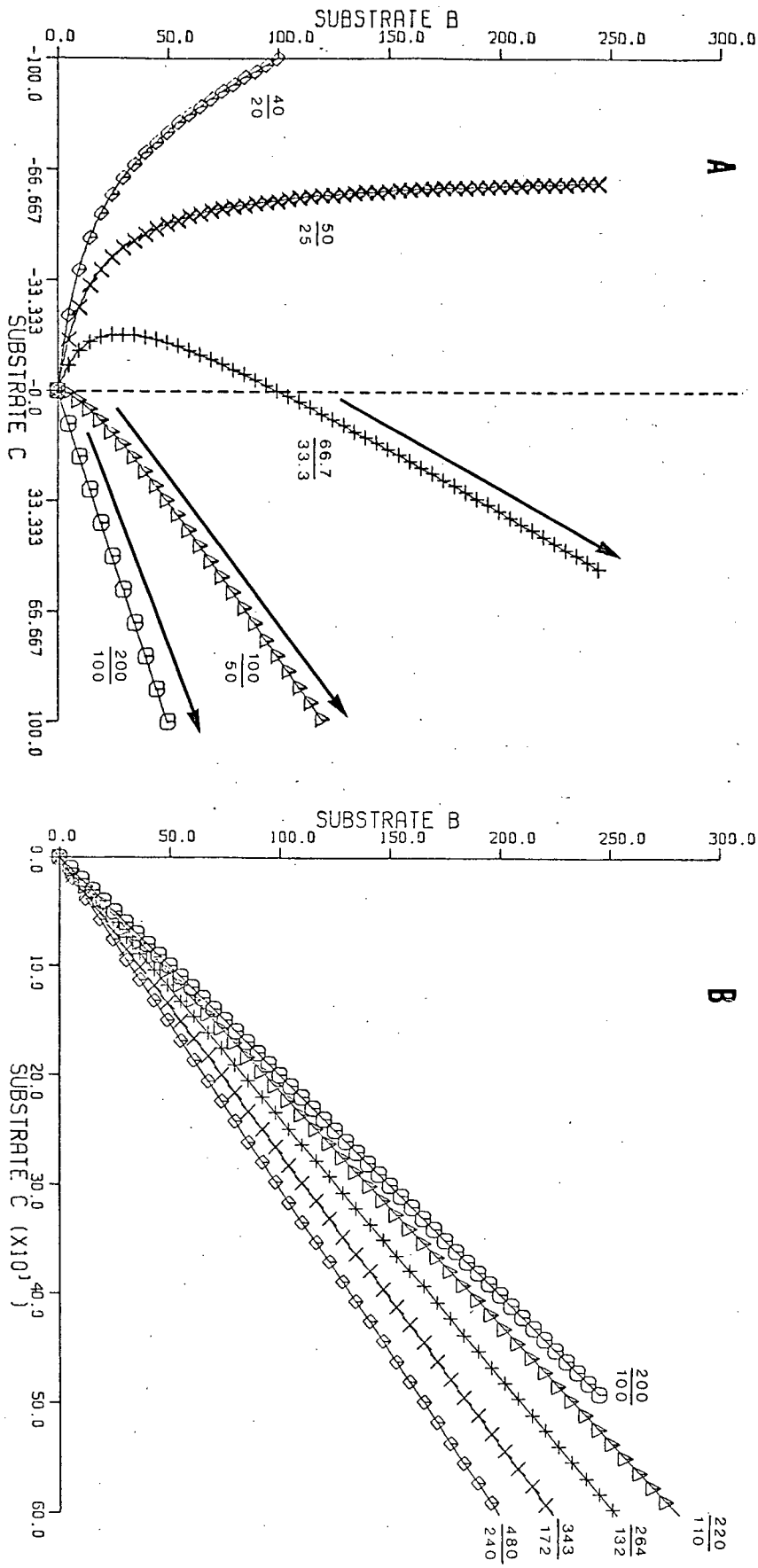
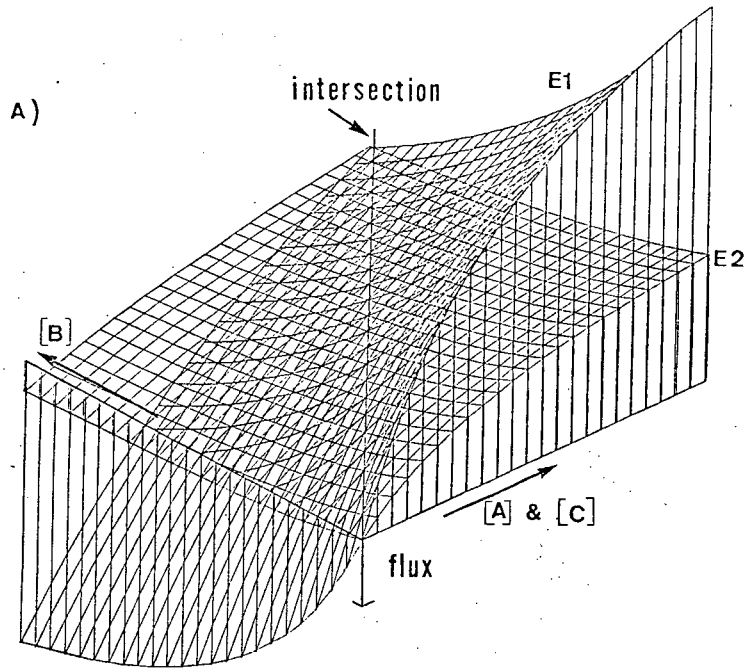

SUBSTRATE B
0.0
50.0
100.0
150.0
200.0
250.0
300.0
SUBSTRATE C
-100.0
-66.667
-33.333
-0.0
33.333
66.667
100.0
A
40/20
50/25
66.7/33.3
100/50
200/100
SUBSTRATE B
0.0
50.0
100.0
150.0
200.0
250.0
300.0
SUBSTRATE C (X10³)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
B
200/100
264/132
343/172
480/240
220/110

Figure5-6 a) Three dimensional plot of two simple uni-uni mechanisms.

b) Two dimensional plot of a) viewed from x-axis.


A)
intersection
E1
E2
[B]
[A] & [C]
flux

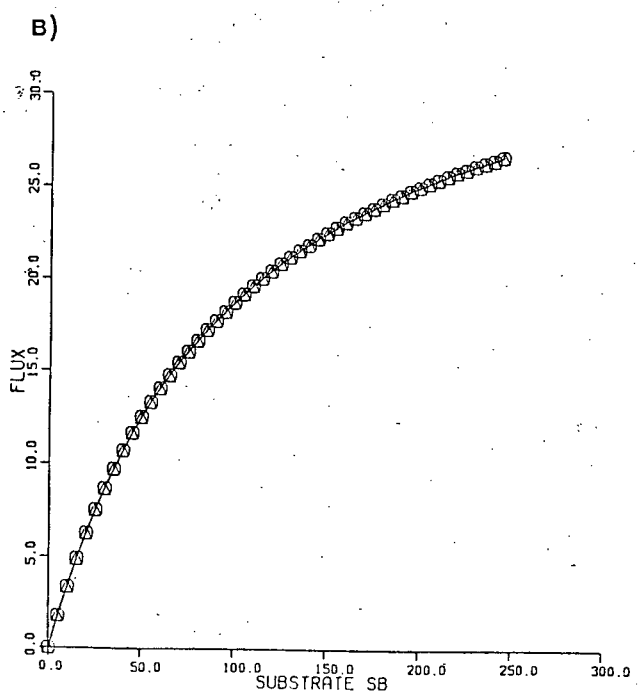
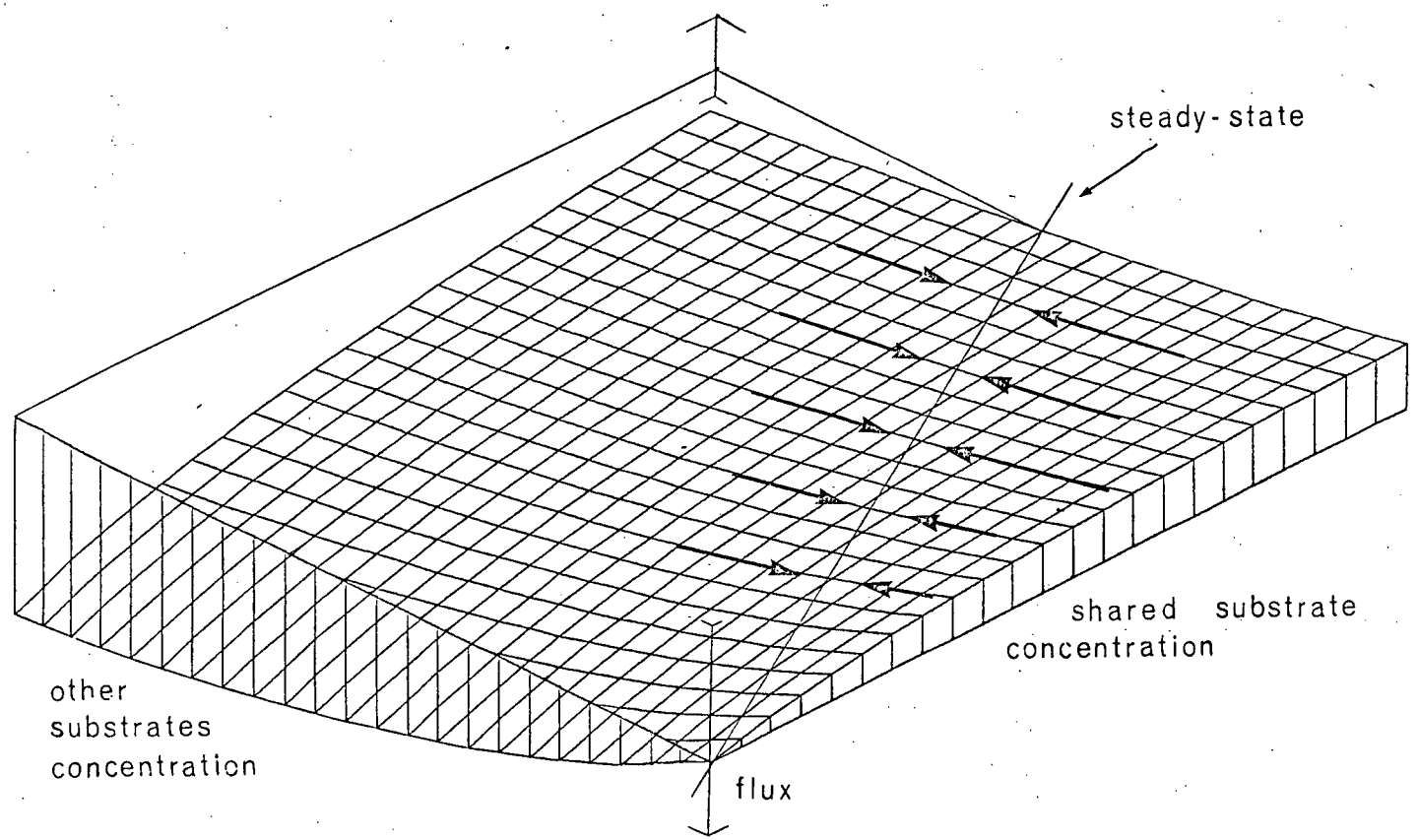

B)
FLUX
0.0
5.0
10.0
15.0
20.0
25.0
30.0
0.0
50.0
100.0
150.0
200.0
250.0
300.0
SUBSTRATE SB

Figure 5-7 Stability of the steady-state.


steady-state
shared substrate concentration
other substrates concentration
flux

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ABBREVIATIONS USED

v	net rate of production of substrate or product
Vmaxf,Vmaxr	maximal velocity of enzyme catalyzed reaction in forward or reverse direction.
V1,V2	maximal velocity of enzyme catalyzed reaction in forward (1) or reverse (2) direction.
kcatf,kcatr	catalytic rate constant for forward or reverse reaction
k1,k-1	rate constant for chemical reaction in forward (1) or reverse (-1) direction
Ka,Kb,Kp,Kq	Michaelis constant for substrate A,B,P or Q
Kia,Kib,Kip, Kiq	Inhibition constant for A,B,P or Q
Keq	Equilibrium constant
R	Gas constant
T	Temperature in °K
k	Boltzmann's constant
h	Planck's constant
$\Delta G_f^{\neq}, \Delta G_r^{\neq}$	Gibbs free enrgy change of activation in forward (f) or reverse (r) direction
$\Delta G_a^b, \Delta G_b^b, \Delta G_p^b, \Delta G_q^b$	Gibbs free energy change of binding of substrate A,B,P, or Q.
$\Delta G_a^{ib}, \Delta G_b^{ib}, \Delta G_p^{ib}, \Delta G_q^{ib}$	Gibbs free energy change of binding of substrate A,B,P,Q in inhibitory mode.

APPENDIX

References to Figure 5-3.

Enzyme	ΔG_R	ΔG	Metabolites
PGI	10	10	9
CPK	16	16	9
PGM	11	11	9 Creatine-2
PK	8	13	9
HK	20	21	12
ald	19	22	9
AK(liver)	24	6	4
AK(muscle)	17	6	9 AMP -2
LDH	3	.3	pyruvate -9
			lactate -24
			NAD -1
			NADH -5
G3PDH	25	7	9 NAD -1
			NADH -5
FDPase	3	15	9
TPI	18	14	9

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