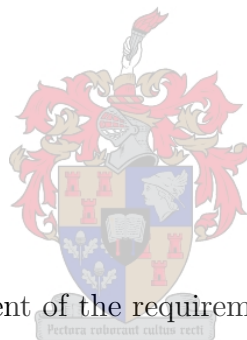


Generic kinetic equations for modelling multisubstrate reactions in computational systems biology

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Biochemistry) at the University of Stellenbosch.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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Signature

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Date

Summary

Systems biology is a rapidly developing field, studying biological systems by methodically perturbing them either chemically, genetically or biologically. The system response is observed and incorporated into mathematical models. These computational models describe the system structure, predicting its behaviour in response to individual perturbations. Metabolic networks are examples of such systems and are modelled *in silico* as kinetic models. These kinetic models consist of the constituent enzyme reactions that make up the different pathways of a metabolic network. Each enzyme reaction is represented as a mathematical equation. The main focus of a kinetic model is to portray as realistically as possible a view *in silico* of physiological behaviour. The equations used to describe model reactions therefore need to make accurate predictions of enzyme behaviour.

Numerous enzymes in metabolic networks are cooperative enzymes and many equations have been put forward to describe these reactions. Examples of equations used to model cooperative enzymes are the Adair equation, the uni-reactant Monod, Wyman and Changeux model, Hill equation, and the recently derived reversible Hill equation. Hill equations fit the majority of experimental data very well and have many advantages over their uni-substrate counterparts. In contrast to the abovementioned equations, the majority of enzyme reactions in metabolism are of a multisubstrate nature. Moreover, these multisubstrate reactions should be modelled as reversible reactions, as the contribution of the reverse reaction rate on the net conversion rate can not be ignored [1]. To date, only the bi-substrate reversible MWC equation has been formulated to describe cooperativity for a reversible reaction of more than one substrate. It is, however, difficult to use as a result of numerous parameters, not all of which have clear operational meaning. Moreover, MWC equations do not predict realistic allosteric modifier behaviour [2, 3]. Hofmeyr & Cornish-Bowden [3] showed how the uni-reactant reversible Hill equation succeeds in predicting realistic allosteric inhibitor behaviour, compared to the uni-reactant MWC equation, which does not. The aim of this study was to therefore derive a reversible Hill equation that can describe multisubstrate cooperative reactions and predicts realistic allosteric modifier behaviour.

In this work, we present a generalised multisubstrate reversible Hill (GRH) equation. The

bi-substrate and three substrate cases of this equation were also extended to incorporate any number of independently binding allosteric modifiers. The derived GRH equation is evaluated against the above mentioned cooperative models and shows good correlation. Moreover, the predicted behaviour of the bi-substrate reversible Hill equation with one allosteric inhibitor is compared to the MWC equation with one allosteric inhibitor *in silico*. This showed how the bi-substrate reversible Hill equation is able to account for substrate-modifier saturation, unlike the MWC equation, which does not. Additionally, the bi-substrate reversible Hill equation behaviour was evaluated against *in vitro* data from a cooperative bi-substrate enzyme which was allosterically inhibited. The experimental data confirm the validity of the behaviour predicted by the bi-substrate reversible Hill equation. Furthermore, we also present here reversible Hill equations for two substrates to one product and one substrate to two products reactions. Reactions of this nature are often found in metabolism and the need to accurately describe their behaviour is as important as reactions with equal substrates and products.

The proposed reversible Hill equations are all independent of underlying enzyme mechanism, they contain parameters that have clear operational meaning and all of the newly derived equations can be transformed to non-cooperative equations by setting the Hill coefficient equal to one. These equations are of great use in computational models, enabling the modeller to accurately describe the behaviour of a vast number of cooperative and non-cooperative enzyme reactions with only a few equations.

Opsomming

Sisteembioologie is 'n vinnig ontwikkelende veld. Dit bestudeer biologiese sisteme deur hulle chemies, geneties of biologies te perturbeer. Die sisteem se respons word waargeneem en geïnkorporeer in wiskundige modelle. Hierdie rekenaarmodelle beskryf die sisteem se struktuur en voorspel sy gedrag wanneer die komponente daarvan afsonderlik geperturbeer word. Metaboliese netwerke is voorbeelde van sulke sisteme en hulle word *in silico* gemodelleer as kinetiese modelle. Die kinetiese modelle bevat die afsonderlike ensiemreaksies waaruit die verskillende paaie van 'n metaboliese netwerk bestaan. Elke ensiemreaksie word voorgestel deur 'n wiskundige vergelyking. Die hoofdoel van enige kinetiese model is om fisiologiese gedrag so getrou as moontlik *in silico* na te boots. Die vergelykings wat gebruik word om die model se ensiemreaksies voor te stel moet die ensiemgedrag dus akkuraat voorspel.

Verskeie ensieme in metaboliese netwerke is koooperatief en 'n aantal vergelykings is voorgestel om hierdie tipe reaksies te beskryf. Voorbeelde hiervan is die Adair-vergelyking, die een-substraat Monod-Wyman-Changeux model, die Hill-vergelyking, en die omkeerbare Hill-vergelyking wat onlangs afgelei is. Hill-vergelykings pas die meeste eksperimentele data baie goed en het heelwat voordele teenoor die ander een-substraat vergelykings. Teenstrydig met al die bogenoemde vergelykings, egter, is die feit dat die meerderheid van ensiemreaksies in metabolisme veelsubstraat-reaksies is. Voorts behoort hierdie veelsubstraat-reaksies gemodelleer word as omkeerbare reaksies, aangesien die effek van die terugreaksie op die netto-reaksiesnelheid nie geïgnoreer kan word nie [1]. Tot op hede is slegs die bi-substraat omkeerbare MWC-vergelyking geformuleer om koooperatiewe vir omkeerbare reaksies van meer as een substraat te beskryf. Hierdie vergelyking is egter moeilik om te gebruik, aangesien dit baie parameters bevat waarvan nie almal 'n duidelike operasionele betekenis het nie. Verder is die MWC-vergelykings nie daartoe in staat om korrekte allosteriese effektor-gedrag te voorspel nie. Hofmeyr & Cornish-Bowden [3] het gewys hoe die een-substraat omkeerbare Hill-vergelyking allosteriese effektor gedrag korrek voorspel, in teenstelling tot die een-substraat MWC-vergelyking wat dit nie doen nie. Die doel van hierdie studie was dus om 'n omkeerbare Hill-vergelyking af te lei vir veelsubstraat koooperatiewe reaksies wat voorts die allosteriese effektore se gedrag korrek voorspel.

In hierdie tesis stel ons 'n algemene veelsubstraat omkeerbare Hill (AOH) vergelyking voor. Die bi-substraat en drie-substraat gevalle van hierdie vergelyking word uitgebrei om 'n willekeurige aantal allosteriese effektore, wat onafhanklik bind, te inkorporeer. Die afgeleide AOH-vergelyking word suksesvol gevalideer deur dit met bogenoemde koöperatiewe modelle te vergelyk. Voorts word die voorspelde gedrag van die bi-substraat omkeerbare Hill-vergelyking met een allosteriese inhibitor *in silico* vergelyk met die gedrag van die MWC-vergelyking vir een allosteriese inhibitor. Dit wys hoe die bi-substraat omkeerbare Hill-vergelyking daarin slaag om substraat-effektor versadiging aan te toon, in teenstelling tot die MWC-vergelyking wat nie hierdie versadigingseffek toon nie. Verder word die bi-substraat omkeerbare Hill-vergelyking se gedrag ook geëvalueer teenoor *in vitro* data van 'n koöperatiewe, allosteries geïnhibeerde bi-substraat ensiem. Die eksperimentele data bevestig die geldigheid van die gedrag soos deur die bi-substraat omkeerbare Hill-vergelyking voorspel. Ten slotte stel ons ook omkeerbare Hill-vergelykings voor vir reaksies van twee substrate na een produk en van een substraat na twee produkte. Sulke reaksies word algemeen aangetref in die metabolisme en dit is dus net so belangrik om hul gedrag akkuraat te kan voorspel as vir reaksies met 'n gelyke aantal substrate en produkte.

Die voorgestelde omkeerbare Hill-vergelykings is almal onafhanklik van die onderliggende ensiemeganisme, hulle bevat parameters wat 'n duidelike operasionele betekenis het, en al die nuut afgeleide vergelykings kan getransformeer word na nie-koöperatiewe vergelykings deur die Hill-koëffisiënt gelyk aan één te stel. Hierdie vergelykings is baie nuttig vir rekenaarmodelle aangesien dit die modeleerder daartoe in staat stel om die gedrag van 'n wye verskeidenheid van koöperatiewe en nie-koöperatiewe reaksies met slegs 'n paar vergelykings akkuraat te kan beskryf.

For Johnny

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1 Introduction

Mathematical modelling and simulation have emerged as important tools to study biological systems, as not all hypotheses can be confirmed or rejected by experimental observation alone. Computational modelling facilitates theoretical hypotheses by asking ‘what if’ questions, and is thus an essential ingredient in the scientific method for the development of new theory from empirical observation. The main challenge in answering such ‘what if’ questions is to understand and predict system behaviour as a whole, reproducing observed experimental phenomena *in silico*. The crux of computational modelling is first to accurately predict the physiological behaviour of the parts of a system, and then, to account for the interplay between these system parts in a mathematical model of the whole system. Metabolic networks are a prime example of a whole system that can be broken down into individual parts. Metabolic networks consist of different pathways, with each pathway consisting of individual enzyme catalysed reactions. Each enzyme catalyses a reaction at a certain rate (flux), and the total pathway flux therefore depends on the constituent reaction fluxes, which are all portrayed as mathematical formulae in a kinetic pathway model. The need to accurately describe individual enzyme reactions mathematically is therefore integral to the endeavour of constructing a realistic kinetic model.

In cellular metabolism many pathway metabolite concentrations do not fluctuate significantly, yet reaction rates do [2]. The enzymes that cause major changes in reaction rate within small tolerances of metabolite fluctuations possess the property of cooperativity. Cooperative enzymes therefore play a key role in metabolism. The mathematical formulae that describe such cooperative enzyme behaviour in *in silico* kinetic models were the main focus of this thesis.

Several formulae are used to describe cooperative enzyme behaviour in theoretical kinetic models. The most common ones are the Hill equation [4], Adair equation [5], Monod, Wyman and Changeux model (MWC) [6] and more recently, the reversible Hill equation [3]. Other equations such as the Koshland, Nemethy and Filmer model [7] do exist; their use has, however, been limited to a few cases, if any.

The MWC model requires knowledge of lower level enzyme mechanism prior to its application, which makes its use in kinetic models difficult. Computational biology is concerned with predict-

ing accurate high level physiological response rather than delineating sub-level enzyme mechanism. The Hill equation is independent of underlying enzyme mechanism and fits a plethora of experimental data sets in the 10–90% physiological concentration range extremely well. As with the other models mentioned, it only describes irreversible reactions, a limitation for modelling application as all enzyme catalysed reactions should be regarded as reversible [1, 8]. It has to date been common practice to model enzyme reactions with high equilibrium constants as irreversible, but the dangers of doing so was shown by Cornish-Bowden & Cárdenas [1]. To be complete, the equations used to model cooperative enzyme reactions in kinetic models must incorporate reversibility to allow for both reaction thermodynamics and substrate/product saturation. Popova & Sel'kov [9] generalised the MWC model to its reversible form, though its use is hampered by numerous parameter definitions, many of which cannot be determined empirically [2, 3, 10]. Furthermore, Hofmeyr & Cornish-Bowden [3] showed how the MWC model is unable to allow for allosteric modifier saturation as predicted by the uni-reactant reversible Hill equation. These limitations, present in each model in some form or another, prompted the derivation of the reversible Hill equation by Hofmeyr & Cornish-Bowden [3]. From a modelling perspective, the reversible Hill equation has many advantages: it is independent of enzyme mechanism, it has fewer parameters with each parameter having operational meaning, it incorporates reversibility and predicts more realistic allosteric modifier behaviour. It can, however, only be applied to one-substrate reactions. In contrast, the majority of enzyme catalysed reactions in metabolic networks are of a multisubstrate nature, many of which are allosterically regulated cooperative conversions. The reversible bi-substrate MWC model was derived by Popova & Sel'kov [11], though the same modelling inadequacies of the one substrate case also apply to the more complex formulation of the bi-substrate case. It has, to the best of our knowledge, never been used in an experimental or theoretical description of bi-substrate cooperative kinetics.

To summarise, the mathematical formulae currently used to describe cooperative kinetics all have some shortcomings. No generic equation is currently available to describe cooperative kinetics of multisubstrate reactions, and no multisubstrate equations are available that incorporate and predict realistic allosteric modifier behaviour. In addition, not all reactions have the same number of substrates and products, and no equations are available to describe such reactions should they show cooperativity. To complicate matters even further, in non-cooperative kinetics, numerous mechanistic equations are present to describe multisubstrate reactions, with parameter definitions that are at best confusing. A generic equation for multisubstrate non-cooperative kinetics is likewise unavailable, nor are there any non-cooperative equations available that incorporate allosteric modifier behaviour.

1.1 Aim and outline of this thesis

The **aim** of this thesis was to derive a generalised reversible Hill equation that incorporates an arbitrary but equal number of substrates and products. The derived equation will be a multisubstrate formulation with all the advantages of the reversible Hill equation. Furthermore, the reversible Hill equations for one, two and three substrate reactions will be extended to incorporate an arbitrary number of modifiers. Setting the Hill coefficient (h) equal to one will transform all the proposed Hill equations into their non-cooperative counterparts. Two additional reversible Hill equations will be derived for one substrate to two products and two substrates to one product reactions, where setting $h = 1$ will transform these two equations to non-cooperative formulations. For computational modelling purposes, these equations are attractive alternatives to the limited and complex models currently available.

The **outline** of this thesis will be as follows: In chapter two, a short description of cooperativity and allostery is given, as well as a brief overview of currently available cooperative models. Chapter three shows the derivation of the multisubstrate reversible Hill equation and the derivation of the two equations for one substrate to two products and two substrates to one product reactions. The multisubstrate reversible Hill equation is then evaluated against known cooperative and non-cooperative models in chapter four, where the general reversible Hill equation is also rewritten into a non-cooperative generic formulation. In chapter five, the two and three substrate reversible Hill equations are extended to incorporate any number of allosteric modifiers. This is followed by the experimental validation of the bi-substrate reversible Hill equation with one modifier in chapter six. Chapter seven concludes with a general discussion of the thesis as well as future prospects of the work.

2 Cooperativity and allostery

Cooperative enzymes play a key role in regulating cellular metabolism. They respond with high sensitivity to small changes in metabolite concentrations. In addition, many cooperative enzymes are also sensitive to metabolites other than their own substrates or products. These metabolites are called allosteric modifiers and can be either activatory or inhibitory. Most often allosteric modifiers act through an effect on the binding affinity of the enzyme for its substrate(s).

This chapter examines the different ways in which cooperativity and allosterism have been incorporated into the mathematical expressions that describe enzyme-catalysed reaction rates.

2.1 Determining the presence of cooperativity

Michaelis-Menten kinetic data typically exhibit hyperbolic saturation curves (Figure 2.1). These hyperbolic curves can be transformed to linear plots from which kinetic parameters such as K_m and V_{\max} can be calculated. Hyperbolic curves are linearised in many ways; below are a few examples.

1. The Lineweaver-Burk plot, $1/v$ vs $1/[\text{substrate}]$.
2. The Eadie-Hofstee plot, v vs $v/[\text{substrate}]$.
3. The Woolf or Hanes plot, $[\text{substrate}]/v$ vs $[\text{substrate}]$.
4. The Eisenthal & Cornish-Bowden *direct linear plot*.

Cooperative enzymes show sigmoidal saturation curves, which when transformed by one of the above mentioned methods do not yield straight lines. Deviations from these linearised plots are typically an indication of the presence of cooperativity (see insert, Figure 2.1).

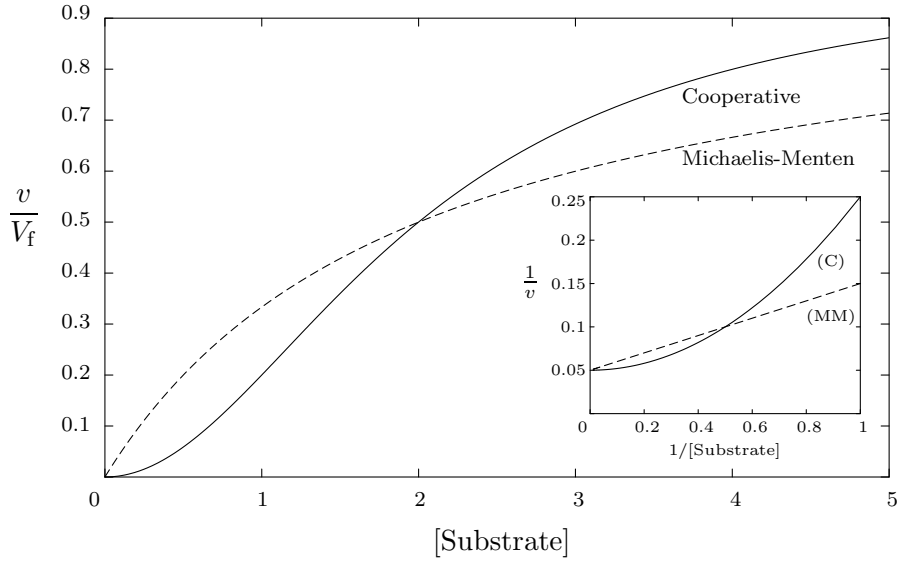
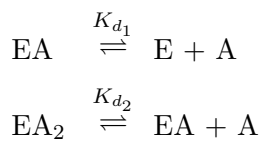


Figure 2.1: Comparison of sigmoidal curve found by Bohr [12] with v/V_f ($\approx \bar{Y}$) as a function of substrate concentration to the hyperbolic curve of Michaelis-Menten kinetics (eq. 8.2). The inserted cooperative kinetics plot (C) clearly deviates from the linear Lineweaver-Burk plot for Michaelis-Menten kinetics (MM).

2.2 The Adair equation

At the turn of the nineteenth century, work by Bohr *et al.* [12] on the oxygen equilibrium of haemoglobin (Hb) showed that this equilibrium is affected by the hydrogen ion concentration. Their work was one of the first to show a sigmoidal curve for the O₂-Hb interaction. A few years later, Adair used osmotic pressure experiments to show that a Hb molecule contains four hemes, and that the Hb molecule adds four oxygen molecules in succession, each with different equilibrium constants [13]. Following this observation, Adair proposed a four-constant equation to describe this behaviour (eq. 8.1 in Appendix). Here the Adair equation will be derived for a dimeric enzyme.

Consider a dimeric enzyme with two identical binding sites that bind substrate A independently (the binding of a substrate to one site does not affect the binding to the other site). The site-dissociation constants K_{d_1} and K_{d_2} refer to the following equilibria:



These dissociation constants do not refer to any particular binding site, but simply to the

position in the reaction sequence. The fraction of protomers occupied by A can be expressed in terms of the fractional saturation (\bar{Y}), where:

$$\bar{Y} = \frac{\text{number of protomers bound to A}}{\text{total number of protomers}}$$

All protomers are part of a dimer, it is therefore necessary to express \bar{Y} in terms of the enzyme-substrate complexes present, where [E] = two empty protomers, [EA] has one empty and one bound protomer and [EA₂] has two fully liganded protomers. The total concentration of substrate-bound protomers is [EA] + 2[EA₂] and the total concentration of protomers is then 2([E] + [EA] + [EA₂]). The fraction of bound protomers can now be expressed as:

$$\bar{Y} = \frac{[\text{EA}] + 2[\text{EA}_2]}{2([\text{E}] + [\text{EA}] + [\text{EA}_2])} \quad (2.1)$$

The dissociation constants K_{d_1} and K_{d_2} are defined as:

$$K_{d_1} = \frac{[\text{E}][\text{A}]}{[\text{EA}]}, \text{ and } K_{d_2} = \frac{[\text{EA}][\text{A}]}{[\text{EA}_2]}$$

Substituting for [EA] and [EA₂] in eq. 2.1 and eliminating [E] from both the numerator and denominator gives:

$$\bar{Y} = \frac{\frac{[\text{A}]}{K_{d_1}} + \frac{2[\text{A}]^2}{K_{d_1}K_{d_2}}}{2\left(1 + \frac{[\text{A}]}{K_{d_1}} + \frac{[\text{A}]^2}{K_{d_1}K_{d_2}}\right)} \quad (2.2)$$

The intrinsic dissociation constant K_a for each site can be defined as the dissociation constant of a single protomer, i.e. when all other binding sites on the protein are absent. Since the sites are identical, K_a will be the same for each site. The site-dissociation constants K_{d_1} and K_{d_2} are related to the intrinsic dissociation constant K_a by so-called statistical binding factors. A substrate molecule is twice as likely to bind to an empty dimer molecule, which has two active sites, than it is to bind to an empty protomer molecule, which has only one active site. Hence the *association* rate constant for binding to the dimer has to be multiplied by 2, giving $K_{d_1} = K_a/2$ for the first binding equilibrium. Similarly, for the second binding equilibrium, a substrate molecule is twice as likely to dissociate from a fully liganded dimer molecule, which has two substrates bound, than from a fully liganded protomer, which has only one substrate bound. Here, the *dissociation* rate constant has to be multiplied by 2, giving $K_{d_2} = 2K_a$. Substituting these relationships into eq. 2.2 gives:

$$\bar{Y} = \frac{\frac{[\text{A}]}{K_a} + \frac{[\text{A}]^2}{K_a^2}}{1 + \frac{2[\text{A}]}{K_a} + \frac{[\text{A}]^2}{K_a^2}} \quad (2.3)$$

If we abandon the assumption of independent binding and assume that the occupancy of an already bound A affects the subsequent binding of A to the empty site by a factor γ , then eq. 2.3 can be rewritten to give:

$$\bar{Y} = \frac{\frac{[A]}{K_a} + \frac{[A]^2}{\gamma K_a^2}}{1 + \frac{2[A]}{K_a} + \frac{[A]^2}{\gamma K_a^2}} \quad (2.4)$$

where $\gamma > 1$ indicates negative cooperativity (second binding of A is hindered), $\gamma < 1$ indicates positive cooperativity (second binding of A is facilitated) and $\gamma = 1$ indicates independent binding (no-cooperativity). Setting $\bar{Y} = 0.5$ (50% saturation with substrate), we can define $A_{0.5} = K_a \gamma^{0.5}$ as the half-saturating concentration of substrate A. $A_{0.5}$ operationally has the same definition as the K_m of the Michaelis-Menten equation, but since the K_m notation is reserved solely for Michaellean kinetics, $A_{0.5}$ is used when referring to cooperative kinetics.

For the case of a catalytic conversion reaction, v is proportional to the number of substrate bound protomers and V_f is proportional to the total number of sub-units, so that $\bar{Y} = v/V_f$. Equation 2.4 can then be rewritten to give the kinetic version of the Adair equation as:

$$\frac{v}{V_f} = \frac{\frac{[A]}{K_a} + \frac{[A]^2}{\gamma K_a^2}}{1 + \frac{2[A]}{K_a} + \frac{[A]^2}{\gamma K_a^2}} \quad (2.5)$$

The Adair equation can also be derived *de novo* from a kinetic point of departure. Figure 2.2 shows the binding of substrate A to a dimeric enzyme with two separate sites. Again assuming

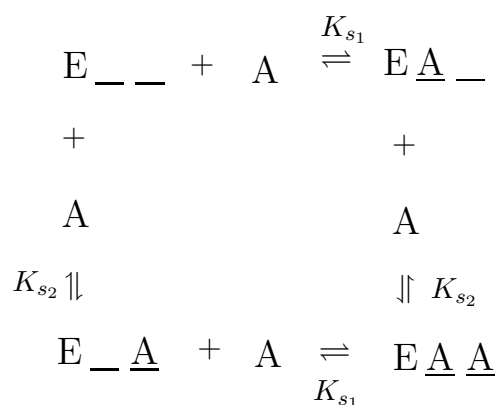


Figure 2.2: An illustration of how binding of A to any of the two available sites on the free enzyme E gives $E\underline{A}$.

the binding sites are independent, the binding of A to site-1 has a dissociation constant of K_{s_1} and to site-2 has a dissociation constant of K_{s_2} . If the reaction rate of $A \rightarrow \text{product}$ at each site is independent with rate constants k_1 and k_2 , the total reaction rate can be written to be the sum of the individual rates [2]:

$$v = \frac{k_1 E_T [A]}{K_{s_1} + [A]} + \frac{k_2 E_T [A]}{K_{s_2} + [A]} \quad (2.6)$$

where E_T is the total enzyme concentration. When $k_1 = k_2$, $k_1 E_T = k_2 E_T = V_f/2$ holds. The limiting rate (V_f) can then be written as $V_f = k_1 E_T + k_2 E_T$, which gives

$$= \frac{(V_f/2)[A]}{K_{s_1} + [A]} + \frac{(V_f/2)[A]}{K_{s_2} + [A]} \quad (2.7)$$

Multiplying eq. 2.7 out gives:

$$\frac{v}{V_f} = \frac{[A](K_{s_1} + K_{s_2}) + 2[A]^2}{2K_{s_1}K_{s_2} + 2[A](K_{s_1} + K_{s_2}) + 2[A]^2} \quad (2.8)$$

$$= \frac{\frac{[A](K_{s_1} + K_{s_2})}{2K_{s_1}K_{s_2}} + \frac{[A]^2}{K_{s_1}K_{s_2}}}{1 + \frac{[A](K_{s_1} + K_{s_2})}{K_{s_1}K_{s_2}} + \frac{[A]^2}{K_{s_1}K_{s_2}}} \quad (2.9)$$

$$= \frac{\frac{[A]}{K_1} + \frac{[A]^2}{K_1 K_2}}{1 + \frac{2[A]}{K_1} + \frac{[A]^2}{K_1 K_2}} \quad (2.10)$$

where, from Figure 2.2, K_1 and K_2 are the molecular dissociation constants for the first and second binding of A, defined as $K_1 = 2(K_{s_1}K_{s_2})/(K_{s_1} + K_{s_2})$, and $K_2 = (K_{s_1} + K_{s_2})/2$. The ratio of molecular dissociation constants is given by $K_2/K_1 = (2 + K_{s_2}/K_{s_1} + K_{s_1}/K_{s_2})/4$ [2]. From the right hand side of the ratio, the sum of the two dissociation constant terms will never be smaller than two. This is to say, the sum of any fraction of numbers (x/y) and its inverse (y/x) will always be bigger than the larger of the two ratios, with a minimum sum of 2 when $x/y = 1$. The K_2/K_1 ratio can therefore never be less than 1, which results in $K_2 \geq K_1$. This shows that the reaction scheme in Figure 2.2 is unable to account for positive cooperativity. Negative cooperativity is therefore always present when the second molecule binds weaker than the first. The assumption of independent binding by Adair shows that whenever positive cooperativity is observed, binding is definitely dependent.

If the molecular dissociation constants are equal ($K_1 = K_2 = K_a$), this is, A binds to each identical site independently (the Adair assumption), eq. 2.10 can be rewritten to give the Adair

equation as (see also eq. 2.5 with $\gamma = 1$):

$$\frac{v}{V_f} = \frac{\frac{[A]}{K_a} + \frac{[A]^2}{K_a^2}}{1 + \frac{2[A]}{K_a} + \frac{[A]^2}{K_a^2}} \quad (2.11)$$

The Adair equation has often been used to describe positive cooperativity, implying a K_2/K_1 ratio smaller than one, and therefore implying dependent binding. Cornish-Bowden [2] showed that in the Adair equation for more than two binding sites, the additional molecular dissociation constants may give rise to relationships between the different K parameters, with some relationships indicating the presence of positive cooperativity between one pair and negative cooperativity between a different pair of constants. When such contradicting relationships are observed, the Adair model cannot be applied. For these cases an alternate operational description of cooperativity is needed that could describe physiological observation independent of binding mechanism. Whitehead [14] proposed that the Hill coefficient was the definition that could fulfil this need.

2.3 The Hill equation

Returning to oxygen binding to haemoglobin, as was the case for the Adair model, Hill [4] also proposed an equation to describe this cooperative nature of the O₂-Hb interaction. The Hill equation is written as:

$$v = \frac{V_f[A]^h}{A_{0.5}^h + [A]^h} \quad (2.12)$$

where h is the Hill coefficient. $h > 1$ indicates positive cooperativity (already bound A facilitates subsequent binding of A), $h < 1$ indicates negative cooperativity (already bound A hinders subsequent binding of A) and for $h = 1$, no cooperativity is present and the Hill equation simplifies to the Michaelis-Menten equation (eq. 8.2 in Appendix). Although Hill assigned no operational meaning to h , it can be used as the upper limit for the number of subunits in physical models, though h is seldomly found to be an integer. V_f remains the maximum (limiting) velocity, as was the case for the Adair equation (eq. 2.5). Although an empirical formulation, the Hill equation has been found to fit a wide range of cooperative data extremely well [3, 2, 15, 16]. The Hill equation can account for both positive and negative cooperativity, as can the Adair model. However, for more than two binding sites, cooperativity in terms of the Hill coefficient is no longer equivalent to its definition in terms of Adair constants. Cornish-Bowden and Koshland

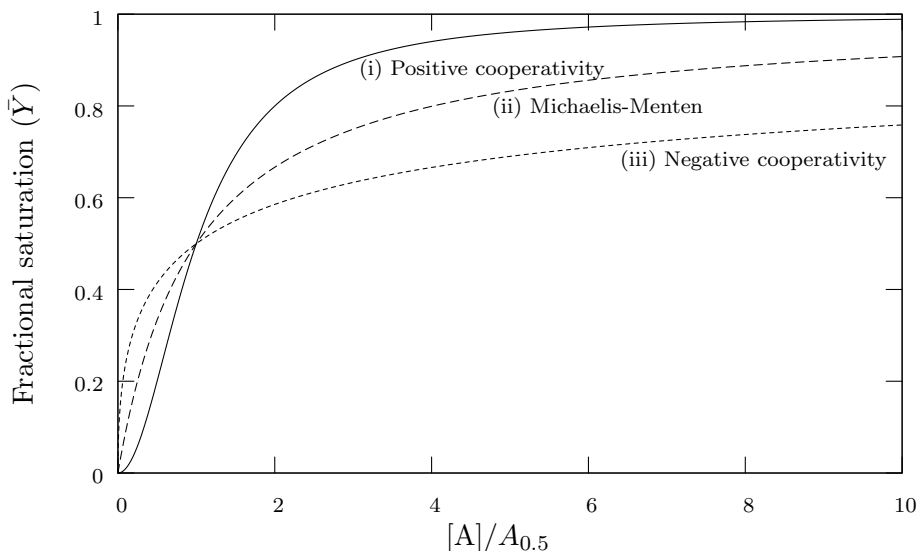


Figure 2.3: Substrate saturation curves showing \bar{Y} as a function of $[A]/A_{0.5}$. This illustrates how positive cooperative -, negative cooperative - and Michaelis-Menten kinetics differ in response to changes in metabolite concentration. The values of the Hill coefficients are: (i) 2.0, (ii) 1 and (iii) 0.5. $A_{0.5} = 2.0$.

[17] investigated this point and found no specific correlation between h and the Adair constants. Although the Adair model adds a more physical meaning to the instances where it can be used, the Hill equation is a more general formulation of cooperativity with a much wider application in cooperative kinetics.

Figure 2.3 shows that for an enzyme exhibiting positive cooperativity, changing the fractional saturation, \bar{Y} , from 10% to 90% requires a 9-fold increase in substrate concentration. The same increase in \bar{Y} , for a Michaelis-Menten system requires an 81-fold increase in ligand concentration. This ‘cooperation’ between sites enhances the enzyme’s sensitivity to small changes in metabolite concentration. The Hill coefficient indicates the type and degree of cooperativity from experimental kinetic data. h is determined by rewriting the Hill equation and taking the logarithm both sides:

$$\log \left[\frac{v}{V_f - v} \right] = h \log[A] - h \log A_{0.5} \quad (2.13)$$

where $v/(V_f - v)$ is correlated to $[EA]/[E]$. The plot drawn from eq. 2.13 is a straight line as a function of $\log[A]$ with slope h (Figure 2.4). This representation of the Hill equation is known as the *Hill plot*, and is an effective means of estimating the Hill coefficient and $A_{0.5}$ for an enzyme in the 10–90% saturation range. The Hill plot has been the standard method used by experimenters to approximate a value of h . The slope of the Hill plot is unity at the extrema, i.e. very low

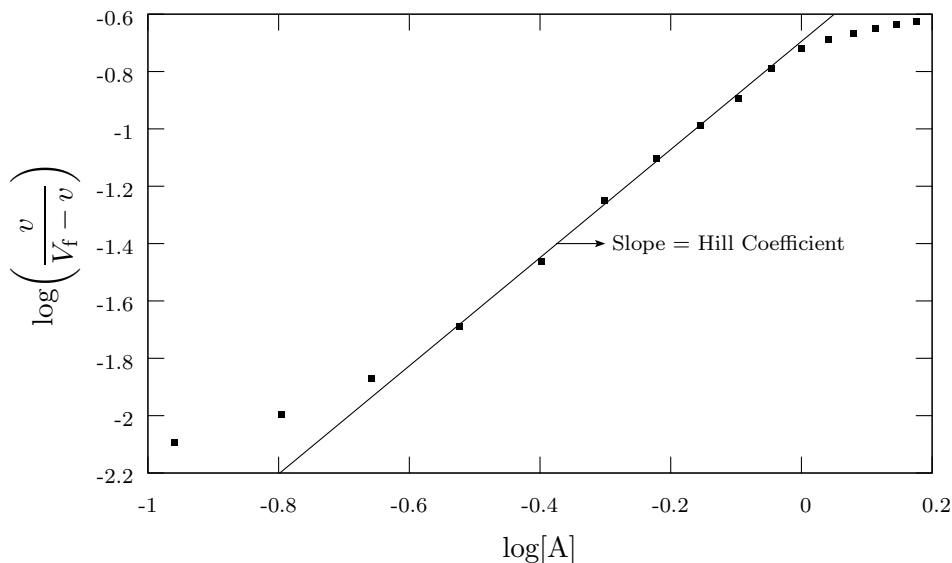


Figure 2.4: An illustration of how the Hill plot can be used to determine the degree of cooperativity from experimental data. (—) = Linear Hill slope, (■) = arbitrary experimental data points.

binding site occupancy and complete saturation. Almost every study that has characterised cooperative enzyme behaviour has used the Hill slope to transform sigmoidal data to estimate h . The advantage of using the Hill plot is its ability to quantify binding parameters without committing to any particular binding mechanism. The irreversible Hill equation has been used extensively in constructing and evaluating simulated cooperativity of *in silico* metabolic models [18, 19].

Other measures of cooperativity have been described. Acerenza & Mizraji [15] defined an alternative index, the ‘global dissociation quotient’, which can be used to establish a quantitative measure of cooperativity. Wyman, and more recently Forsén and Linse, proposed an alternate, quantitative measurement of cooperativity [20, 21]. They proposed using the free energy of interaction between substrate binding sites ($\Delta\Delta G$) as a more accurate measure of cooperativity, where $\Delta\Delta G$ is a direct measurement of inter-binding site cooperativity. The use of $\Delta\Delta G$ in experimental data analysis has, however, been limited. More recently, an alternative approach to analyse cooperative data was suggested by Kurganov [22], eq. 8.3 in Appendix. Kurganov assumed that the interactions between substrate-binding sites responsible for deviations from Michaelian kinetics result in the effective Michaelis constant, K_m^{eff} , changing in response to an increase in v/V_f . The uni-substrate velocity equation proposed can account for both positive and negative cooperativity, as shown by the relationship between the Hill coefficient and Kurganov’s ϱ parameter. This empirical equation by Kurganov gives an accurate description for $v/V_f = 0$

and 1 where the Hill equation does not. The K_m values at these extremes K_0 and K_{lim} are, however, not empirically realistic.

The existence of negative cooperativity was disputed at first and was deemed an artifact of the sequential model of Koshland, Nemethy and Filmer [7]. Subsequent observations of negative cooperativity in enzymes [23, 24] have led to different views as to its significance, cause and physiological purpose [25–28]. More recently, it has become apparent that negative cooperativity plays an integral role in metabolic systems [29]. Nearly 50% of cooperative enzymes that have been characterised show negative cooperativity.

The importance of negative cooperativity is evident as shown in a study by Gerhart & Pardee [30]. They investigated the allosteric inhibition of carbamoyl-phosphate-synthetase by CTP. Carbamoyl-phosphate-synthetase (CPS) is at a branchpoint that leads to numerous other pathway products beside CTP. When CTP is in excess, it is important that CPS activity is not completely inhibited, as this would result in all other pathways at this branchpoint being shut down. The negative cooperativity that CTP shows for CPS is a failsafe measure to ensure CPS is not entirely inhibited when CTP concentration is high [29].

Allosterism is an important enzyme property where, in addition to substrate and product binding sites, an additional binding site is present for an effector ligand (modifier). The modifier can either inhibit or activate the enzyme. (Enzymes that are subject to allosteric regulation can be categorised as either K-series, V-series or K-V series enzymes, see Appendix 8.1). These modifiers cause a measurable conformational change (concerted) in certain regions of the protein [29] that often result in a significant change in pathway flux. It is essential that reaction formulae in computational models be able to accurately account for cooperativity and allosteric effects. Many such formulae have been used to date, none more popular than the Monod, Wyman and Changeux model.

2.4 Triple-J paving the way: The concerted model of Jacques Monod, Jeffries Wyman and Jean-Pierre Changeux

In an attempt to describe the cooperative nature of oxygen-binding to haemoglobin, Pauling was the first to suggest that the oxygen binding sites on haemoglobin would have to be in close proximity to allow for electronic interaction [13]. This was later shown not to be the case following the elucidation of haemoglobin's three dimensional structure [31]. Preceding this idea, Fischer adopted a lock and key principle for explaining enzyme-substrate interactions [32]. The

binding site was assumed to be the exact imprint of the substrate. Contrary to Fischer and Pauling, Koshland proposed that instead of a rigid, non-dynamic binding site, the binding site changes into the correct conformation upon substrate binding [33]. This was called the theory of ‘induced fit’ and could explain long-range interactions between active sites.

The observation that conformational changes in substrate binding can explain cooperativity, resulted in two different theories being proposed. The first of these was postulated in 1965 by Monod, Wyman and Changeux (MWC). It was named the symmetry or concerted model [6]. The MWC model assumed that the binding of a single ligand will cause an identical conformational change in each enzyme subunit, thus conserving protein symmetry. The model construction was based on various postulates:

1. each subunit can exist in only one of two states, designated R- (relaxed) and T- (tense) state. The R-state binds substrate strongly and T-state binds substrate weakly, or not at all,
2. all the subunits (protomers) of an oligomeric enzyme must be in the same conformational state, being either in the T-form, which is the predominant conformation for unliganded protein, or in the R-form, which is the predominant conformation for liganded species. Hybrids, such as the RT-state of a dimer are not allowed.
3. The two conformational states of the enzyme are always in equilibrium, with an equilibrium constant $L = [T_n]/[R_n]$, where T_n and R_n represent the unliganded T and R states.
4. A substrate can bind to any one of these two states, but the dissociation constants K_R and K_T are of the order $K_R \ll K_T$.
5. Allosteric inhibitors bind exclusively to the T-form of the enzyme, leading to an increase in L . Allosteric activators bind solely to the R-form and as a result, decrease L .

The rate equation derived by MWC is:

$$\frac{v}{V_f} = \frac{L'c\frac{[A]}{K_R} \left(1 + c\frac{[A]}{K_R}\right)^{n-1} + \frac{[A]}{K_R} \left(1 + \frac{[A]}{K_R}\right)^{n-1}}{L' \left(1 + c\frac{[A]}{K_R}\right)^n + \left(1 + \frac{[A]}{K_R}\right)^n}$$

where $L' = L\frac{(1 + \beta)^n}{(1 + \gamma)^n}$, $\beta = \frac{[I]}{K_I}$, $\gamma = \frac{[Z]}{K_Z}$; $[A]$, $[I]$ and $[Z]$ are substrate, inhibitor and activator concentrations respectively; K_R , K_I and K_Z are the corresponding intrinsic dissociation constants for the subunit monomer-ligand complexes; n is the number of subunits; $c = K_R/K_T$;

L is the equilibrium constant for the transition $T_n \rightleftharpoons R_n$ in absence of ligands (T_n/R_n); v is the initial reaction velocity and V_f is the limiting forward reaction velocity [2, 6, 34]. When $c = 1$ eq. 2.14 simplifies to the Michaelis-Menten equation (eq. 8.2). The degree of cooperativity depends on the values of L and c and is higher when L is large and c is very small. Setting c , β and γ all equal to 0, gives the MWC equation most commonly used for irreversible cooperative enzyme reactions:

$$\bar{Y} = \frac{\frac{[A]}{K_R} \left(1 + \frac{[A]}{K_R}\right)^{n-1}}{L + \left(1 + \frac{[A]}{K_R}\right)^n} \quad (2.14)$$

When L increases, so does the sigmoidicity of the v/V_f vs $[A]$ plot. If $L = 0$ the curve becomes hyperbolic. Cooperativity in the MWC model is based on a reaction mechanism response upon perturbation of the T_n/R_n equilibrium. When L is large the T_n conformation is favoured. Consider only one molecule of R_n being present. When ligand A is added, R_n will momentarily be liganded with only one A, disturb the $T_n:R_n$ equilibrium, and introduce one more R_n by decreasing the T_n pool. The two R_n molecules now have three open sites. When the next A binds to any of them, another R_n will be drawn from the T_n pool, giving three R_n molecules with four open sites. Thus, upon every one site being bound with A, one R_n (two sites) are introduced. This shows that after the initial lagphase, the rate at which ligand is bound increases exponentially as the amount of open sites (R_n) increase. When the T_n pool is depleted, it indicates enzyme saturation and the catalytic rate will be at a maximum. From the above explanation, it can be seen that the saturation curve must be sigmoidal, and thus confirms the ability of MWC model to account for positive homotropic (interactions between identical ligands) cooperativity. However, it is quite evident from this analogy that the MWC model is unable to predict negative homotropic cooperativity.

A mathematical approach may provide more concrete evidence of the MWC model's inability to predict negative cooperativity. Cornish-Bowden showed that the MWC equation for a dimer can be rewritten to take the shape of the Adair equation [2]:

$$\bar{Y} = \frac{\left(\frac{1/K_R + L/K_T}{1 + L}\right) [A] + \left(\frac{1/K_R^2 + L/K_T^2}{1 + L}\right) [A]^2}{1 + 2\left(\frac{1/K_R + L/K_T}{1 + L}\right) [A] + \left(\frac{1/K_R^2 + L/K_T^2}{1 + L}\right) [A]^2} \quad (2.15)$$

From eq. 2.15 the ratio of the two molecular dissociation constants (see p. 18) can be written as:

$$\frac{K_2}{K_1} = \frac{\frac{1}{K_R^2} + \frac{2L}{K_R K_T} + \frac{L^2}{K_T^2}}{\frac{1}{K_R^2} + \frac{L}{K_T^2} + \frac{L}{K_T^2} + \frac{L^2}{K_T^2}} \quad (2.16)$$

The ratio of K_2/K_1 will always be less than 1 since $2L/K_R K_T \leq (L/K_T^2 + L/K_R^2)$. This relationship holds for any pair of molecular Adair constants, whether the enzyme is a dimer or an oligomer. K_2 is never larger than K_1 which indicates the subsequent binding of the substrate molecule will always be stronger. This shows that the MWC model only incorporates positive cooperativity.

One of the advantages of the MWC model is its ability to accommodate both homotropic and heterotropic (interactions between different ligands) allosteric effects, without an increase in complexity of the mechanism. Moreover, in the MWC model, binding of substrate analogues to enzyme increase the molecules present in the R-form, resulting in non-allosteric activation. The need to accommodate such activation effects in the enzyme model followed from the finding that certain enzymes exhibit activation by low concentrations of substrate analogues; D-lactate dehydrogenase [35], dCMP deaminase [36], threonine dehydratase [37] and lactate dehydrogenase [38] are examples. On the other hand, binding exclusively to the T-form results in a decrease in enzyme molecules in the R-state, therefore in inhibition.

A study by Changeux & Rubin investigated the allosteric interactions in aspartate transcarbamylase III [39]. Predictions from the MWC model were compared to experimental data to determine whether the data set supports the model postulates on which the MWC equation is based. The main focus was to compare allosteric observations with allosteric predictions proposed by the model. Quantitative data analysis showed good correlation to the model saturation functions, and model parameters were estimated using the Scatchard coordinate system [2, 40]. From these parameter values, Changeux & Rubin suggested that the substrate analog binds the R-form exclusively and the allosteric inhibitor shows preference for the T-form of the enzyme. The conformational changes in protein over wide ranges of substrate and allosteric inhibitor concentrations were found to be compatible with the two-state MWC model. They did, however, find that although the data appeared to coincide with the concerted model, the presence of a model mechanism different to the symmetry model could not be ruled out.

The above example rests solely on the interpretation of data with respect to the saturation functions of MWC, and does not incorporate catalytic conversion. The postulates of the MWC model make its application to kinetics difficult, as shown by Dalziel [41], where the difference between a K-enzyme and V-enzyme system proposed by Monod, Wyman and Changeux necessitates derivation of two separate velocity expressions. Moreover, Kurganov [42] made a detailed kinetic analysis of the generalised MWC model, assuming that the equilibrium between the R- and T-forms is rapid in comparison with rate of catalysis, an assumption nearly always made when deriving rate equations. His analysis showed how at low concentrations of substrates or

substrate-analogs, the MWC equation gives an excellent result. A study by Yon [43] of aspartate transcarbamylase prompted a modification of the kinetic MWC model to account for several reaction anomalies. Khan *et al.* [44] did not make use of the modified MWC equation, proposing that the purified enzyme's behaviour can be readily explained in simple MWC model terms. Both studies conclude that the kinetic MWC model fits the experimental data. A rigorous test of the kinetic MWC model's predictive ability was done by Henry *et al.* [45]. A model of 85 coupled differential equations was constructed to determine haemoglobin bimolecular rebinding as well as tertiary and quaternary conformational change kinetics. Their conclusion was that, although not perfect, the MWC model can explain equilibrium and kinetic data equally well.

The Hill coefficient is often used as a measure of cooperativity inside MWC models. A conversion of the MWC saturation function into an expression for the Hill coefficient was proposed by Kegeles [46]. This expression can be written for an n -site enzyme as:

$$h = n \frac{K_A [A]_m}{1 + K_A [A]_m} \quad (2.17)$$

where $[A]_m$ is the substrate concentration at maximum slope in the Hill plot and K_A is an intrinsic binding constant to the R-form. Equation 2.17 shows that h is equal to the number of binding sites per molecule, n , multiplied by the degree of saturation at the maximum slope in the Hill plot. This is a means of quickly correlating Hill coefficients to the degree of saturation of the cooperative enzyme. An example of such a collaboration between the MWC model and its approximation with Hill coefficients was given by Waser *et al.* [47] for the modelling of phosphofructokinase kinetics *in silico*. Their random order, two-state allosteric MWC model provided the best fit to the for the experimental data for muscle PFK. Waser's conclusions correlated very well with previous findings by Pettigrew & Frieden [48, 49] for the same enzyme.

An analytical solution to the MWC model parameters was proposed by Zhou and co-workers [50]. Two ideas were summarised, i) the use of a Kegeles Hill coefficient and ii) the modelling of experimental data shown by Waser *et al.* Zhou *et al.* extended the Kegeles h -estimation. They presented an expression to determine the slope of the Hill plot at any substrate concentration. Furthermore, an equation for substrate concentration at maximum Hill slope ($[A]_0$) was derived. Together with the general equation for approximating the slope of the Hill plot, they were able to provide formulae to calculate all the parameters of the MWC model from h , $[A]_0$ and the value of $\bar{v}/(n - \bar{v})$ at $[A]_0$. These formulae were then used to determine the set of MWC model parameters that best describe a set of experimental oxygen binding data by fitting the model to the data. The MWC model was found to correlate well with the experimental data.

There are circumstances where the model fails as a result of the postulates it is based upon. An example is the allosteric inhibitory effect of PEP on glucose-6-phosphate dehydrogenase from

Zymomonas mobilis [51]. This study by Scopes showed how the Hill equation gives a better approximation, leaving the binding mechanism in the reaction unresolved. In such cases where the explanation for data falls outside the ‘scope’ of the MWC model, an alternative to the concerted model must be considered.

2.5 The sequential model of Koshland, Nemethy and Filmer

An alternative model to that of Monod, Wyman and Changeux was proposed by Koshland, Nemethy and Filmer (KNF) [7]. The KNF model is based on the induced fit theory of Koshland [33]. This model is different from the MWC model in that it includes, and insists on, the existence of hybrid species between the conformational R- and T-forms proposed by Monod, Wyman and Changeux. It postulates that each subunit changes shape upon ligand binding, causing a perturbation in the shape of the unliganded subunits or the interactions between them. The KNF model therefore abolishes the MWC postulate that all subunits in an enzyme must exist in either the R- or T-form. Furthermore it also considers geometry of subunit association [2] because different configurations of subunits result in different binding equations. The model permits ligands to bind to both the R- and T-forms with different binding affinity, consistent with the induced fit theory. It suggested an induced conformational change from T \rightarrow R as the ligand binds to the T-conformation, resulting in sequential changes in subunit geometry. The model became known as the *sequential model* and could account for positive and negative cooperativity equally well. The equation KNF proposed can be written as follows:

$$\bar{Y} = \frac{c[A]/\bar{K} + [A]^2/\bar{K}^2}{1 + 2c[A]/\bar{K} + [A]^2/\bar{K}^2} \quad (2.18)$$

where $\bar{K} = K_t K_A / K_{R:R}^{0.5}$, $c = K_{R:T} / K_{R:R}^{0.5}$, $K_t = [T]/[R]$, $K_A = [R][A]/[RA]$, $[A]$ = concentration of substrate A, $K_{R:T} = [R:T][T:T]$, where $K_{R:T}$ is the equilibrium constant that depicts the stability of the [R:T] hybrid interface relative to the standard [T:T] interface and $K_{R:R} = [R:R]/[T:T]$ is the obvious relative [R:R] interface equilibrium constant.

This model shows that the range of binding behaviour of a single ligand is independent of association geometry and quaternary protein structure, and is determined by two parameters. The first parameter is a relative stability interface ratio which compares the intermediate [R:T]-state to the [R:R]- and [T:T]-states. The second parameter is the mean Adair dissociation constant for binding from unliganded to completely liganded enzyme. Comparing the Adair equation (eq. 2.5) to the KNF equation, it can be shown that $K_1 = \bar{K}/c$ and $K_2 = c\bar{K}$. Once again looking at the Adair ratio for dissociation constants: $K_2/K_1 = c^2$. This shows that the degree of cooperativity depends only on the value of c . For $K_2 < K_1$, the second substrate

molecule binds with a higher affinity than the first substrate molecule, which indicates positive cooperativity and $c < 1$. Conversely, should $K_2 > K_1$ then a value of $c > 1$ will indicate negative cooperativity.

This observation shows that the KNF model can account for negative cooperativity, setting it apart from the MWC model. It is worth noting that for dimeric enzymes showing positive cooperativity, distinguishing between the MWC and KNF mechanism is difficult as both models give equally good fits.

A theoretical approach to distinguish between these two models was proposed by Henis & Levitzki [52]. The approach was based on the introduction of a second ligand that competes for the same binding site as the native substrate. This second ligand can bind either non-cooperatively or cooperatively. Both the MWC and KNF models specifically predict the nature and extent of such a competing substrate and this approach can be applied to separate the two binding models. Henis & Levitzki showed that for a non-cooperative competing ligand the MWC model can be excluded. The introduction of a competing ligand that changes the saturation behaviour to give a Hill coefficient less than 1, i.e. negatively cooperative, also excludes the MWC model. Seydoux *et al.* [53] followed a similar approach to Henis & Levitzki in their study of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. The binding of NAD^+ was shown to be negatively cooperative. Their main focus was to investigate the binding of the NAD^+ analogues; ADP-ribose and ATP and 3-acetylpyridine-adenine dinucleotide. They concluded that NAD^+ binding behaviour was due to induced conformational changes and served as support for the KNF model. Branlant [54] did a similar study of glyceraldehyde-3-phosphate dehydrogenase from sturgeon muscle. He adopted an alternative approach to data fitting and obtained good fits to all the proposed KNF models. Branlant's findings correlated well with those of Seydoux and Henis & Levitzki [55].

These above mentioned studies only serve as examples to show that previously the enzyme mechanism present was first determined prior to enzyme characterisation. However, the use of KNF models has generally been few and far between. The MWC model has been the predominant choice to describe cooperativity in kinetic models. For modelling purposes, these two models share a common disadvantage of being formulations that do not incorporate reaction reversibility.

2.6 The uni-reactant reversible Hill equation

Many cooperative enzymes have high reaction equilibrium constants and can be regarded as essentially irreversible catalytic events. Reactions catalysed by these enzymes have been modelled accordingly. However, to accept these reactions as completely irreversible is not always correct. Theoretically, all reactions should be considered as reversible to some degree [1, 3, 2, 8]. In practice the use of reversible rate equations has been hampered by the lack of kinetic data available for the reverse reaction of an enzyme since the main focus of most kinetic studies relies on characterising the forward catalytic rate.

Popova & Sel'kov [9] generalised the uni-reactant MWC model to its reversible form. Its use is hampered by complicated parameter definitions, with the parameters not only being numerous (see eq. 8.10), but also being outside the scope of experimental determination [3, 10]. This observation, together with the fact that the Hill equation gives an excellent approximation to cooperative kinetics, without prior knowledge of the substrate binding mechanism, prompted the search for a reversible cooperative equation based on the Hill equation. In 1997, Hofmeyr & Cornish-Bowden [3] generalised the Hill equation incorporating allosteric modifier (M) effects for uni-uni reactions of the form $A \rightleftharpoons P$ to its reversible form,

$$v = \frac{V_f \alpha \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^{h-1}}{\frac{1 + \mu^h}{1 + \sigma^{2h} \mu^h} + (\alpha + \pi)^h} \quad (2.19)$$

with $\alpha = [A]/A_{0.5}$, $\pi = [P]/P_{0.5}$ and $\mu = [M]/M_{0.5}$. $A_{0.5}$, $P_{0.5}$ and $M_{0.5}$ are half-saturating constants, defined on p. 17, and V_f is the maximum forward rate. σ is a measure of how much the modifier affects the dissociation constant of the substrate and product. The generalised reversible Hill equation is a simple and accurate alternative to the complex models of MWC and KNF. The parameters therein all have the same meaning as the original irreversible Hill equation. Additionally, the reversible Hill equation simplifies to the irreversible Hill equation when $[A]$ or $[P] = 0$. It incorporates a thermodynamic term, which solely depends on metabolite concentrations, separating the reversible property from the kinetic properties of the enzyme. Although only a uni-reactant formulation of cooperativity, the reversible Hill equation has clear advantages in kinetic modelling compared to the MWC and lesser used KNF models.

2.7 Cooperativity models under non-equilibrium conditions

All the models discussed to this point have been equilibrium models that can only be applied to kinetic data by assuming that v/V_f is a true measure of fractional saturation \bar{Y} . However, if one

could measure binding at equilibrium and if these direct binding studies showed that substrate-binding were not a cooperative process, but corresponding kinetic studies showed sigmoidal plots and departures from linearity in other primary plots (see section 2.1), then it would have to be concluded that the kinetic mechanism of the reaction is responsible for the cooperative effect. This is termed kinetic cooperativity.

2.7.1 Kinetic cooperativity of monomeric enzymes

Mechanistic kinetic cooperativity can be present in a monomeric enzyme if the free enzyme exists in two or more conformations with each conformation able to react with the substrate at different rates [56]. This type of cooperativity arises as a result of cooperation in time of two or more different conformations of the same enzyme in the overall conversion reaction. Kinetic cooperativity of monomeric enzymes can therefore not exist under equilibrium conditions and in turn requires the conformational transitions between the free enzyme forms to be ‘slow’. Such enzymes are termed *hysteretic* enzymes [57–59]. Additionally, Ferdinand [60] was one of the first to show that a random-order ternary-complex mechanism for a bi-substrate monomeric enzyme-catalysed reaction can lead to sigmoidal kinetics in the absence of cooperative binding. The main assumption is that, e.g. for the mechanism $E + A + B \rightleftharpoons \text{products}$, one of the pathways (e.g. $E \rightleftharpoons EA \rightleftharpoons EAB \rightarrow \text{products}$) is kinetically preferred and will proceed faster than the other. Moreover, the affinity of E for B is less than that of EA for B. Jensen & Trentini [61] showed that the enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase from *Rhodomicrobium vannielli* may catalyse its reaction according to the Ferdinand type mechanism.

Rabin [62] has showed how a uni-substrate reaction catalysed by an enzyme with a single binding site for the substrate can show sigmoidal kinetics, provided that the enzyme can exist in more than one conformation. An example of such a mechanism is $E \rightleftharpoons ES \rightleftharpoons E'S \rightleftharpoons E' \rightleftharpoons E$ where E is assumed to be thermodynamically more stable than E' and the catalytic conversions are $ES \rightleftharpoons EP$ and $E'S \rightleftharpoons E'P$. The ‘slow’ transition between E and E' upon the desorption of the last product from the active site, will result in the enzyme retaining the conformation stabilised by that product for a short period of time before relaxing to a different conformation. The enzyme is then said to show mnemonical behaviour. The most attractive feature of the mnemonical model is its simplicity and the fact that it introduces no new hypothesis, only one new idea that desorption of a product may be faster than the corresponding conformational change of the enzyme. For a complete discussion of mnemonical behaviour and systems, see Ricard & Cornish-Bowden [57], pp. 261–264.

2.7.2 Kinetic cooperativity of polymeric enzymes

Ricard and co-workers [63–65] pioneered the development of a new theory to explain oligomeric enzyme cooperativity. This theory was termed *structural kinetics* and was aimed at understanding how subunit interactions and conformational constraints of cooperative enzymes may allow the precise tuning of the conversion of substrate to its product. Structural kinetics does not adhere to the assumption of proportionality between the substrate-binding isotherm and the reaction rate, as assumed in the MWC model. Moreover, it is a more general theory than the KNF model and also simplifies to the KNF formulation should the catalytic reaction rate be regarded as ‘slow’.

The principle behind structural kinetics is that subunit interactions in an oligomeric enzyme can have two different types of effect on the reaction rate. The rate of conformational changes may be altered by subunit interactions during the catalytic process, i.e. in the absence of inter-subunit stress, subunits packed together may undergo faster or slower conformational changes than one would expect should the subunits be isolated. The resultant free energy contribution is termed the protomer arrangement energy contribution [64]. In addition, subunit interactions may also be responsible for changes in three dimensional subunit structure should the subunits be closely coupled. The corresponding energy contribution is termed the quaternary constraint energy contribution [64].

A main principle of structural kinetics is partitioning the Gibbs free energy of activation of a catalytic conversion reaction into the above protomer arrangement and quaternary constraint energy contributions. This allows the derivation of any kind of rate equation and binding isotherm on the basis of structural terms. These rate equations are usually very complex and assumptions regarding the subunit interaction and coupling in the ground state and transition state are made to simplify them considerably. These are: i) the relaxation of quaternary constraints in the transition states, ii) should no quaternary constraint be present, a subunit can exist in only two possible conformations, and iii) every subunit bound to the transition state is unique [57, 64]. These three postulates proposed by Ricard and co-workers allow the derivation of simplified structural kinetic models that are, to a certain degree, a good approximation of reality. They concluded that the possibilities for regulation of polymeric enzyme systems offered by the kinetic cooperativity are more delicate and diverse than one would have anticipated from direct equilibrium-binding studies.

2.8 Multisubstrate reactions inside metabolism

All discussions to this point have only considered uni-substrate models or equations. The majority of key regulatory metabolic enzymes that exhibit cooperative behaviour catalyse reactions involving more than one substrate. Popova & Sel'kov [11] generalised the MWC model to a reversible bi-substrate reaction (eq. 8.10 in Appendix). Certain parameters in eq. 8.10 do not convey a clear mechanistic meaning and are impossible to determine empirically. To our knowledge, no investigator has applied this equation in a study of cooperative enzyme kinetics.

2.9 Motivation

The MWC and KNF models currently used to describe cooperativity are based on enzyme mechanism and derived from postulates that are not entirely convincing. These models are difficult to work with because:

- the reversible uni-substrate and bi-substrate MWC and uni-reactant KNF equations contain many constants and parameter definitions,
- the binding mechanism dominates the choice of model to be applied,
- the MWC model is unable to account for negative binding- or negative kinetic cooperativity or both,
- the MWC R- and T-forms have vastly different binding affinities, but identical catalytic properties (perfect K -system),
- certain MWC model parameters have no clear operational meaning,
- the MWC model is unable to account for realistic modifier effects,
- the equation formulation depends on the number of enzyme-subunits, which is especially cumbersome when deciding on a KNF model (i.e. tetrahedral, square or linear),
- there is no easy means of converting the KNF model parameter for degree of cooperativity, c , to the Hill coefficient,
- the generalised KNF model only predicts irreversible behaviour,
- allosteric regulation by the KNF model allows for allosteric effectors to act in numerous ways, where the overall effect depends on the interaction constants involved.

The above observations show that the MWC and KNF models, from a modelling perspective, are not obvious choices when attempting to describe multi-substrate cooperative kinetics. As a matter of fact, no real model is an obvious choice since no real usable models for multisubstrate cooperative reactions exist. The Hill and reversible Hill equations fit cooperative experimental data very well in the 10–90% saturation range, without commitment to any mechanism. Moreover, the parameters present in the two Hill equations all have clear operational meaning and can be determined experimentally. From the aims set out in section 1.1, we aim to derive multi-substrate Hill equations that will

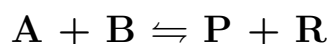
- be independent of underlying binding mechanism,
- have fewer parameters to resolve,
- give a direct indication to the degree of cooperativity,
- encompass parameters within the realm of experimental determination,
- include the contribution of reversible reactions, and
- separate the thermodynamic and kinetic properties of the enzyme.

Moreover, the generalised multisubstrate reversible Hill equation will incorporate any arbitrary but equal number of substrates and products. The reversible Hill equations for two and three substrate reactions incorporating any number of modifiers will give accurate allosteric modifier behaviour. This study will attempt to validate the derived bi-substrate reversible Hill equation with one modifier by comparing *in silico* predicted and experimentally determined enzyme-modifier behaviour. Furthermore, we shall also derive the reversible Hill equations for one substrate to two products and two substrates to one product reactions, since reactions of these orders are often found in metabolic pathways.

3 Generalising the reversible Hill equation for multisubstrate reactions

3.1 Bi-substrate bi-product reactions

When attempting to generalise any irreversible equation to its reversible form, care must be taken not to violate the thermodynamic constraints of the system. For this derivation the first system considered is defined as follows:



1. A dimeric enzyme with two binding sites, one for substrate A (site-A) and its product P with concentrations [A] and [P], and a second for substrate B (site-B) and its product R with concentrations [B] and [R].
2. Binding to sites A and B is independent (the binding of A or P does not affect the binding of B or R and *vice versa*).
3. The binding reactions are all at equilibrium, i.e. the rate at which for example species EA₂B₂ dissociates is orders of magnitude faster than that of its conversion to EAPBR.

Before deriving the reversible Hill equation for the above mentioned bi-substrate reaction, for three substrate reactions and finally, for reactions of any number of substrates, we shall first explore the basic case of a one-substrate (uni-uni) conversion.

Consider a one-substrate conversion reaction where substrate A gets converted to its product P, $A \rightleftharpoons P$. The mass action ratio at any instant during the reaction for this one-substrate and the above bi-substrate conversion can be written as:

$$\Gamma_{uni-uni} = \frac{[P]}{[A]}, \quad \text{and} \quad \Gamma_{bi-bi} = \frac{[P][R]}{[A][B]}$$

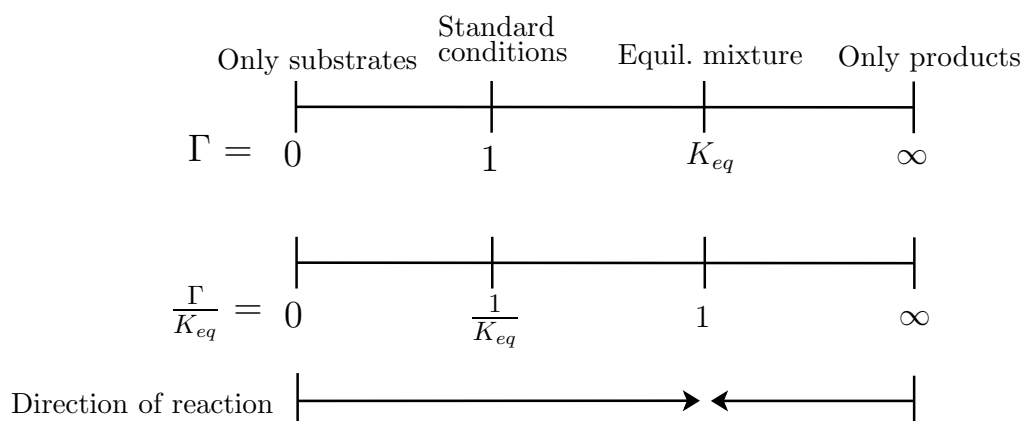


Figure 3.1: An illustration of the different stages of substrate to product conversion for an arbitrary enzyme catalysed reaction. Γ = the mass action ratio and K_{eq} is the equilibrium constant. The position of K_{eq} on the continuum can be anywhere between 0 and ∞ .

Figure 3.1 shows that at equilibrium $\Gamma = K_{eq}$. As any reaction will always tend towards equilibrium, if Γ lies to the right of K_{eq} , the reaction will proceed to the left (the reverse reaction). Similarly, if Γ lies to the left of K_{eq} , the reaction will proceed to the right (forward reaction). Assuming the forward catalytic rate to be positive, Figure 3.1 shows that:

- $\Gamma/K_{eq} < 1$ indicates the forward reaction,
- $\Gamma/K_{eq} > 1$ indicates the reverse reaction,
- $\Gamma/K_{eq} = 1$ indicates equilibrium.

Any reversible reaction involving metabolites [A], [B], [P] and [R] can be written as a function which contains $(1 - \Gamma/K_{eq})$ [3]. Such a function can be considered to be:

$$v = \left(1 - \frac{\Gamma}{K_{eq}}\right) g([A],[B],[P],[R]....) \tag{3.1}$$

where g is a function of the concentrations affecting the reaction rate. Γ/K_{eq} is the disequilibrium ratio for any reaction, and it then follows that when $\Gamma/K_{eq} > 1$, $(1 - \Gamma/K_{eq}) < 0$ indicates the reverse reaction and for $\Gamma/K_{eq} < 1$, $(1 - \Gamma/K_{eq}) > 0$ indicates the forward reaction.

The enzyme rate equation that depends on substrate and product concentrations can be written as:

$$\begin{aligned} v_{net} &= v_f - v_r \\ v_f &= k_{f_i} [\text{Reactive enzyme-substrate complex}]_i + \dots + k_{f_{i+1}} [\text{Reactive enzyme-substrate complex}]_{i+1} \\ v_r &= k_{r_i} [\text{Reactive enzyme-product complex}]_i + \dots + k_{r_{i+1}} [\text{Reactive enzyme-product complex}]_{i+1} \end{aligned}$$

where k_{f_i} is the respective forward catalytic rate constant of a species and k_{r_i} is the respective reverse catalytic rate constant of a species. For $i+1 = n$, n represents the number of reactive enzyme-metabolite complexes that can take part in the forward and reverse conversion reaction. Following formulation of the rate equation, both sides are divided by E_T (the total concentration of all the possible enzyme-metabolite complexes that can result from binding). The rate equation is then rewritten as:

$$\frac{v}{E_T} = \frac{\sum_{i=1}^n k_{f_i} [\text{reactive substrate}]_i - \sum_{j=1}^n k_{r_j} [\text{reactive product}]_j}{[E] + [E\text{-substrate}] + [E\text{-substrate/product}] + [E\text{-product}]} \quad (3.2)$$

Expressing all the enzyme species concentrations in terms of $[E]$ (unliganded free enzyme) and free metabolite concentrations will result in $[E]$ being cancelled from both the numerator and denominator. Following this analogy the Michaelis-Menten equation for the conversion of $A \rightleftharpoons P$ can be written as a result of the Haldane relationship, eq. 3.3,

$$K_{eq}^{uni} = \frac{V_f}{V_r} \cdot \frac{K_p}{K_a} \quad (3.3)$$

to give the reversible Michaelis-Menten equation as:

$$v = \frac{V_f \alpha - V_r \pi}{1 + \alpha + \pi} = \frac{\left(1 - \frac{\Gamma}{K_{eq}}\right) V_f \alpha}{1 + \alpha + \pi} \quad (3.4)$$

where $V_f = k_f \cdot E_T$, $V_r = k_r \cdot E_T$, $\alpha = [A]/K_a$ and $\pi = [P]/K_p$. α and π are the substrate- and product concentrations scaled to their Michaelis constants. It can now be seen from the right hand side of eq. 3.4 that the earlier proposed function g (eq. 3.1) depends solely on positive values. The direction in which a reversible reaction will proceed is therefore only dependent on the sign of the $(1 - \Gamma/K_{eq})$ term. The uni-reactant reversible Hill equation (eq. 2.19) shows the function g and the thermodynamic term $(1 - \Gamma/K_{eq})$. The reversible Hill uni-reactant equation simplifies to the rewritten form of the Michaelis-Menten equation (eq. 3.4) when the Hill coefficient = 1. It also simplifies to the irreversible Hill equation when the product concentration = 0. A reversible bi-substrate Hill equation will therefore have to:

1. be written in the form of eq. 3.1,
2. simplify to the uni-reactant reversible Hill equation when binding site-A is absent or binding site-B is absent,
3. show good correlation to one-substrate reversible Hill kinetics at saturating levels of one of its substrates A or B,
4. show a good approximation to the irreversible Hill equation when $[P]$ and $[R] = 0$ and $[B]$ is saturating,
5. show a good approximation to non-cooperative bi-substrate equations when the Hill coefficient h is 1.

3.1.1 Derivation for 2 subunits

The kinetic Adair equation for a dimeric enzyme was derived in chapter two and is written as:

$$\frac{v}{V_f} = \frac{\frac{[A]}{K_a} + \frac{[A]^2}{\gamma K_a^2}}{1 + \frac{2[A]}{K_a} + \frac{A_2}{\gamma K_a^2}}$$

where $V_f = 2k_f \cdot [E_T]$. From p. 17, $A_{0.5} = K_a \gamma_a^{0.5}$. $A_{0.5}$ was defined as the concentration of substrate A needed for half-maximal velocity. For the 2-site enzyme reaction $A + B \rightleftharpoons P + R$ considered here, the respective half maximal saturation concentrations of molecules B, P, and R can be written as $B_{0.5} = K_b \gamma_b^{0.5}$, $P_{0.5} = K_p \gamma_p^{0.5}$ and $R_{0.5} = K_r \gamma_r^{0.5}$. From this point onwards, $X_{0.5}$ will serve as a general term for the substrate and product half-saturating concentrations. Figure 3.2 illustrates the binding model for molecules A and P binding to the free enzyme. Once more, the statistical factor of 1/2 comes from the observation that the first molecule of A can bind to any one of two sites on E and only dissociate from one. Similarly, the second molecule of A can only bind to one site, but dissociate from two, resulting in a statistical factor of 2. Moreover, the binding of the alternate P molecule to the second site, should the first site be occupied by A, will result in no statistical factors, but an additional interaction factor γ_{ap} . This interaction factor is the measure by how much an already bound A molecule changes the dissociation constant for the following binding of P to the second site. The interaction factor γ_{pa} is then a measure of change in dissociation constant K_a should the first site be occupied by a P molecule.

Figures 3.2 and 3.3 show the rewriting of dissociation constants for binding of only molecules A and P in terms of their substrate $X_{0.5}$ notation.

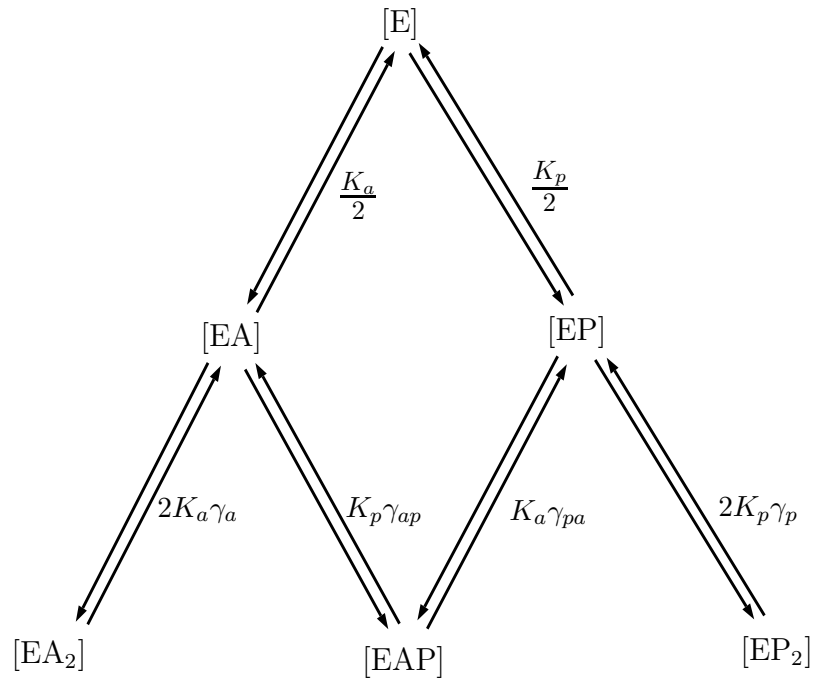


Figure 3.2: Illustration of molecules A + P binding to free enzyme and the respective dissociation constant for each binding step.

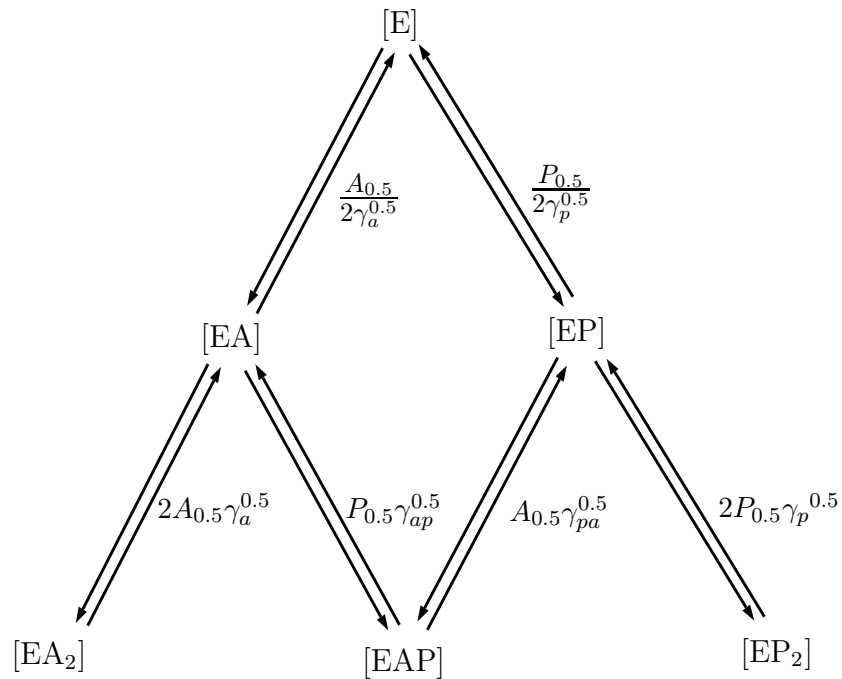


Figure 3.3: Molecules A and P binding to free enzyme with the dissociation constants rewritten in terms of their $X_{0.5}$ concentrations.

Similarly, the binding of B and R to site-B gives $B_{0.5}$ and $R_{0.5}$. Consider the case of binding of both substrates A and B to the free enzyme. From section 3.1, assuming these substrates bind independently, the binding of molecule A has no effect on the subsequent binding of molecule B and *vice versa*. Substrates only affect the dissociation constant for molecules binding at the same site. The interaction parameter for A–A binding is called γ_{aa} and for B–B interactions γ_{bb} where $\gamma_{aa} \neq \gamma_{bb}$. Microscopic reversibility requires that the product of equilibrium constants around any catalytic cycle should be 1. The interaction parameters for each site are therefore equal, resulting in $\gamma_{aa} = \gamma_{ap} = \gamma_{pa} = \gamma_{pp}$. γ_a can therefore be regarded as the interaction parameter for A–A, A–P and P–P interactions. The same assumption holds for B–B, B–R and R–R interactions at site-B which gives the common interaction parameter γ_b . The binding scheme for molecules A and B is shown in Figure 3.4.

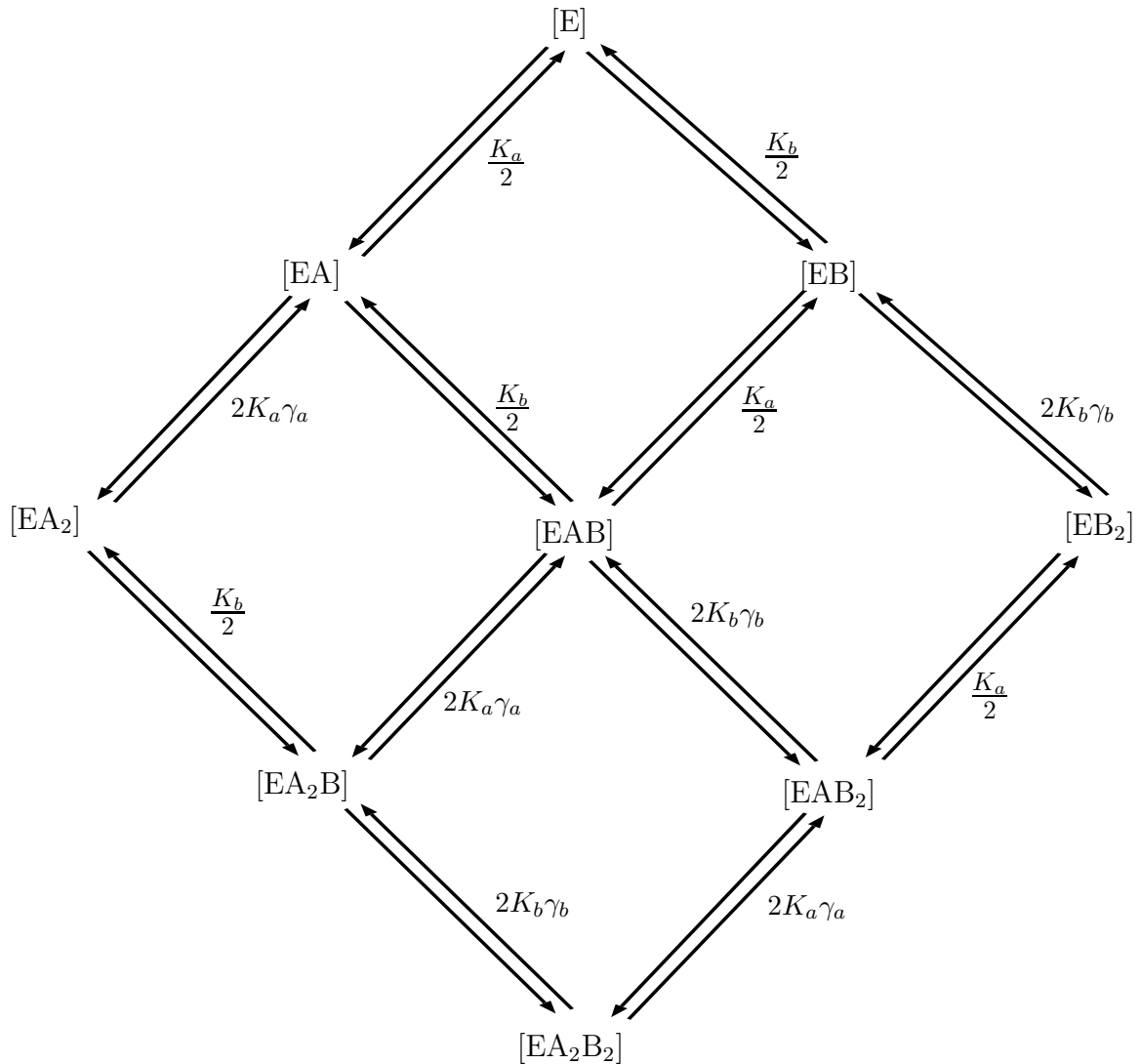


Figure 3.4: Bound enzyme forms for molecules A and B binding to free enzyme.

Figure 3.4 serves only as a template, since there are of course many more possible binding combinations between the four different molecules. All the possible enzyme-complexes assuming completely random binding can be summarised in Table 3.1.

Table 3.1: All the species that can result from binding for the $A + B \rightleftharpoons P + R$ reaction.

E	EA	EA ₂	EAP	EP ₂	EP
EB	EAB	EA ₂ B	EAPB	EP ₂ B	EPB
EB ₂	EAB ₂	EA ₂ B ₂	EAPB ₂	EP ₂ B ₂	EPB ₂
EBR	EABR	EA ₂ BR	EAPBR	EP ₂ BR	EPBR
ER ₂	EAR ₂	EA ₂ R ₂	EAPR ₂	EP ₂ R ₂	EPR ₂
ER	EAR	EA ₂ R	EAPR	EP ₂ R	EPR

From Figure 3.3 it can be seen that the dissociation constant for each species listed in Table 3.1 is one or a combination of several of the following:

$$K_a = A_{0.5}/\gamma_a^{0.5},$$

$$K_p = P_{0.5}/\gamma_a^{0.5},$$

$$K_b = B_{0.5}/\gamma_b^{0.5} \text{ and}$$

$$K_r = R_{0.5}/\gamma_b^{0.5}$$

where the constants have been written in terms of their $X_{0.5}$ concentrations for half-maximal velocity. Scaling the metabolite concentrations to their $X_{0.5}$ values simplifies the notation and can be written as:

$$\alpha = \frac{[A]}{A_{0.5}}, \beta = \frac{[B]}{B_{0.5}}, \pi = \frac{[P]}{P_{0.5}}, \text{ and } \rho = \frac{[R]}{R_{0.5}} \quad (3.5)$$

Each of the entries in Table 3.1 (except E) is rewritten in terms of its dissociation constants from Figure 3.2. These dissociation constants are then converted to their $X_{0.5}$ concentrations and entered into Table 3.2 with [E] factored out.

Table 3.2: Summary of the enzyme—dimer—species considered for E_T , rewritten as an equilibrium expression (Equil. exp.) in terms of free metabolite concentrations, and their scaled notation.

[Species]	Equil. exp.	Scaled notation	[Species]	Equil. exp.	Scaled notation
[E]	$\frac{[E]}{[E]}$	1	[EA ₂ BR]	$\frac{2[A]^2[R]^2}{\gamma_a\gamma_bK_a^2K_r^2}$	$2\alpha^2\rho^2$
[EA]	$\frac{2[A]}{K_a}$	$2\alpha\gamma_a^{0.5}$	[EA ₂ R ₂]	$\frac{[A]^2[R]^2}{\gamma_a\gamma_bK_a^2K_r^2}$	$\alpha^2\rho^2$
[EA ₂]	$\frac{[A]^2}{\gamma_aK_a^2}$	α^2	[EA ₂ R]	$\frac{2[A]^2[R]}{\gamma_aK_a^2K_r}$	$2\alpha^2\rho\gamma_b^{0.5}$
[EAP]	$\frac{2[A][P]}{\gamma_aK_pK_a}$	$2\alpha\pi$	[EAPB]	$\frac{4[A][P][B]}{\gamma_aK_aK_pK_b}$	$4\alpha\pi\beta\gamma_b^{0.5}$
[EP ₂]	$\frac{[P]^2}{\gamma_aK_p^2}$	π^2	[EAPB ₂]	$\frac{2[A][P][B]^2}{\gamma_a\gamma_bK_aK_pK_b^2}$	$2\alpha\pi\beta^2$
[EP]	$\frac{2[P]}{K_p}$	$2\pi\gamma_a^{0.5}$	[EAPBR]	$\frac{4[A][P][B][R]}{\gamma_a\gamma_bK_aK_bK_pK_r}$	$4\alpha\pi\beta\rho$
[EB]	$\frac{2[B]}{K_b}$	$2\beta\gamma_b^{0.5}$	[EAPR ₂]	$\frac{2[A][P][R]^2}{\gamma_a\gamma_bK_aK_pK_r^2}$	$2\alpha\pi\rho^2$
[EB ₂]	$\frac{[B]^2}{\gamma_bK_b^2}$	$2\beta^2$	[EAPR]	$\frac{4[A][P][R]}{\gamma_aK_aK_pK_r}$	$4\alpha\pi\rho\gamma_b^{0.5}$
[EBR]	$\frac{2[A][R]}{\gamma_bK_bK_r}$	$2\beta\rho$	[EP ₂ B]	$\frac{2[P]^2[B]}{\gamma_aK_p^2K_b}$	$2\pi^2\beta\gamma_b^{0.5}$
[ER ₂]	$\frac{[R]^2}{\gamma_bK_r}$	α^2	[EPR]	$\frac{4[P][R]}{K_pK_r}$	$4\pi\rho\gamma_a^{0.5}\gamma_b^{0.5}$
[ER]	$\frac{2[R]}{K_r}$	$2\rho\gamma_b^{0.5}$	[EP ₂ B ₂]	$\frac{[P]^2[B]^2}{\gamma_a\gamma_bK_p^2K_b^2}$	$\pi^2\beta^2$
[EAB]	$\frac{4[A][B]}{K_aK_b}$	$4\alpha\beta\gamma_a^{0.5}\gamma_b^{0.5}$	[EP ₂ BR]	$\frac{2[P]^2[B][R]}{\gamma_a\gamma_bK_p^2K_bK_r}$	$2\alpha^2\beta\rho$
[EAB ₂]	$\frac{2[A][B]^2}{\gamma_bK_aK_b^2}$	$2\alpha\beta^2\gamma_a^{0.5}$	[EP ₂ R ₂]	$\frac{[P]^2[R]^2}{\gamma_a\gamma_bK_p^2K_r^2}$	$\pi^2\rho^2$
[EABR]	$\frac{4[A][B][R]}{\gamma_bK_aK_bK_r}$	$4\alpha\beta\rho\gamma_a^{0.5}$	[EP ₂ R]	$\frac{2[P]^2[R]}{\gamma_aK_p^2K_r}$	$2\pi^2\rho\gamma_b^{0.5}$
[EAR ₂]	$\frac{2[A][R]^2}{\gamma_bK_aK_r^2}$	$2\alpha\rho^2\gamma_a^{0.5}$	[EPB]	$\frac{4[P][B]}{K_pK_b}$	$4\pi\beta\gamma_a^{0.5}\gamma_b^{0.5}$
[EAR]	$\frac{4[A][R]}{K_aK_r}$	$4\alpha\rho\gamma_a^{0.5}\gamma_b^{0.5}$	[EPB ₂]	$\frac{2[P][B]^2}{\gamma_bK_pK_b^2}$	$2\pi\beta^2\gamma_a^{0.5}$
[EA ₂ B]	$\frac{2[A]^2[B]}{\gamma_aK_a^2K_b}$	$2\alpha^2\beta\gamma_b^{0.5}$	[EPBR]	$\frac{4[P][B][R]}{\gamma_bK_pK_bK_r}$	$4\pi\beta\rho\gamma_a^{0.5}$
[EA ₂ B ₂]	$\frac{[A]^2[B]^2}{\gamma_a\gamma_bK_a^2K_b^2}$	$\alpha^2\beta^2$	[EPR ₂]	$\frac{2[P][R]^2}{\gamma_bK_pK_r^2}$	$2\pi\rho^2\gamma_a^{0.5}$

From Table 3.2, analogous to the derivation of eq. 3.4, the denominator will comprise all enzyme species that sum to E_T . All the species in the third and sixth columns of Table 3.2 can now be grouped and factorised to give E_T —the denominator—as:

$$\begin{aligned}
 D_i &= 1 + 2\alpha\gamma_a^{0.5} + \alpha^2 + 2\alpha\pi + 2\pi\gamma_a^{0.5} + \pi^2 = 1 + (\alpha + \pi)(2\gamma_a^{0.5} + \alpha + \pi) \\
 D_{ii} &= 2\beta\gamma_b^{0.5} + \beta^2 + 2\beta\rho + 2\gamma_b^{0.5}\rho + \rho^2 = (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho) \\
 D_{iii} &= 4\gamma_a^{0.5}\gamma_b^{0.5}\alpha\beta + 2\gamma_a^{0.5}\alpha\beta^2 + 2\gamma_a^{0.5}\alpha\beta\rho + 2\gamma_b^{0.5}\alpha^2\beta + \alpha^2\beta^2 + \alpha^2\beta\rho + 2\gamma_b^{0.5}\alpha\pi\beta + \alpha\pi\beta^2 + \alpha\pi\beta\rho + \\
 &\quad 4\gamma_a^{0.5}\gamma_b^{0.5}\alpha\rho + 2\gamma_a^{0.5}\alpha\beta\rho + 2\gamma_a^{0.5}\alpha\rho^2 + 2\gamma_b^{0.5}\alpha^2\rho + \alpha^2\beta\rho + \alpha^2\rho^2 + 2\gamma_b^{0.5}\alpha\pi\rho + \alpha\pi\beta\rho + \alpha\pi\rho^2 + \\
 &\quad 4\gamma_a^{0.5}\gamma_b^{0.5}\pi\beta + 2\gamma_a^{0.5}\pi\beta^2 + 2\gamma_a^{0.5}\pi\beta\rho + 2\gamma_b^{0.5}\alpha\pi\beta + \alpha\pi\beta^2 + \alpha\pi\beta\rho + 2\gamma_b^{0.5}\pi^2\beta + \pi^2\beta^2 + \pi^2\beta\rho + \\
 &\quad 4\gamma_a^{0.5}\gamma_b^{0.5}\pi\rho + 2\gamma_a^{0.5}\pi\beta\rho + 2\gamma_a^{0.5}\pi\rho^2 + 2\gamma_b^{0.5}\alpha\pi\rho + \alpha\pi\beta\rho + \alpha\pi\rho^2 + 2\gamma_b^{0.5}\pi^2\rho + \pi^2\beta\rho + \pi^2\rho^2 \\
 &= (\alpha\beta + \alpha\rho + \pi\beta + \pi\rho)(4\gamma_a^{0.5}\gamma_b^{0.5} + 2\gamma_a^{0.5}\beta + 2\gamma_a^{0.5}\rho + 2\gamma_b^{0.5}\alpha + \alpha\beta + \alpha\rho + 2\gamma_b^{0.5}\pi + \pi\beta + \pi\rho) \\
 &= (\alpha + \pi)(2\gamma_a^{0.5} + \alpha + \pi)(\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho)
 \end{aligned}$$

From the factorised terms, the denominator (D) can now be written as:

$$D = D_i + D_{ii} + D_{iii} \quad (3.6)$$

which gives

$$D = 1 + (\alpha + \pi)(2\gamma_a^{0.5} + \alpha + \pi) + (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho) + (\alpha + \pi)(2\gamma_a^{0.5} + \alpha + \pi)(\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho) \quad (3.7)$$

The conversion rate for reaction $A + B \rightleftharpoons P + R$ can be formulated as:

$$v = k_f[\text{EAxBy}] - k_r[\text{EPxRy}]$$

where x can either be A, P or vacant and y can either be B, R or vacant. Species EA_2B_2 is fully liganded with substrate only and will therefore give the highest forward catalytic rate, 2^*k_f , where 2 is the forward catalytic factor (FCF). Similarly, species EP_2R_2 is fully liganded with product only and will give the highest reverse catalytic rate, 2^*k_r , where 2 is the reverse catalytic factor (RCF). Any other species considered for catalysis will have a FCF or RCF equal to 1. One catalytic event converts one A and one B molecule, **bound to the same subunit**, simultaneously to P and R. The statistical factors introduced for catalysis are the product of i) statistical factors from binding (BF) as can be seen from the scaled notation in columns 3 and 6 of Table 3.2 and ii) the FCF and RCFs. Furthermore it is important to note that some species are dead-end complexes. For example, species EAPBR and EPBR each have a total binding factor (TBF) of 4, yet only two of the four possible conformations for each species will be catalytically active yielding a net BF of 2.

The species that will take part in the forward and reverse conversion are listed in Table 3.3. The final scaled notations to be entered into the rate equation are given. From Table 3.3 the rate equation can be written as:

$$\begin{aligned} v_{net} &= v_f - v_r \\ v_f &= 2k_f\alpha\beta(\gamma_a^{0.5}\gamma_b^{0.5} + \beta\gamma_a^{0.5} + \rho\gamma_a^{0.5} + \alpha\gamma_b^{0.5} + \pi\gamma_b^{0.5} + \alpha\beta + \alpha\rho + \pi\beta + \pi\rho) \\ v_r &= 2k_r\pi\rho(\gamma_a^{0.5}\gamma_b^{0.5} + \beta\gamma_a^{0.5} + \rho\gamma_a^{0.5} + \alpha\gamma_b^{0.5} + \pi\gamma_b^{0.5} + \alpha\beta + \alpha\rho + \pi\beta + \pi\rho) \end{aligned}$$

Factorising v_f and v_r and dividing both sides of $v_{net} = v_f - v_r$ by $[E_T]$ gives:

$$\frac{v}{[E_T]} = \frac{(V_f\alpha\beta - V_r\pi\rho)(\gamma_a^{0.5}(\gamma_b^{0.5} + \beta + \rho) + \alpha(\gamma_b^{0.5} + \beta + \rho) + \pi(\gamma_b^{0.5} + \beta + \rho))}{D} \quad (3.8)$$

where $V_f = 2k_f[E_T]$ and $V_r = 2k_r[E_T]$. From the Haldane relationship and mass action ratio given below:

$$K_{eq} = \frac{V_f}{V_r} \cdot \frac{P_{0.5}R_{0.5}}{A_{0.5}B_{0.5}} \quad \text{and} \quad \Gamma = \frac{[P][R]}{[A][B]}$$

eq. 3.8 can now be rewritten with a factorised numerator and its denominator D—eq. 3.7—to give the rate equation as follows:

$$v = \frac{V_f\alpha\beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\gamma_a^{0.5} + \alpha + \pi)(\gamma_b^{0.5} + \beta + \rho)}{1 + (\alpha + \pi)(2\gamma_a^{0.5} + \alpha + \pi) + (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho) + (\alpha + \pi)(2\gamma_a^{0.5} + \alpha + \pi)(\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho)} \quad (3.9)$$

Table 3.3: Summary of the enzyme species considered for catalysis. TBF = total binding factor, BF = binding factor for separate productive and dead-end configurations, FCF = forward catalytic factor, RCF = reverse catalytic factor, TSF = total statistical factor for term, and Rate eq. term = the scaled notation of species entered into the rate equation.

Forward conversion						
Species	TBF	BF	FCF	RCF	TSF	Rate eq. term
[EAB]	4	2	1	0	2	$2k_f \cdot \alpha\beta\gamma_a^{0.5}\gamma_b^{0.5}$
		2	0	0	0	-
[EAB ₂]	2	2	1	0	2	$2k_f \cdot \alpha\beta^2\gamma_a^{0.5}$
[EABR]	4	2	1	0	2	$2k_f \cdot \alpha\beta\rho\gamma_a^{0.5}$
		2	0	0	0	-
[EA ₂ B]	2	2	1	0	2	$2k_f \cdot \alpha^2\beta\gamma_b^{0.5}$
[EA ₂ B ₂]	1	1	2	0	2	$2k_f \cdot \alpha^2\beta^2$
[EA ₂ BR]	2	2	1	0	2	$2k_f \cdot \alpha^2\beta\rho$
[EAPB]	4	2	1	0	2	$2k_f \cdot \alpha\pi\beta\gamma_b^{0.5}$
		2	0	0	0	-
[EAPB ₂]	2	2	1	0	2	$2k_f \cdot \alpha\pi\beta^2$
[EAPBR]	4	2	1	1	2	$2k_f \cdot \alpha\pi\beta\rho$
		2	0	0	0	-
Reverse conversion						
[EPR]	4	2	0	1	2	$2k_r \cdot \pi\rho\gamma_a^{0.5}\gamma_b^{0.5}$
		2	0	0	0	-
[EPBR]	4	2	0	1	2	$2k_r \cdot \pi\beta\rho\gamma_a^{0.5}$
		2	0	0	0	-
[EPR ₂]	2	2	0	1	2	$2k_r \cdot \pi\rho^2\gamma_a^{0.5}$
[EAPR]	4	2	0	1	2	$2k_r \cdot \alpha\pi\rho\gamma_b^{0.5}$
		2	0	0	0	-
[EP ₂ R ₂]	1	1	0	2	2	$2k_r \cdot \pi^2\rho^2$
[EAPR ₂]	2	2	0	1	2	$2k_r \cdot \alpha\pi\rho^2$
[EP ₂ R]	2	2	0	1	2	$2k_r \cdot \pi^2\rho\gamma_b^{0.5}$
[EP ₂ BR]	2	2	0	1	2	$2k_r \cdot \pi^2\beta\rho$
[EAPBR]	4	2	1	1	2	$2k_r \cdot \alpha\pi\beta\rho$
		2	0	0	0	-

Until this point the equation formulation was based on $\gamma_a \neq \gamma_b$. Rewriting the equation with $\gamma_a = \gamma_b$ gives eq. 3.10. The only difference from eq. 3.9 is in the interaction parameter notation where all the γ parameters are now the same, $\gamma^{0.5}$.

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\gamma^{0.5} + \alpha + \pi)(\gamma^{0.5} + \beta + \rho)}{1 + (\alpha + \pi)(2\gamma^{0.5} + \alpha + \pi) [1 + (\beta + \rho)(2\gamma^{0.5} + \beta + \rho)] + (\beta + \rho)(2\gamma^{0.5} + \beta + \rho)} \quad (3.10)$$

Inferring different and equal effects of A at site-A and B at site-B shows that the equation formulation accounts for all ranges of interaction effects at both sites. Eqs. 3.9 and 3.10 can be regarded as an alternate ways of writing the Adair equation for bi-substrate catalysis. In eq. 3.10 γ has the same definition as in the kinetic Adair equation (eq. 2.5). It is thus a measure of the degree of cooperativity of the Adair bi-substrate equation. From this it can be seen that interaction parameter γ shows positive cooperativity for $\gamma < 1$. It then becomes clear that if $\gamma = 1$, there will be no cooperative effect at any site as the dissociation constant will then equal the $X_{0.5}$ value. Setting γ_a and $\gamma_b = 1$ in eq. 3.9 will give,

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (1 + \alpha + \pi)(1 + \beta + \rho)}{1 + (\alpha + \pi)(2 + \alpha + \pi) + (\beta + \rho)(2 + \beta + \rho) + (\alpha + \pi)(2 + \alpha + \pi)(\beta + \rho)(2 + \beta + \rho)} \quad (3.11)$$

Stepwise regrouping of the denominator is done as follows:

$$\begin{aligned} v &= \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (1 + \alpha + \pi)(1 + \beta + \rho)}{(\alpha + \pi)(2 + \alpha + \pi) [1 + (\beta + \rho)(2 + \beta + \rho)] + [(\beta + \rho)(2 + \beta + \rho) + 1]}, \\ &= \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (1 + \alpha + \pi)(1 + \beta + \rho)}{[1 + (\alpha + \pi)(2 + \alpha + \pi)] [1 + (\beta + \rho)(2 + \beta + \rho)]}, \\ &= \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (1 + \alpha + \pi)(1 + \beta + \rho)}{(1 + \alpha + \pi)^2 (1 + \beta + \rho)^2} \end{aligned}$$

Cancelling $(1 + \alpha + \pi)$ and $(1 + \beta + \rho)$ from both the numerator and denominator gives the reversible non-cooperative random order equation for two substrate conversions as:

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right)}{(1 + \alpha + \pi)(1 + \beta + \rho)} \quad (3.12)$$

The Adair bi-substrate equation also accounts for the interaction parameter, γ , e.g. for site-A to equal one and for site-B not to equal one. This is commonly observed in cooperative enzyme behaviour for bi-substrate catalysis [23, 66, 67], where the enzyme exhibits cooperative

saturation curves for one substrate and hyperbolic Michaelis curves for saturation of the other. Setting one interaction parameter $\gamma_a = 1$, stepwise analysis of the equation denominator (D_A) is done as follows:

$$\begin{aligned} D_A &= (\alpha + \pi)(2 + \alpha + \pi) [1 + (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho)] + [(\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho) + 1], \\ &= [1 + (\alpha + \pi)(2 + \alpha + \pi)] [1 + (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho)], \\ &= (1 + \alpha + \pi)^2 [1 + (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho)] \end{aligned}$$

Hence, the Adair bi-substrate equation with $\gamma_a = 1$ and rewritten denominator D_A can be written as:

$$\begin{aligned} v &= \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (1 + \alpha + \pi)(\gamma_b^{0.5} + \beta + \rho)}{(1 + \alpha + \pi)^2 [1 + (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho)]} \\ &= \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\gamma_b^{0.5} + \beta + \rho)}{(1 + \alpha + \pi) [1 + (\beta + \rho)(2\gamma_b^{0.5} + \beta + \rho)]} \end{aligned}$$

This shows that the cooperativity is retained for binding to site-B when site-A exhibits Michaelian kinetics, which is consistent with the assumption (section 3.1) that binding to site-A and site-B is independent. The limiting degree of cooperativity for this model will be reached when $\gamma = 0$, which when entered into eq. 3.9 gives eq. 3.13.

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)(\beta + \rho)}{[1 + (\alpha + \pi)^2] [1 + (\beta + \rho)^2]} \quad (3.13)$$

Equation 3.13 shows that if the cooperative effect is at the limit, any intermediate species that result from binding will not be present in E_T . It is therefore possible to construct a binding scheme for a cooperative bi-substrate model for infinite cooperativity. Figure 3.5 illustrates this binding model with only empty or fully liganded binding sites depicted. From Figure 3.5, eq. 3.13 can be derived directly without considering the contribution of intermediate species. This will be shown in the following section.

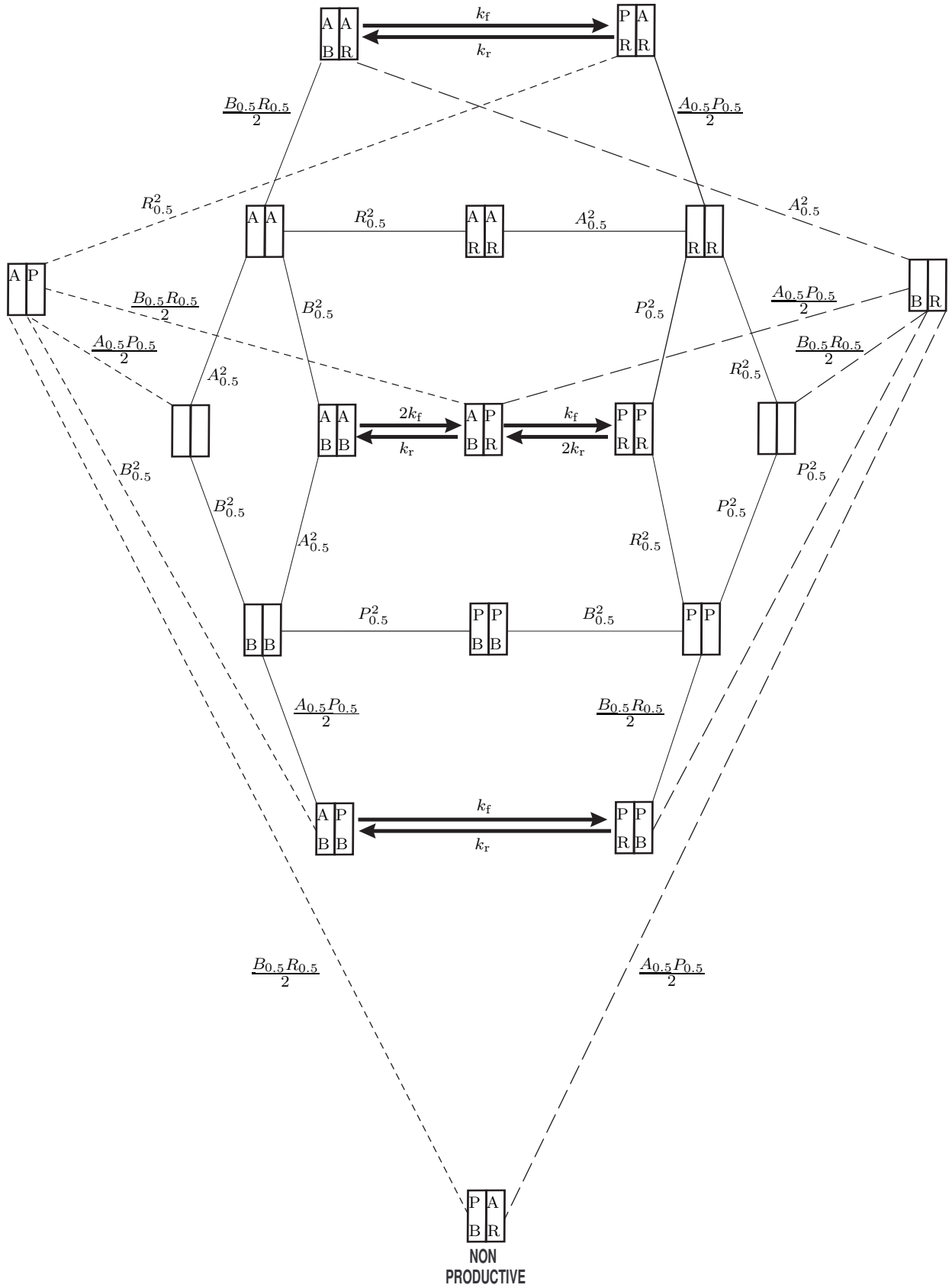


Figure 3.5: Representation of bound enzyme forms for reaction $A + B \rightleftharpoons R + P$. No intermediate bound enzyme species are present as these species make a negligible contribution to E_T when assuming infinite cooperativity. 47

3.1.2 Derivation for 2 subunits assuming infinite cooperativity

For this derivation infinite cooperativity is assumed, i.e. following the binding of A to an unliganded enzyme subunit, the second A or P molecule binds immediately. This results in no intermediate complexes being present in the species considered for E_T . Scaling the concentrations of these enzyme species to their respective substrate $X_{0.5}$ concentrations results in Table 3.4.

Table 3.4: Dimeric enzyme-complex species for infinite cooperativity, rewritten as equilibrium expressions (Equil. exp.) in terms of their free metabolite concentrations and their scaled notations. α , β , π and ρ are defined in the main text.

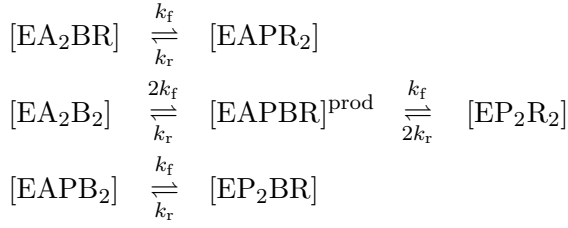
[Species]	Equil. exp.	Scaled notation	[Species]	Equil. exp.	Scaled notation
[EA ₂]	$\frac{[E][A]^2}{A_{0.5}^2}$	α^2	[EB ₂]	$\frac{[E][B]^2}{B_{0.5}^2}$	β^2
[EP ₂]	$\frac{[E][P]^2}{P_{0.5}^2}$	π^2	[ER ₂]	$\frac{[E][R]^2}{R_{0.5}^2}$	ρ^2
[EAP]	$\frac{2[E][A][P]}{A_{0.5}P_{0.5}}$	$2\alpha\pi$	[EBR]	$\frac{2[E][B][R]}{B_{0.5}R_{0.5}}$	$2\beta\rho$
[EA ₂ B ₂]	$\frac{[E][A]^2[B]^2}{A_{0.5}^2B_{0.5}^2}$	$\alpha^2\beta^2$	[EP ₂ R ₂]	$\frac{[E][P]^2[R]^2}{P_{0.5}^2R_{0.5}^2}$	$\pi^2\rho^2$
[EA ₂ R ₂]	$\frac{[E][A]^2[R]^2}{A_{0.5}^2R_{0.5}^2}$	$\alpha^2\rho^2$	[EP ₂ B ₂]	$\frac{[E][P]^2[B]^2}{P_{0.5}^2B_{0.5}^2}$	$\pi^2\beta^2$
[EP ₂ BR]	$\frac{2[E][P]^2[B][R]}{P_{0.5}^2B_{0.5}R_{0.5}}$	$2\pi^2\beta\rho$	[EA ₂ BR]	$\frac{2[E][A]^2[B][R]}{A_{0.5}^2B_{0.5}R_{0.5}}$	$2\alpha^2\beta\rho$
[EAPB ₂]	$\frac{2[E][A][P][B]^2}{A_{0.5}P_{0.5}B_{0.5}^2}$	$2\alpha\pi\beta^2$	[EAPR ₂]	$\frac{2[E][A][P][R]^2}{A_{0.5}P_{0.5}R_{0.5}^2}$	$2\alpha\pi\rho^2$
[EAPBR]	$\frac{4[E][A][P][B][R]}{A_{0.5}P_{0.5}B_{0.5}R_{0.5}}$	$4\alpha\pi\beta\rho$	[E]	[E]	1

Writing the concentrations of the species in column 3 of Table 3.4 with E factored out, gives the denominator $[E_T]/[E]$ of the rate equation as:

$$\begin{aligned}
 \frac{[E_T]}{[E]} &= 1 + \alpha^2 + \beta^2 + \pi^2 + \rho^2 + 2\alpha\pi + 2\beta\rho + \alpha^2\beta^2 + 2\alpha^2\beta\rho + \\
 &\quad \alpha^2\rho^2 + 2\alpha\pi\beta^2 + 4\alpha\pi\beta\rho + 2\alpha\pi\rho^2 + \pi^2\beta^2 + 2\pi^2\beta\rho + \pi^2\rho^2 \\
 &= D_2
 \end{aligned}$$

The species that will take part in the conversion reaction can be summarised together with their

respective rate constants as follows:



The notation for species $\text{EAPBR}^{\text{prod}}$, indicates the active configurations of this species. Two of the four possible configurations for this species are dead-end complexes.

The rate equation for conversion $v_{\text{net}} = v_f - v_r$ can be formulated as:

$$\begin{aligned} \frac{v_{\text{net}}}{[\text{E}_T]} &= \frac{k_f([\text{EA}_2\text{BR}] + 2[\text{EA}_2\text{B}_2] + [\text{EAPBR}]^{\text{prod}} + [\text{EAPB}_2])}{[\text{E}_T]} - \\ &\frac{k_r([\text{EAPR}_2] + [\text{EAPBR}]^{\text{prod}} + 2[\text{EP}_2\text{R}_2] + [\text{EP}_2\text{BR}])}{[\text{E}_T]} \\ &= \frac{k_f(2\alpha^2\beta\rho + 2\alpha^2\beta^2 + 2\alpha\pi\beta\rho + 2\alpha\pi\beta^2) - k_r(2\alpha\pi\rho^2 + 2\alpha\pi\beta\rho + 2\pi^2\rho^2 + 2\pi^2\beta\rho)}{D_2} \\ &= \frac{2k_f\alpha\beta(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta) - 2k_r\pi\rho(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta)}{D_2} \end{aligned}$$

Let $V_f = 2k_f \cdot \text{E}_T$ and $V_r = 2k_r \cdot \text{E}_T$. The rate equation is rewritten to show:

$$v = \frac{(V_f\alpha\beta - V_r\pi\rho)(\alpha + \pi)(\beta + \rho)}{D_2} \quad (3.14)$$

Factorising D_2 to determine the denominator terms of the rate equation gives:

$$\begin{aligned} D_2 &= 1 + (\alpha + \pi)^2 + (\beta + \rho)^2 + \alpha^2(\beta + \rho)^2 + 2\alpha\pi(\beta + \rho)^2 + \pi^2(\beta + \rho)^2 \\ &= 1 + (\alpha + \pi)^2 + (\alpha + \pi)^2(\beta + \rho)^2 + (\beta + \rho)^2 \end{aligned}$$

Substitution into eq. 3.14 results in eq. 3.15.

$$v = \frac{(V_f\alpha\beta - V_r\pi\rho)(\alpha + \pi)(\beta + \rho)}{1 + (\alpha + \pi)^2 + (\alpha + \pi)^2(\beta + \rho)^2 + (\beta + \rho)^2} \quad (3.15)$$

Using the Haldane relationship and mass action ratio as:

$$K_{eq} = \frac{V_f}{V_r} \cdot \frac{P_{0.5}R_{0.5}}{A_{0.5}B_{0.5}} \quad \text{and} \quad \Gamma = \frac{[\text{P}][\text{R}]}{[\text{A}][\text{B}]}$$

now leads to eq. 3.13:

$$v = \frac{V_f\alpha\beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)(\beta + \rho)}{[1 + (\alpha + \pi)^2][1 + (\beta + \rho)^2]}$$

which is the same as the Adair bi-substrate equation with $\gamma^{0.5} = 0$. Equation 3.13 is therefore the reversible rate equation for a dimeric infinitely cooperative enzyme catalysing a bi-substrate reaction.

3.1.3 Derivation for 3 subunits: Towards a general bi-substrate reversible equation for n subunits

Equation 3.13 is the rate equation derived for an enzyme consisting of two subunits. Many enzymes inside metabolic pathways are, however, oligomeric proteins consisting of more than two protomers. Deriving a rate equation for an enzyme consisting of three subunits will path the way to generalising eq. 3.13 to an enzyme consisting of any number of subunits (n).

As before, I shall first identify the enzyme species that will be considered for E_T . The same system definitions that were proposed for a dimeric enzyme also hold for the derivation of a cooperative bi-substrate rate equation catalysed by a trimeric enzyme. Taking into account infinite cooperativity, only enzyme species with fully liganded binding sites are considered. Table 3.5 shows these species.

Table 3.5: All possible binding species for 3 subunit enzyme assuming infinite cooperativity.

E	EA ₃	EA ₂ P	EAP ₂	EP ₃
EB ₃	EA ₃ B ₃	EA ₂ PB ₃	EAP ₂ B ₃	EP ₃ B ₃
EB ₂ R	EA ₃ B ₂ R	EA ₂ PB ₂ R	EAP ₂ B ₂ R	EP ₃ B ₂ R
EBR ₂	EA ₃ BR ₂	EA ₂ PBR ₂	EAP ₂ BR ₂	EP ₃ BR ₂
ER ₃	EA ₃ R ₃	EA ₂ PR ₃	EAP ₂ R ₃	EP ₃ R ₃

Following the same reasoning from which Figures 3.2–3.5 were constructed for a dimeric enzyme, Figure 3.6 can be constructed for substrates and products binding to a trimeric enzyme. Figure 3.6 serves only as an example of a more complex total binding mechanism, though it summarises the main idea of three subunit binding and the species involved.

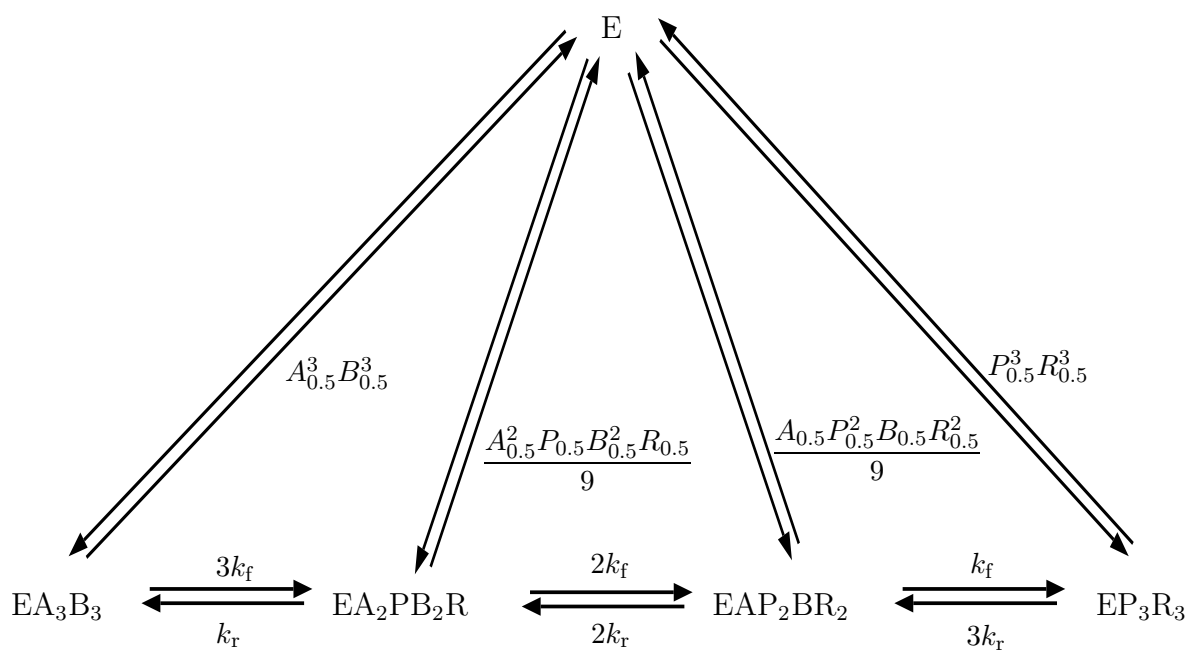


Figure 3.6: Condensed illustration of substrates and products binding (diagonal lines) to free trimeric enzyme and the conversion (horizontal lines) of these species.

Table 3.6: Summary of the enzyme—trimer—species considered for E_T , rewritten as equilibrium expressions (Equil. exp.) in terms of their free metabolite concentrations and their scaled notations.

[Species]	Equil. exp.	Scaled notation	[Species]	Equil. exp.	Scaled notation
[E]	$\frac{[E]}{[E]}$	1			
[EA ₃]	$\frac{[A]^3}{A_{0.5}^3}$	α^3	[A ₂ P]	$\frac{3[A]^2[P]}{A_{0.5}^2 P_{0.5}}$	$3\alpha^2\pi$
[EAP ₂]	$\frac{3[A][P]^2}{A_{0.5} P_{0.5}^2}$	$3\alpha\pi^2$	[EP ₃]	$\frac{[P]^3}{P_{0.5}^3}$	π^3
[EB ₃]	$\frac{[B]^3}{B_{0.5}^3}$	β^3	[EB ₂ R]	$\frac{3[B]^2[R]}{B_{0.5}^2 R_{0.5}}$	$3\beta^2\rho$
[EBR ₂]	$\frac{3[B][R]^2}{B_{0.5} R_{0.5}^2}$	$3\beta\rho^2$	[ER ₃]	$\frac{[R]^3}{R_{0.5}^3}$	ρ^3
[EA ₃ B ₃]	$\frac{[A]^3[B]^3}{A_{0.5}^3 B_{0.5}^3}$	$\alpha^3\beta^3$	[EA ₃ B ₂ R]	$\frac{3[A]^3[B]^2[R]}{A_{0.5}^3 B_{0.5}^2 R_{0.5}}$	$3\alpha^3\beta^2\rho$
[EA ₃ BR ₂]	$\frac{3[A]^3[B][R]^2}{A_{0.5}^3 B_{0.5} R_{0.5}^2}$	$3\alpha^3\beta\rho^2$	[EA ₃ R ₃]	$\frac{[A]^3[R]^3}{A_{0.5}^3 R_{0.5}^3}$	$\alpha^3\rho^3$
[EA ₂ PB ₃]	$\frac{3[A]^2[P][B]^3}{A_{0.5}^2 P_{0.5} B_{0.5}^3}$	$3\alpha^2\rho\beta^3$	[EA ₂ PB ₂ R] [#]	$\frac{9[A]^2[P][B]^2[R]}{A_{0.5}^2 P_{0.5} B_{0.5}^2 R_{0.5}}$	$9\alpha^2\pi\beta^2\rho$
[EA ₂ PBR ₂] [*]	$\frac{9[A]^2[P][B][R]^2}{A_{0.5}^2 P_{0.5} B_{0.5} R_{0.5}^2}$	$9\alpha^2\pi\beta\rho^2$	[EA ₂ PR ₃]	$\frac{3[A]^2[P][R]^3}{A_{0.5}^2 P_{0.5} R_{0.5}^3}$	$3\alpha^2\pi\rho^3$
[EAP ₂ R ₃]	$\frac{3[A][P]^2[R]^3}{A_{0.5} P_{0.5}^2 R_{0.5}^3}$	$3\alpha\pi^2\rho^3$	[EAP ₂ B ₂ R] [*]	$\frac{9[A][P]^2[B]^2[R]}{A_{0.5} P_{0.5}^2 B_{0.5}^2 R_{0.5}}$	$9\alpha\pi^2\beta^2\rho$
[EAP ₂ BR ₂] [†]	$\frac{9[A][P]^2[B][R]^2}{A_{0.5} P_{0.5}^2 B_{0.5} R_{0.5}^2}$	$9\alpha\pi^2\beta\rho^2$	[EAP ₂ R ₃]	$\frac{3[A][P]^2[R]^3}{A_{0.5} P_{0.5}^2 R_{0.5}^3}$	$3\alpha\pi^2\rho^3$
[EP ₃ B ₃]	$\frac{[P]^3[B]^3}{P_{0.5}^3 B_{0.5}^3}$	$\pi^3\beta^3$	[EP ₃ B ₂ R]	$\frac{3[P]^3[B]^2[R]}{P_{0.5}^3 B_{0.5}^2 R_{0.5}}$	$3\pi^3\beta^2\rho$
[EP ₃ BR ₂]	$\frac{3[P]^3[B][R]^2}{P_{0.5}^3 B_{0.5} R_{0.5}^2}$	$3\pi^3\beta\rho^2$	[EP ₃ R ₃]	$\frac{[P]^3[R]^3}{P_{0.5}^3 R_{0.5}^3}$	$\pi^3\rho^3$

In Table 3.6 and Figure 3.6 different statistical factors (SF) are present. This can readily be explained by three examples.

EA₃B₃: for the first molecule of A binding to E, A can bind to any one of three sites, but dissociate only from one giving a SF of 1/3, i.e. any site has a one in three chance of being occupied. The second A molecule can bind to one of two sites but also dissociate from two giving a SF of 2/2 = 1. For the third molecule of A binding, A can bind to only one site but dissociate from three, giving a SF of 3. The product of these three statistical factors results in the site SF (SSF) for site-A = 1. Applying the same reasoning to site-B, it becomes clear that the product of the SSFs gives the total binding factor (TBF) for **EA₃B₃** = 1. From this, any homotrimer (each site fully liganded by only one molecule type) will then have a TBF of 1.

EA₃B₂R: From the previous example, when one site is fully liganded by only one substrate or product the SSF for that site is equal to 1. For **EA₃B₂R** one change was made to site-B where a substrate B is substituted by its product R. The first binding of B gives a SF of 1/3. The second binding of B results in a SF of 1. R can now only bind to one site and dissociate from one giving a SF of 1. The product of site-B SFs gives its SSF = 3. Taking the product of site-A SSF and site-B SSF gives an overall TBF for **EA₃B₂R** = 3. From this, any mono-hybrid (one site liganded by only one molecule, second site a combination of molecules) will have a TBF of 3. (Note that the order in which the monohybrid molecules bind has no effect on the TBF).

EA₂PB₂R: From the example for **EA₃B₂R**, it can be seen that the SSF for a binding site fully liganded with different molecules is always 3. This example shows a species with two mono-hybrid sites. Taking the product of the two mono-hybrid site SSFs gives the overall TBF for a double-hybrid species = 9. The following binding scheme illustrates the formulation of TBF factors.

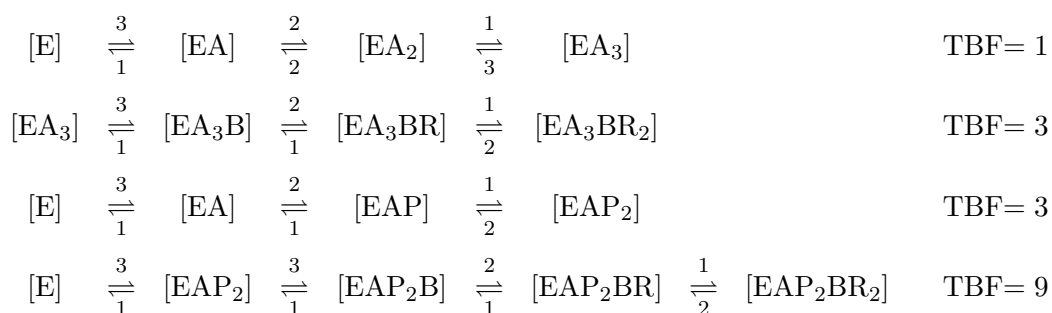


Table 3.6 summarizes all the enzyme-complexes written in terms of their respective dissociation constants and scaled substrate $X_{0.5}$ notations with [E] factored out. The denominator is the sum of all the complexes in Table 3.6. Written in terms of scaled notations the denominator is

formulated as follows:

$$\begin{aligned}
 [E_T]/[E] = & 1 + \alpha^3 + \beta^3 + \pi^3 + \rho^3 + 3\alpha^2\pi + 3\alpha\pi^2 + 3\beta^2\rho + 3\beta\rho^2 + \alpha^3\beta^3 + \\
 & 3\alpha^3\beta^2\rho + 3\alpha^3\beta\rho^2 + \alpha^3\rho^3 + 3\alpha^2\pi\beta^3 + 9\alpha^2\pi\beta^2\rho + 9\alpha^2\pi\beta\rho^2 + \\
 & 3\alpha^2\pi\rho^3 + 3\alpha\pi^2\beta^3 + 9\alpha\pi^2\beta^2\rho + 9\alpha\pi^2\beta\rho^2 + 3\alpha\pi^2\rho^3 + \pi^3\beta^3 + \\
 & 3\pi^3\beta^2\rho + 3\pi^3\beta\rho^2 + \pi^3\rho^3,
 \end{aligned}$$

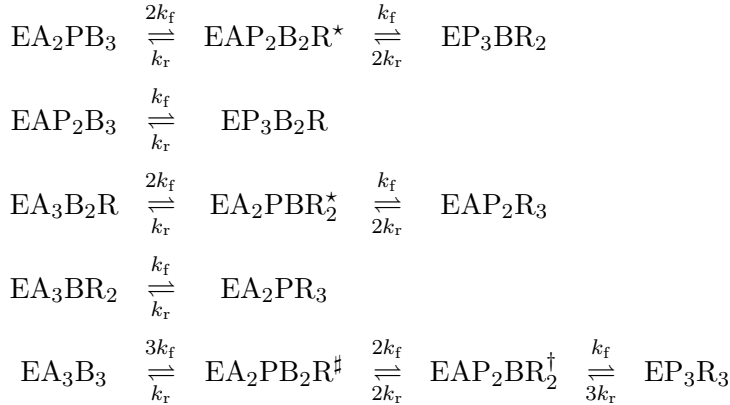
Grouping these terms gives

$$\begin{aligned}
 (\alpha + \pi)^3 &= \alpha^3 + 3\alpha^2\pi + 3\alpha\pi^2 + \pi^3 \\
 (\beta + \rho)^3 &= \beta^3 + 3\beta^2\rho + 3\beta\rho^2 + \rho^3 \\
 (\alpha + \pi)^3(\beta + \rho)^3 &= \alpha^3\beta^3 + 3\alpha^3\beta^2\rho + 3\alpha^3\beta\rho^2 + \alpha^3\rho^3 + 3\alpha^2\pi\beta^3 + 9\alpha^2\pi\beta^2\rho + 9\alpha^2\pi\beta\rho^2 + \\
 & 3\alpha^2\pi\rho^3 + 3\alpha\pi^2\beta^3 + 9\alpha\pi^2\beta^2\rho + 9\alpha\pi^2\beta\rho^2 + 3\alpha\pi^2\rho^3 + \pi^3\beta^3 + 3\pi^3\beta^2\rho \\
 & + 3\pi^3\beta\rho^2 + \pi^3\rho^3
 \end{aligned}$$

This leaves the denominator for three subunits (D_3) as follows:

$$D_3 = 1 + (\alpha + \pi)^3 + (\alpha + \pi)^3(\beta + \rho)^3 + (\beta + \rho)^3 = (1 + (\alpha + \pi)^3)(1 + (\beta + \rho)^3) \quad (3.16)$$

As before, the conversion rate for the rate equation can be formulated as $v_{net} = v_f - v_r$. The species that will be converted to either product from the forward reaction or to substrate from the reverse reaction can be illustrated in the catalytic conversion scheme given below:



The species that will undergo conversion for the 3-subunit case must first be evaluated more closely. The determination and role of statistical factors from binding was shown earlier. The presence of dead-end complexes and inactive species in certain 3-subunit complexes prompted the use of binding factors (BF).

From the catalytic conversion scheme give above for three subunits, it can be seen that certain complexes have labels assigned to them. The labeled complexes can exist in multiple binding

configurations. This behaviour is explained below in terms of the species' label and Figures 3.7, 3.8, 3.9 and 3.10.

$EAP_2B_2R^*$ (from Figure 3.7) and $EA_2PBR_2^*$ (from Figure 3.8):

Only 6 of the 9 configurations are productive, all following $\xrightleftharpoons[k_r]{k_f}$ catalysis. : BF = 6

$EA_2PB_2R^\ddagger$ (from Figure 3.9):

All configurations are productive, where 3 of 9 configurations follow $\xrightleftharpoons[k_r]{2k_f}$ catalysis : BF = 3

and remaining 6 configurations follow $\xrightarrow{k_f}$ catalysis. : BF = 6

$EAP_2BR_2^\dagger$ (from Figure 3.10):

All configurations are productive, where 3 of 9 configurations follow $\xrightleftharpoons[2k_r]{k_f}$ catalysis : BF = 3

and remaining 6 configurations follow $\xleftarrow{k_r}$ catalysis. : BF = 6

This scheme and the scheme for the **total binding factors**, p. 58, show two things. First, that the BFs of a species' different configurations sum to its TBF, and second, several species that are entered into the conversion rate carry both a BF and a FCF or RCF (FCF and RCF defined on p. 42) with them. Species TBFs only get used in construction of the denominator (E_T). The total enzyme concentration (E_T) takes into account active and dead-end complexes. Dead-end configurations, though catalytically inactive, still contribute to the total concentration of that species. Therefore, the TBF of a species does not have to be separated into its BF components when formulating E_T . Table 3.7 shows the trimeric species considered for catalysis, its respective TBFs, separated BFs, the FCF and RCF of separate species configurations and the total statistical factor (TSF) for each configuration. The TSF for each configuration is the product of its BF and FCF for the forward conversion, and its BF and RCF for the reverse conversion. Table 3.7 also indicates the final scaled notation of species entered into the rate equation, where each species is the product of its TSF and scaled free substrate and product concentrations. From this the conversion reaction of a trimeric cooperative enzyme in terms of its forward rate constants (k_f) and reverse rate constants (k_r) can be written as follows:

Figure 3.7: Possible configurations for EAP_2B_2R resulting from random binding and their corresponding catalytic rate constants.

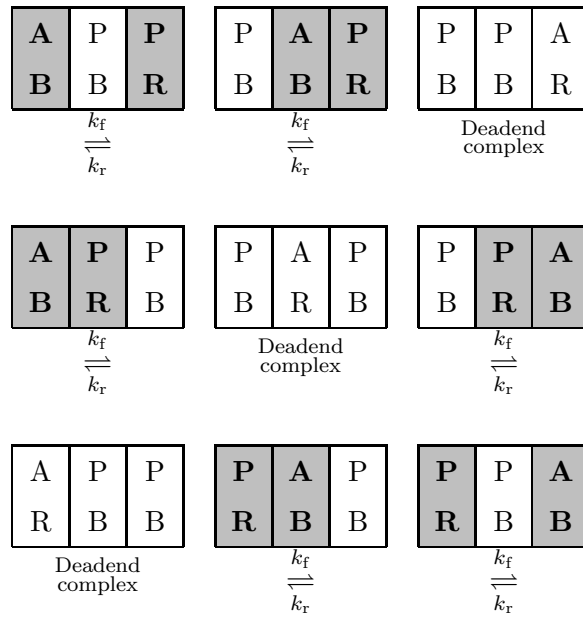


Figure 3.8: Possible configurations for EA_2PBR_2 resulting from random binding and their corresponding catalytic rate constants.

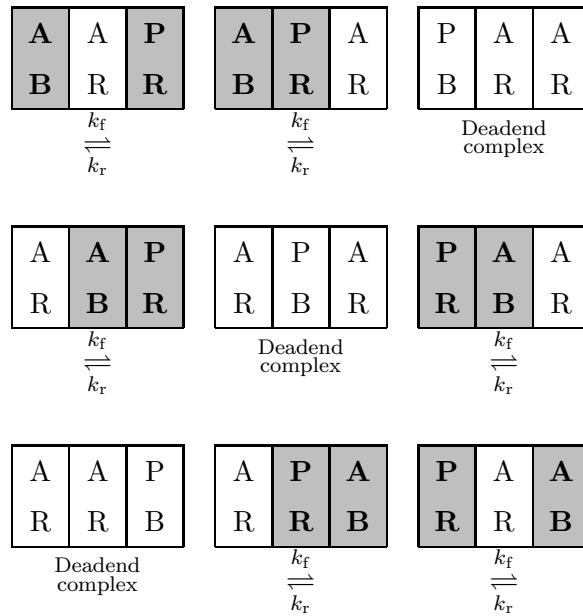


Figure 3.9: Possible configurations for EA₂PB₂R resulting from random binding and their corresponding catalytic rate constants.

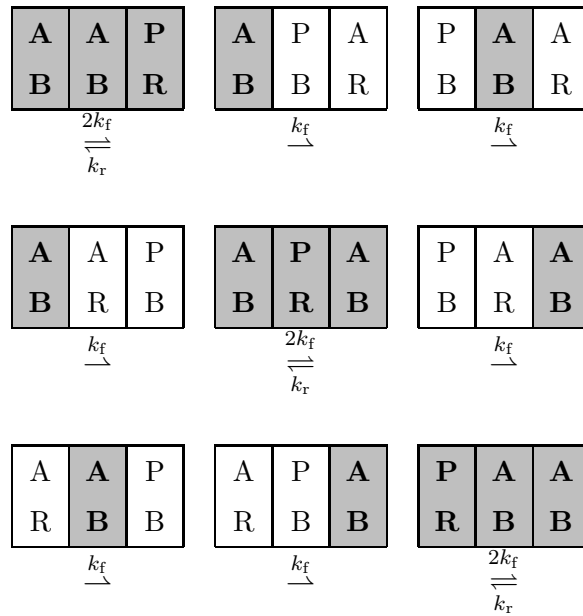


Figure 3.10: Possible configurations for EAP₂BR₂ resulting from random binding and their corresponding catalytic rate constants.

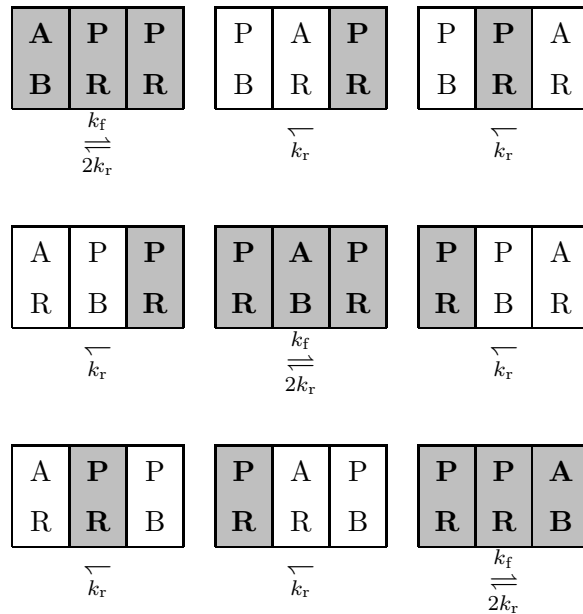


Table 3.7: Summary of the enzyme species considered for catalysis. TBF = total binding factor, FCF = forward catalytic factor, RCF = reverse catalytic factor, TSF = total statistical factor for term (BF*FCF for forward conversion, BF*RCF for reverse conversion), and Rate eq. term = the scaled notation of species entered into the rate equation (TSF*scaled free metabolite concentrations).

Forward conversion						
Species	TBF	BF	FCF	RCF	TSF	Rate eq. term
[EA ₂ PB ₃]	3	3	2	0	6	$6\alpha^2\pi\beta^3$
[EAP ₂ B ₂ R]	9	6	1	1	6	$6\alpha\pi^2\beta^2\rho$
		3	0	0	0	-
[EAP ₂ B ₃]	3	3	1	0	3	$6\alpha\pi^2\beta^3$
[EA ₃ B ₂ R]	3	3	2	0	6	$6\alpha^3\beta^2\rho$
[EA ₂ PBR ₂]	9	6	1	1	6	$6\alpha^2\pi\beta\rho^2$
		3	0	0	0	-
[EA ₃ BR ₂]	3	3	1	0	3	$3\alpha^3\beta\rho^2$
[EA ₃ B ₃]	1	1	3	0	3	$3\alpha^3\beta^3$
[EA ₂ PB ₂ R]	9	3	2	1	6	$6\alpha^2\pi\beta^2\rho$
		6	1	0	6	$6\alpha\pi^2\beta\rho^2$
[EAP ₂ BR ₂]	9	3	1	2	3	$3\alpha\pi^2\beta\rho^2$
		6	0	1	0	-
Reverse conversion						
[EP ₃ BR ₂]	3	3	0	2	6	$6\pi^3\beta\rho^2$
[EAP ₂ B ₂ R]	9	6	1	1	6	$6\alpha\pi^2\beta^2\rho$
		0	0	0	0	-
[EP ₃ B ₂ R]	3	3	0	1	3	$3\pi^3\beta^2\rho$
[EAP ₂ R ₃]	3	3	0	2	6	$6\alpha\pi^2\rho^3$
[EA ₂ PBR ₂]	9	6	1	1	6	$6\alpha^2\pi\beta\rho^2$
		0	0	0	0	-
[EA ₂ PR ₃]	3	3	0	1	3	$3\alpha^2\pi\rho^3$
[EP ₃ R ₃]	1	1	0	3	3	$3\pi^3\rho^3$
[EAP ₂ BR ₂]	9	3	1	2	6	$6\alpha\pi^2\beta\rho^2$
		6	0	1	6	-
[EA ₂ PB ₂ R]	9	3	1	2	6	$6\alpha^2\pi\beta^2\rho$
		6	0	1	6	$6\alpha^2\beta^2\pi\rho$

The forward conversion rate is:

$$\begin{aligned}
 &= \frac{k_f(2[\text{EA}_2\text{PB}_3] + [\text{EAP}_2\text{B}_2\text{R}]_{\text{BF } 6}^* + [\text{EAP}_2\text{B}_3] + [\text{EA}_3\text{B}_2\text{R}] + [\text{EA}_2\text{PBR}_2]_{\text{BF } 6}^* +)}{D_3} \\
 &= \frac{k_f([\text{EA}_3\text{BR}_2] + 3[\text{EA}_3\text{B}_3] + (2[\text{EA}_2\text{PB}_2\text{R}]_{\text{BF } 3}^\# + [\text{EA}_2\text{PB}_2\text{R}]_{\text{BF } 6}^\#) + [\text{EAP}_2\text{BR}_2]_{\text{BF } 3}^\dagger)}{D_3} \\
 &= \frac{k_f(6\alpha^2\pi\beta^3 + 6\alpha\pi^2\beta^2\rho + 3\alpha\pi^2\beta^3 + 6\alpha^3\beta^2\rho + 6\alpha^2\pi\beta\rho^2) +}{D_3} \\
 &= \frac{k_f(3\alpha^3\beta\rho^2 + 3\alpha^3\beta^3 + 12\alpha^2\pi\beta^2\rho + 3\alpha\pi^2\beta\rho^2)}{D_3} \\
 &= \frac{3k_f\alpha\beta(2\alpha\pi\beta^2 + 2\pi^2\beta\rho + \pi^2\beta^2 + 2\alpha^2\beta\rho + 2\alpha\pi\rho^2) + 3k_f\alpha\beta(\alpha^2\rho^2 + \alpha^2\beta^2 + 4\alpha\pi\beta\rho + \pi^2\rho^2)}{D_3}
 \end{aligned}$$

and the reverse conversion rate is:

$$\begin{aligned}
 &= \frac{k_r(2[\text{EP}_3\text{BR}_2] + [\text{EAP}_2\text{B}_2\text{R}]_{\text{BF } 6}^* + [\text{EP}_3\text{B}_2\text{R}] + 2[\text{EAP}_2\text{R}_3] + [\text{EA}_2\text{PBR}_2]_{\text{BF } 6}^* +)}{D_3} \\
 &= \frac{k_r([\text{EA}_2\text{PR}_3] + 3[\text{EP}_3\text{R}_3] + (2[\text{EAP}_2\text{BR}_2]_{\text{BF } 3}^\dagger + [\text{EAP}_2\text{BR}_2]_{\text{BF } 6}^\dagger) + [\text{EA}_2\text{PB}_2\text{R}]_{\text{BF } 3}^\#)}{D_3} \\
 &= \frac{k_r(6\pi^3\beta\rho^2 + 6\alpha\pi^2\beta^2\rho + 3\pi^3\beta^2\rho + 6\alpha\pi^2\rho^3 + 6\alpha^2\pi\beta\rho^2) +}{D_3} \\
 &= \frac{k_r(3\alpha^2\pi\rho^3 + \pi^3\rho^3 + 12\alpha\pi^2\beta\rho^2 + 3\alpha^2\pi\beta^2\rho)}{D_3} \\
 &= \frac{3k_r\pi\rho(2\pi^2\beta\rho + 2\alpha\pi\beta^2 + \pi^2\beta^2 + 2\alpha\pi\rho^2 + 2\alpha^2\beta\rho) + 3k_r\pi\rho(\alpha^2\rho^2 + \pi^2\rho^2 + 4\alpha\pi\beta\rho + \alpha^2\beta^2)}{D_3}
 \end{aligned}$$

Following the same method that was used for the derivation of the Adair bi-substrate equation, let $V_f = 3k_f \cdot E_T$, and $V_r = 3k_r \cdot E_T$. The rate equation can now be written as:

$$v = \frac{(V_f\alpha\beta - V_r\pi\rho)(\phi)}{D_3} \quad (3.17)$$

where

$$\phi = (2\pi^2\beta\rho + 2\alpha\pi\beta^2 + \pi^2\beta^2 + 2\alpha\pi\rho^2 + 2\alpha^2\beta\rho + \alpha^2\rho^2 + \pi^2\rho^2 + 4\alpha\pi\beta\rho + \alpha^2\beta^2).$$

Factorising ϕ gives

$$\phi = (\alpha + \pi)^2(\beta + \rho)^2$$

resulting in the rate equation taking the shape of

$$v = \frac{(V_f\alpha\beta - V_r\pi\rho)(\alpha + \pi)^2(\beta + \rho)^2}{D_3}$$

Once again using the Haldane relationship and mass action ratio as:

$$K_{eq} = \frac{V_f}{V_r} \cdot \frac{P_{0.5}R_{0.5}}{A_{0.5}B_{0.5}} \quad \text{and} \quad \Gamma = \frac{[\text{P}][\text{R}]}{[\text{A}][\text{B}]}$$

and substituting the factorised denominator D_3 back into eq. 3.17 gives the rate equation for a trimeric cooperative enzyme catalysing a bi-substrate reaction as:

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^2 (\beta + \rho)^2}{(1 + (\alpha + \pi)^3)(1 + (\beta + \rho)^3)} \quad (3.18)$$

From eqs. 3.13 and 3.18 we postulate that a general equation for bi-substrate reactions catalysed by a cooperative enzyme with n sites for infinite cooperativity, will take the form of:

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^{n-1} (\beta + \rho)^{n-1}}{(1 + (\alpha + \pi)^n)(1 + (\beta + \rho)^n)} \quad (3.19)$$

3.2 Evaluating and extending the derived bi-substrate reversible equation to the bi-substrate reversible Hill equation

At the beginning of this chapter a number of criteria were stipulated which the proposed bi-substrate reversible Hill equation should satisfy. Equation 3.19 will now be tested against these criteria.

Criterion 1: The proposed equation should be written in the form of eq. 3.1.

It can clearly be seen that eq. 3.19 is written in the exact form of eq. 3.1. Property g is proven to be a function dependent on a positive V_f and substrate and product concentrations which will always have positive values. Moreover, eq. 3.19 shows terms for separate binding to site-A and site-B clearly. These terms combined refer to the fully liganded enzyme and can be seen in both the numerator—simultaneous conversion of A + B—and in the denominator. Equation 3.19 is therefore a reversible bi-substrate rate equation.

Criterion 2: The proposed equation should simplify to the uni-substrate reversible Hill equation when binding to site-A or site-B is absent. Setting n equal to the Hill coefficient in eq. 3.19 gives eq. 3.20.

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^{h-1} (\beta + \rho)^{h-1}}{(1 + (\alpha + \pi)^h)(1 + (\beta + \rho)^h)} \quad (3.20)$$

Assigning $n = h$ applies only for infinite cooperativity as was the case for the derivation of eqs. 3.13 and 3.18. From eq. 3.20 it becomes clear that in the absence of binding to either site-A or site-B, the denominator simplifies to the denominator of the reversible Hill uni-substrate eq. 2.19 with the assignment of $n = h$. The denominator of these rate equations contains all the enzyme species that result from binding. In the absence of one substrate and product the other metabolites will still bind to the free enzyme. Therefore, the species that are considered for the derivation of the one-substrate reversible Hill equation are the only ones present when binding

to one of the two sites of eq. 3.20 is presumed zero. The numerator of eq. 3.20, however, goes to zero when binding to one site is eliminated. This is expected as the bi-substrate equation converts A and B simultaneously, and in the absence of one of the substrate the reaction rate will be zero. The observation that the same species are obtained for both eqs. 2.19 and 3.20 at zero binding of one of the substrates of eq. 3.20, presents proof that should the assumption of simultaneous conversion be abolished, eq. 3.20 simplifies to the one-substrate reversible Hill equation, eq. 2.19.

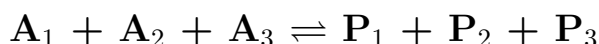
Eq. 3.19 accounts equally well for both criteria 1 and 2. It was derived from the Adair bi-substrate equation assuming the limiting degree of cooperativity. From this it follows that the assignment of $n = h$ can be made for eq. 3.19, which then gives the reversible Hill equation for bi-substrate kinetics, eq. 3.20.

3.3 Three substrate three product reactions

Inside cellular metabolism, enzymes that convert three substrates are often found e.g. pyruvate carboxylase which catalyses $\text{pyruvate} + \text{CO}_2 + \text{ATP} \rightleftharpoons \text{oxaloacetate} + \text{ADP} + \text{P}_i$ in gluconeogenesis and adenylosuccinate synthetase which catalyses $\text{IMP} + \text{GTP} + \text{aspartate} \rightleftharpoons \text{adenylosuccinate} + \text{GDP} + \text{P}_i$ during AMP synthesis [68]. Deriving the reversible Hill equation for three substrates will not only aid the construction of *in silico* models for metabolic pathways, but also show how the reversible Hill equation expands from the one-substrate case to the two- and three-substrate cases. From this expansion, a general reversible Hill equation incorporating any number of substrates or products will be formulated. In order to generalise the reversible Hill equation to multisubstrate reactions, the substrate A and B notation will no longer be used, instead, the notation A_1 , A_2 and A_3 , which refers to substrates one, two and three respectively, will be used, with P_1 , P_2 and P_3 referring to the respective products.

3.3.1 The reversible Hill equation for three substrates assuming infinite cooperativity

Consider the following reaction catalysed by a dimeric-enzyme:



where the subscripts denote different substrates and products. Therefore, fully liganded site-1 species can be written as $(A_1)_2$, $(P_1)_2$ or $(A_1)(P_1)$. The same notation holds for sites 2 and

3. The initial considerations used for deriving the bi-substrate reversible Hill equation (section 3.1) also apply to this derivation. As before, infinite cooperativity is assumed. This assumption results in no intermediate complexes being present in E_T . The species considered for the catalytic term are shown in Table 3.8. α_1 , α_2 and α_3 are the concentrations of A_1 , A_2 and A_3 scaled to their respective half saturation constants ($X_{0.5}$ values). Similarly, π_1 , π_2 and π_3 are the concentrations of P_1 , P_2 and P_3 scaled to their $X_{0.5}$ values.

For the conversion rate $v_{net}/E_T = (v_f - v_r)/E_T$, the forward rate is formulated as:

$$\begin{aligned} v_f/E_T &= 2k_f(\alpha_1^2\alpha_2^2\alpha_3^2 + \alpha_1\pi_1\alpha_2^2\alpha_3^2 + \alpha_1^2\alpha_2\pi_2\alpha_3^2 + \alpha_1\pi_1\alpha_2\pi_2\alpha_3^2 + \alpha_1^2\alpha_2^2\alpha_3\pi_3 + \\ &\quad \alpha_1\pi_1\alpha_2^2\alpha_3\pi_3 + \alpha_1^2\alpha_2\pi_2\alpha_3\pi_3 + \alpha_1\pi_1\alpha_2\pi_2\alpha_3\pi_3) \\ &= 2k_f\alpha_1\alpha_2\alpha_3(\alpha_1\alpha_2\alpha_3 + \pi_1\alpha_2\alpha_3 + \alpha_1\pi_2\alpha_3 + \pi_1\pi_2\alpha_3 + \alpha_1\alpha_2\pi_3 + \pi_1\alpha_2\pi_3 + \alpha_1\pi_2\pi_3 + \pi_1\pi_2\pi_3) \\ v_f &= V_f\alpha_1\alpha_2\alpha_3(\alpha_1 + \pi_1)(\alpha_2 + \pi_2)(\alpha_3 + \pi_3) \end{aligned}$$

where $V_f = 2k_f \cdot E_T$. The reverse rate is formulated as:

$$\begin{aligned} v_r/E_T &= 2k_r(\alpha_1\pi_1\alpha_2\pi_2\alpha_3\pi_3 + \pi_1^2\alpha_2\pi_2\alpha_3\pi_3 + \alpha_1\pi_1\alpha_2\pi_2^2\alpha_3\pi_3 + \pi_1^2\pi_2^2\alpha_3\pi_3 + \alpha_1\pi_1\alpha_2\pi_2\pi_3^2 + \\ &\quad \pi_1^2\alpha_2\pi_2\pi_3^2 + \alpha_1\pi_1\pi_2^2\pi_3^2 + \pi_1^2\pi_2^2\pi_3^2) \\ &= 2k_r\pi_1\pi_2\pi_3(\alpha_1\alpha_2\alpha_3 + \pi_1\alpha_2\alpha_3 + \alpha_1\pi_2\alpha_3 + \pi_1\pi_2\alpha_3 + \alpha_1\alpha_2\pi_3 + \pi_1\alpha_2\pi_3 + \alpha_1\pi_2\pi_3 + \pi_1\pi_2\pi_3) \\ v_r &= V_r\pi_1\pi_2\pi_3(\alpha_1 + \pi_1)(\alpha_2 + \pi_2)(\alpha_3 + \pi_3) \end{aligned}$$

where $V_r = 2k_r \cdot E_T$. Substituting for v_f and v_r , the net conversion rate is written as:

$$v_{net} = \frac{(V_f\alpha_1\alpha_2\alpha_3 - V_r\pi_1\pi_2\pi_3)(\alpha_1 + \pi_1)(\alpha_2 + \pi_2)(\alpha_3 + \pi_3)}{E_T}$$

Using the Haldane relationship and mass action ratio for this case,

$$K_{eq} = \frac{V_f}{V_r} \cdot \frac{(P_1)_{0.5}(P_2)_{0.5}(P_3)_{0.5}}{(A_1)_{0.5}(A_2)_{0.5}(A_3)_{0.5}} \quad \text{and} \quad \Gamma = \frac{[P_1][P_2][P_3]}{[A_1][A_2][A_3]}$$

the final rate equation, with a factorised E_T (factorisation not shown) and generalising from $n = 2$ to $n = h$, can be written as:

$$v = \frac{V_f\alpha_1\alpha_2\alpha_3 \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha_1 + \pi_1)^{h-1}(\alpha_2 + \pi_2)^{h-1}(\alpha_3 + \pi_3)^{h-1}}{(1 + (\alpha_1 + \pi_1)^h)(1 + (\alpha_2 + \pi_2)^h)(1 + (\alpha_3 + \pi_3)^h)} \quad (3.21)$$

Table 3.8: Summary of the enzyme species considered for catalysis by a 3-substrate 3-product dimeric enzyme. TBF = total binding factor, BF = binding factor for separate productive and dead-end configurations, FCF = forward catalytic factor, RCF = reverse catalytic factor, TSF = total statistical factor for term and Rate eq. term = the scaled notation of species entered into the forward or reverse rate equation terms.

Forward conversion						
Species	TBF	BF	FCF	RCF	TSF	Rate eq. term
$[E(A_1)_2(A_2)_2(A_3)_2]$	1	1	2	0	2	$2k_f \cdot \alpha_1^2 \alpha_2^2 \alpha_3^2$
$[E(A_1)_1(P_1)(A_2)_2(A_3)_2]$	2	2	1	0	2	$2k_f \cdot \alpha_1 \pi_1 \alpha_2^2 \alpha_3^2$
$[E(A_1)_2(A_2)(P_2)(A_3)_2]$	2	2	1	0	2	$2k_f \cdot \alpha_1^2 \alpha_2 \pi_2 \alpha_3^2$
$[E(A_1)(P_1)(A_2)(P_2)(A_3)_2]$	4	2	1	0	2	$2k_f \cdot \alpha_1 \pi_1 \alpha_2 \pi_2 \alpha_3^2$
		2	0	0	0	-
$[E(A_1)_2(A_2)_2(A_3)(P_3)]$	2	1	1	0	2	$2k_f \cdot \alpha_1^2 \alpha_2^2 \alpha_3 \pi_3$
$[E(A_1)(P_1)(A_2)_2(A_3)(P_3)]$	4	2	1	0	2	$2k_f \cdot \alpha_1 \pi_1 \alpha_2^2 \alpha_3 \pi_3$
		2	0	0	0	-
$[E(A_1)_2(A_2)(P_2)(A_3)(P_3)]$	4	2	1	0	2	$2k_f \cdot \alpha_1^2 \alpha_2 \pi_2 \alpha_3 \pi_3$
		2	0	0	0	-
$[E(A_1)(P_1)(A_2)(P_2)(A_3)(P_3)]$	8	2	1	1	2	$2k_f \cdot \alpha_1 \pi_1 \alpha_2 \pi_2 \alpha_3 \pi_3$
		6	0	0	0	-
Reverse conversion						
$[E(A_1)(P_1)(A_2)(P_2)(A_3)(P_3)]$	8	2	1	1	2	$2k_r \cdot \alpha_1 \pi_1 \alpha_2 \pi_2 \alpha_3 \pi_3$
		6	0	0	0	-
$[E(P_1)_2(A_2)(P_2)(A_3)(P_3)]$	4	2	0	1	2	$2k_r \cdot \pi_1^2 \alpha_2 \pi_2 \alpha_3 \pi_3$
		2	0	0	0	-
$[E(A_1)(P_1)(P_2)_2(A_3)(P_3)]$	4	2	0	1	2	$2k_r \cdot \alpha_1 \pi_1 \alpha_2 \pi_2^2 \alpha_3 \pi_3$
		2	0	0	0	-
$[E(P_1)_2(P_2)_2(A_3)(P_3)]$	2	2	0	1	2	$2k_r \cdot \pi_1^2 \pi_2^2 \alpha_3 \pi_3$
$[E(A_1)(P_1)(A_2)(P_2)(P_3)_2]$	4	2	0	1	2	$2k_r \cdot \alpha_1 \pi_1 \alpha_2 \pi_2 \pi_3^2$
		2	0	0	0	-
$[E(P_1)_2(A_2)(P_2)(P_3)_2]$	2	2	0	1	2	$2k_r \cdot \pi_1^2 \alpha_2 \pi_2 \pi_3^2$
$[E(A_1)(P_1)(P_2)_2(P_3)_2]$	2	2	0	1	2	$2k_r \cdot \alpha_1 \pi_1 \pi_2^2 \pi_3^2$
$[E(P_1)_2(P_2)_2(P_3)_2]$	1	1	0	2	2	$2k_r \cdot \pi_1^2 \pi_2^2 \pi_3^2$

3.4 Generalising the reversible Hill equation to n substrates

Comparing the different formulations of the reversible Hill equation i.e. one-, two- and three substrate cases (below),

$$\begin{aligned}
 & \text{Uni-reactant reversible Hill equation} \\
 v &= \frac{V_f \alpha_1 \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha_1 + \pi_1)^{h-1}}{(1 + (\alpha_1 + \pi_1)^h)} \\
 & \text{Bi-substrate reversible Hill equation} \\
 v &= \frac{V_f \alpha_1 \alpha_2 \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha_1 + \pi_1)^{h-1} (\alpha_2 + \pi_2)^{h-1}}{(1 + (\alpha_1 + \pi_1)^h)(1 + (\alpha_2 + \pi_2)^h)} \\
 & \text{Three-substrate reversible Hill equation} \\
 v &= \frac{V_f \alpha_1 \alpha_2 \alpha_3 \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha_1 + \pi_1)^{h-1} (\alpha_2 + \pi_2)^{h-1} (\alpha_3 + \pi_3)^{h-1}}{(1 + (\alpha_1 + \pi_1)^h)(1 + (\alpha_2 + \pi_2)^h)(1 + (\alpha_3 + \pi_3)^h)}
 \end{aligned}$$

the pattern of equation expansion upon addition of another substrate and product can be seen. Thus, we postulate that the general reversible Hill equation (GRH) for multisubstrate reactions can be written as:

$$v = V_f \prod_{i=1}^{n_s} \alpha_i \left(1 - \frac{\Gamma}{K_{eq}}\right) \prod_{i=1}^{n_s} \left(\frac{(\alpha_i + \pi_i)^{h-1}}{1 + (\alpha_i + \pi_i)^h}\right) \quad (3.22)$$

where n_s is any number of substrates.

The reversible Hill equation is derived for three substrates, and then generalised to n substrates (3.22). It complies with both criteria 1 and 2. We propose this formulation of the reversible Hill equation as the generalised reversible Hill (GRH) equation for multisubstrate cooperative, and non-cooperative (setting $h = 1$) reactions. It separates individual substrate binding sites, consistent with the assumption from section 3.1. Two weeks prior to submitting this MSc thesis, it came to our attention that Westermarck *et al.* [69] proposed a formulation of the reversible Hill equation for several substrates, shown below.

$$v = \frac{V_f \prod_{i=1}^{n_s} \alpha_i \left(1 - \frac{\Gamma}{K_{eq}}\right) \left(\prod_{i=1}^{n_s} \alpha_i + \prod_{j=1}^{n_p} \pi_j\right)^{h-1}}{\prod_{i=1}^{n_s} (1 + (\alpha_i + \pi_i)^h)} \quad (3.23)$$

This formulation of the multisubstrate reversible Hill equation is, however, incorrect. The numerator fails to separate the individual binding sites. Moreover, the numerator incorporates a different number of substrates (n_s) and products (n_p), though this property is not present in the denominator, which is a clear contradiction. In addition, Westermark's numerator infers that e.g. substrate α_1 and product π_2 can never be simultaneously bound to the enzyme. This is of course incorrect since both these metabolites will most certainly bind in the absence of their respective co-product π_1 or co-substrate α_2 . A detailed discussion falls outside the scope of this thesis, but will be published elsewhere.

3.5 Two substrates to one product (bi-uni) and one substrate to two products (uni-bi) reactions

In cellular metabolism, not all reactions have the same number of substrates and products. Examples of enzymes that catalyse one substrate to two product reactions are fructose biphosphate aldolase which converts fructose-1,6-bisphosphate \rightleftharpoons glyceraldehyde-3-phosphate + dihydroxyacetone phosphate (DHAP) during glycolysis, adenylosuccinate lyase which converts adenylosuccinate \rightleftharpoons fumarate + AMP in the purine nucleoside cycle and fructose-1-phosphate aldolase which converts fructose-1-phosphate \rightleftharpoons DHAP + glyceraldehyde in the liver [68]. Examples of two substrate to one product reactions are the conversion of G3P + DHAP \rightleftharpoons FBP by aldolase in gluconeogenesis and the conversion of erythrose-4-phosphate + DHAP \rightleftharpoons seduheptulose-1,7-bisphosphate also by aldolase in the Calvin cycle of plants [68].

No simple cooperative, or non-cooperative, equations are currently available to describe reactions of the nature of the above two cases.

3.5.1 Derivation of the bi-uni reversible Hill equation

Consider a dimeric enzyme that catalyses the following reaction:



where all the assumptions made for the derivation of the GRH equation still hold. A and B combine to give product P. If P is bound to a subunit, no A or B can bind as their binding sites are blocked. All the possible enzyme species that will result from binding (E_T) can be

summarised as follows:

$$\begin{aligned} E_T &= E + EA_2 + EB_2 + EP_2 + EABP^* + EAP^* + EBP^* + EA_2B_2 \\ \frac{E_T}{E} &= 1 + \alpha^2 + \beta^2 + \pi^2 + 2\alpha\beta\pi + s\beta\pi + \alpha^2\beta^2 \\ &= D_{21} \end{aligned}$$

where species labelled with a (*) have a BF of 2, as these species can exist in two possible configurations. From D_{21} , only the species with both A and B bound are considered for the catalytic term. Following the same method that was used to derive the GRH equation, the rate equation can be written in terms of the species scaled notations as:

$$\frac{v}{E_T} = \frac{2k_f\alpha\beta(\alpha\beta + \pi) - 2k_r\pi(\alpha\beta + \pi)}{D_{21}} \quad (3.24)$$

Rewriting eq. 3.24 with $V_f = 2k_fE_T$, $\Gamma = [P]/[A][B]$, $K_{eq} = \frac{V_f P_{0.5}}{V_r A_{0.5}B_{0.5}}$ (from the Haldane relationship), gives the following expression:

$$v = \frac{V_f \alpha\beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha\beta + \pi)}{1 + \alpha^2 + \beta^2 + 2\alpha\pi + 2\beta\pi + (\alpha\beta + \pi)^2} \quad (3.25)$$

The denominator in eq. 3.25 can be factorised further by simply adding and subtracting $2\pi^2$ to and from it to give the rate equation as:

$$v = \frac{V_f \alpha\beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha\beta + \pi)}{1 + (\alpha + \pi)^2 + (\beta + \pi)^2 + (\alpha\beta + \pi)^2 - 2\pi^2} \quad (3.26)$$

To generalise the above equation to three subunits, we follow the same steps for the derivation of a two substrate to one product reaction for a trimeric enzyme: the denominator (in terms of scaled notations) is given as:

$$D_{213} = 1 + \alpha^3 + \beta^3 + \alpha^3\beta^3 + 3\alpha^2\beta^2\pi + 3\alpha\beta\pi^2 + \pi^3 + 3\alpha^2\pi + 3\beta^2\pi + 3\alpha\pi^2 + 3\beta\pi^2$$

where the factor 3 is the BF. Each species preceded by a BF of 3 can exist in three possible configurations. Adding and subtracting $2\pi^3$ to and from D_{213} , the rate equation can now be formulated as:

$$v = \frac{V_f \alpha\beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha\beta + \pi)^2}{1 + (\alpha + \pi)^3 + (\beta + \pi)^3 + (\alpha\beta + \pi)^3 - 2\pi^3} \quad (3.27)$$

where $V_f = 3k_f E_T$ and Γ and K_{eq} are the mass-action ratio and equilibrium constant defined as before. From eqs. 3.26 and 3.27 we postulate that a two substrate to one product equation for n subunits can be written as :

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha \beta + \pi)^{n-1}}{1 + (\alpha + \pi)^n + (\beta + \pi)^n + (\alpha \beta + \pi)^n - 2\pi^n} \quad (3.28)$$

Setting $n = h$ in eq. 3.28 (as was the case for the bi-substrate and three substrate reversible Hill equations) gives the bi-uni reversible Hill equation as:

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha \beta + \pi)^{h-1}}{1 + (\alpha + \pi)^h + (\beta + \pi)^h + (\alpha \beta + \pi)^h - 2\pi^h} \quad (3.29)$$

3.5.2 Derivation of the uni-bi reversible Hill equation

Consider an enzyme that catalyses the following reaction:



This reaction is of course simply the reverse of the above derived bi-uni reversible Hill equation. The equation must therefore have the same shape, with the substrate and product scaled notations expected to change place. To avoid repetition, this derivation for two and three subunits will not be shown. Note that only one A on a subunit is needed for a species to be reactive. The reversible Hill equation for a one substrate to two product reaction is given as:

$$v = \frac{V_f \alpha \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi \rho)^{h-1}}{1 + (\alpha + \pi)^h + (\alpha + \rho)^h + (\alpha + \pi \rho)^h - 2\alpha^h} \quad (3.30)$$

Setting $h = 1$ in eqs. 3.29 and 3.30, these equations simplify to their non-cooperative form. Both these equations can therefore be used to describe Michaellean uni-bi and bi-uni enzyme kinetics..

The GRH equation will be evaluated in terms of criteria 3, 4 and 5 in chapter four.

4 Comparisons between the proposed reversible Hill equation and other models

The first step to validate any new model of mathematical origin lies in its ability to predict behaviour in good correlation to that of a well accepted standard. The development of computer software to simulate the behaviour of metabolic pathway models has provided the tools necessary to evaluate such new mathematical formulae. Therefore, to evaluate the proposed reversible Hill equation (GRH), the first step is to compare it to models of known validity. The Adair bi-substrate equation (eq. 3.9) was used as reference. Other models such as MWC and KNF do provide equations for comparison to the reversible Hill; however, the multitude of parameters needing be defined and the complexity of these models make them difficult to use.

4.1 Comparing the derived reversible Hill equation to other equations used to describe cooperativity

4.1.1 Adair equation

It should be noted that the Adair bi-substrate equation is itself also newly derived, and its own validity will first be evaluated against the uni-reactant Adair equation. The uni-reactant Adair equation for cooperative kinetics has been used widely to characterize sigmoidal experimental data [2, 3]. Figure 4.1 shows the surface plots drawn for the two different Adair equations. The second substrate concentration for the Adair bi-substrate equation, [B], was fixed at a saturating value of 10^4 and its product [R] was fixed at 0. All other parameters that occur in both equations were given the same values. It is clear from Figure 4.1 that the Adair bi-substrate surface plot shows good agreement to the uni-reactant Adair plot. The only discrepancy occurs at high product concentration when the substrate concentration is low. This discrepancy is accentuated by the logarithmic scale and it can be seen from the low values on the z-axis that this difference between the two Adair equations will physiologically be indistinguishable. Figure 4.1 therefore

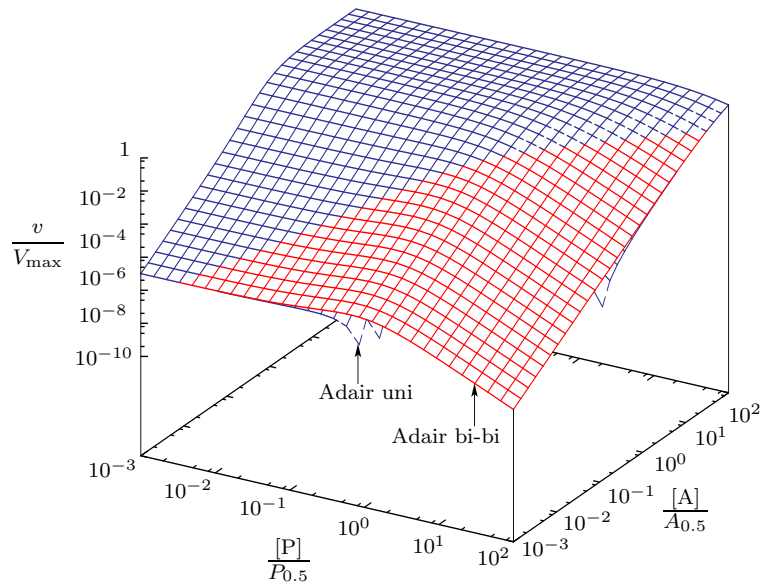
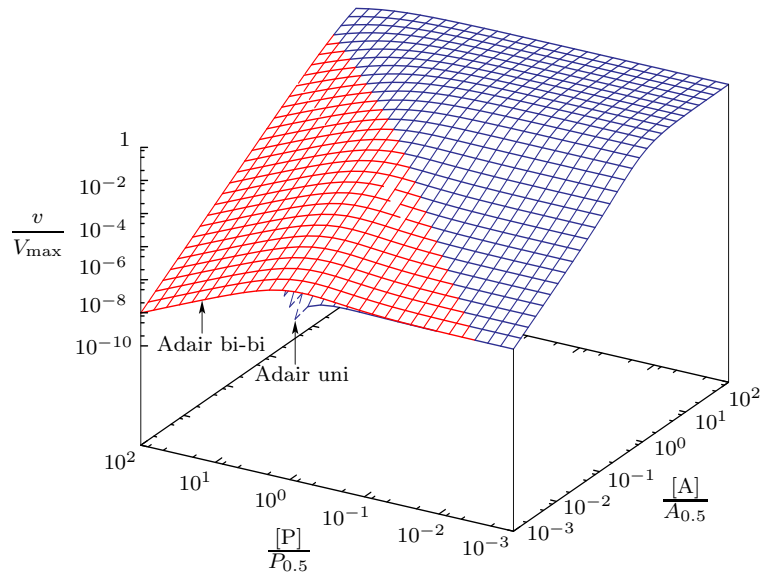


Figure 4.1: Two different angle views of surface plots of the Adair bi-substrate equation (eq. 3.9) and the Adair uni-substrate equation (eq. 8.4). The surfaces are plotted in triple logarithmic space with $K_{eq} = 1000$, all substrate $X_{0.5}$ values = 1 and $\gamma = 0.01$. For the Adair bi-substrate model, $[B] = 10^4$ and $[R] = 0$.

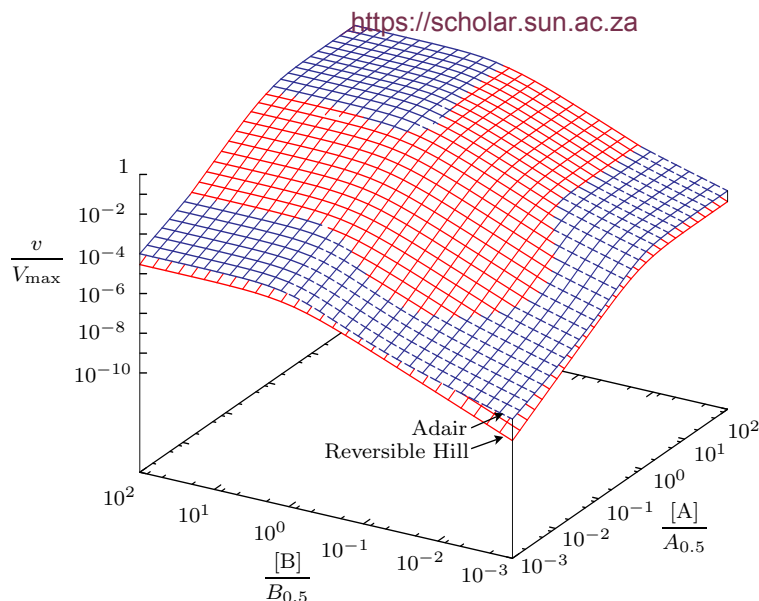


Figure 4.2: 3D plot showing the visual comparison of the bi-substrate reversible Hill equation vs Adair bi-substrate equation for conversion of $A + B \rightleftharpoons P + R$ at $[P]/P_{0.5} = 0$ and $[R]/R_{0.5} = 0$. The surfaces are plotted in triple logarithmic space. The arbitrary data was generated with the Adair bi-substrate equation (3.9) and bi-substrate reversible Hill equation (3.20) with $K_{eq} = 1000$, $\gamma_a = \gamma_b = 0.01$ and $h = 1.513$ (adjusted by hand).

show that the derived Adair bi-substrate equation gives reaction rates that are—for experimental purposes—identical to that of the uni-reactant Adair equation.

Figure 4.2 shows the surface plots for the GRH equation ($n_s = 2$, herein referred to as bi-substrate reversible Hill equation) and Adair bi-substrate equations. The bi-substrate reversible Hill plot agrees very well to the Adair bi-substrate plot. These plots of the two equations in irreversible form give different behaviour only at very low substrate concentrations where the corresponding v/V_f values will be indistinguishable in practice. Figure 4.3 shows the behaviour of the bi-substrate reversible Hill and Adair bi-substrate equations at saturating levels of substrate and increasing product. This plot is the opposite of Figure 4.2 where the products were zero. Figure 4.3 also shows a very good correlation in reaction rates predicted by the reversible Hill equation compared to the Adair bi-substrate equation. Setting the Hill coefficient = 1 in the bi-substrate reversible Hill equation and $\gamma^{0.5} = 1$ in the Adair bi-substrate equation will result in both equations simplifying to their non-cooperative forms. Figure 4.4 shows this under irreversible conditions with h and $\gamma = 1$. Once again these plots agree extremely well.

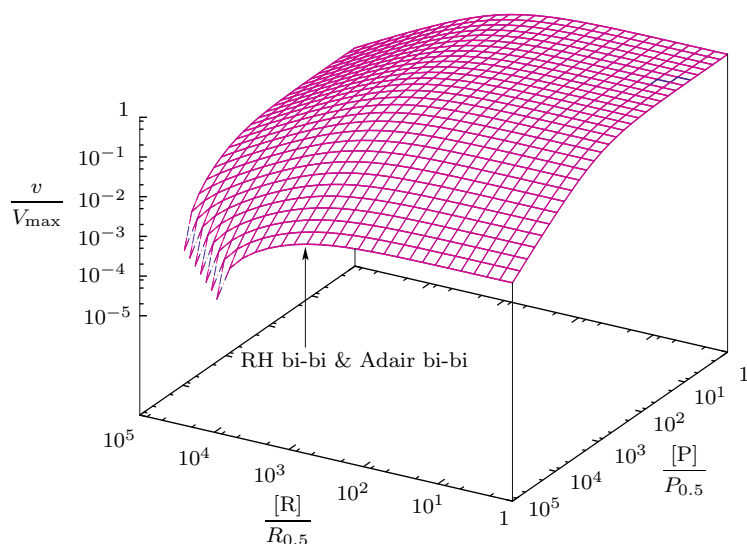


Figure 4.3: 3D plot showing the visual comparison of bi-substrate reversible Hill equation (eq. 3.20) vs the Adair bi-substrate equation (eq. 3.9) for conversion of $A + B \rightleftharpoons P + R$ at saturating A and B and increasing product concentrations. The surfaces are plotted in triple logarithmic space. $[A]/A_{0.5} = 1000$, $[B]/B_{0.5} = 1000$, $K_{eq} = 1000$, $\gamma_a = \gamma_b = 0.01$ and $h = 1.513$ (adjusted by hand).

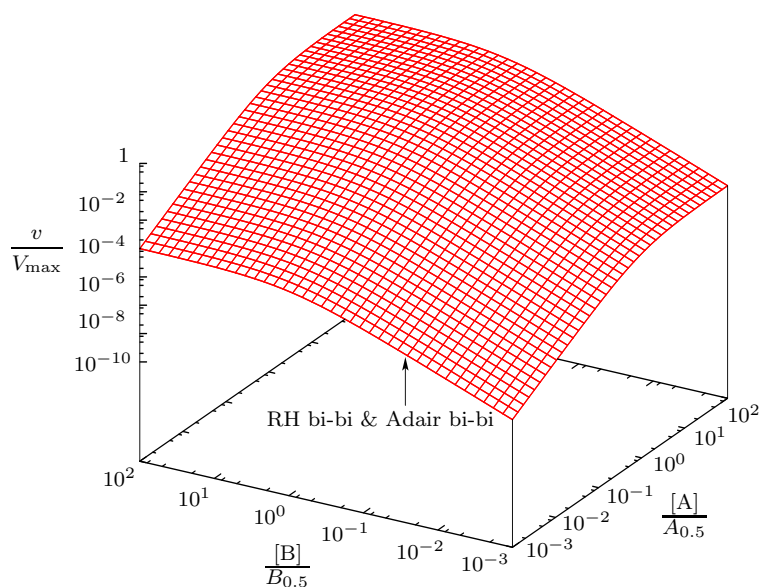


Figure 4.4: Reversible Hill bi-substrate equation vs Adair bi-substrate equation with $h = 1$ and $\gamma = 1$, $[P]$ and $[R] = 0$.

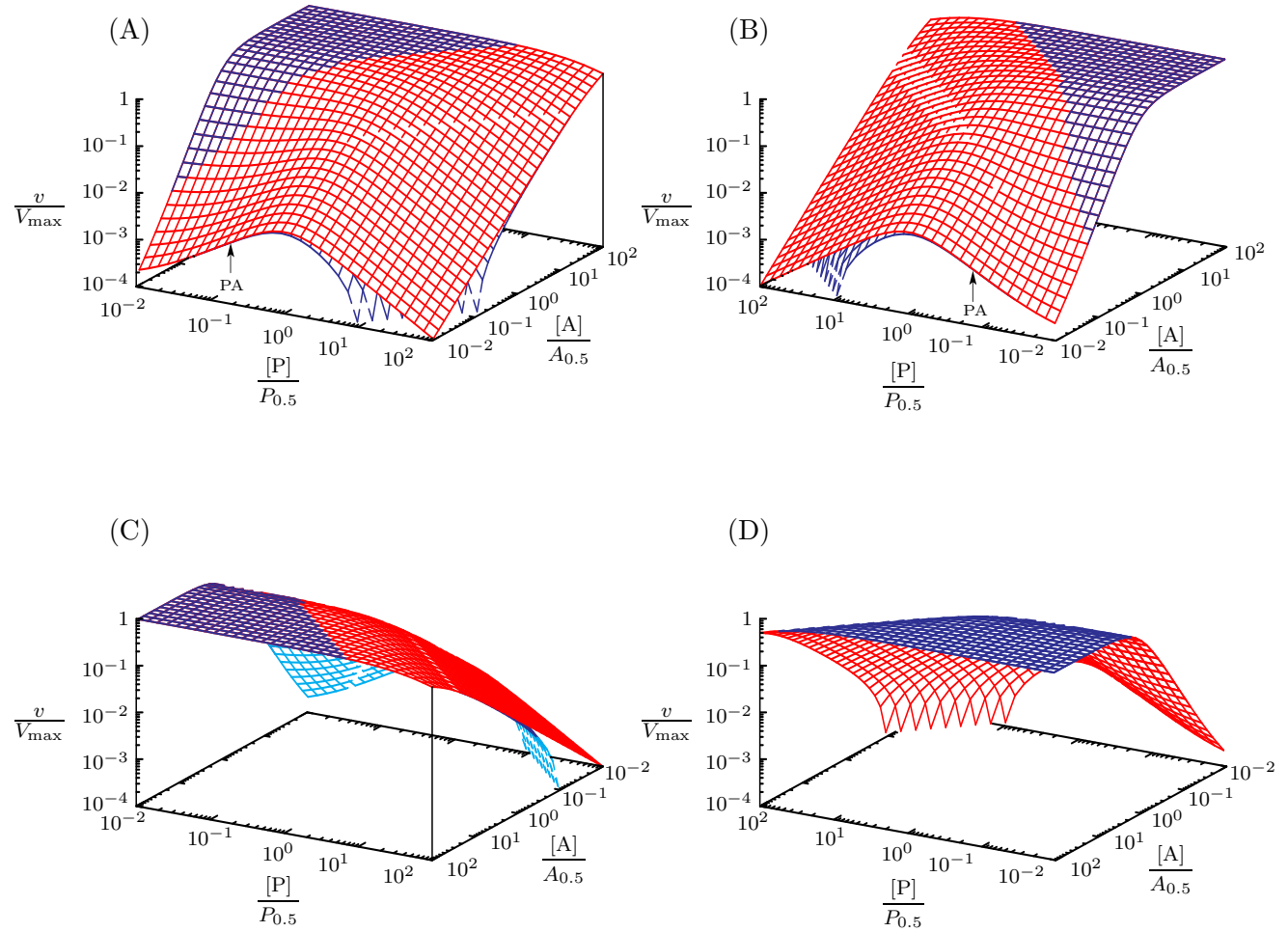


Figure 4.5: Multiple angle views of a surface plot comparing the one-substrate reversible Hill and the bi-substrate reversible Hill equations. Substrate B for the bi-substrate reversible Hill equation was saturated at 10^5 , $h = 2$, $K_{eq} = 1000$ and $[R] = 0$. PA = product activation.

4.1.2 One substrate reversible Hill equation

The bi-substrate reversible Hill equation can also be compared to the uni-reactant reversible Hill equation at saturating substrate B concentrations and setting $[R] = 0$ for the bi-substrate equation. Figure 4.5 shows a multiplot of all the different angles obtained from the comparison between these two reversible Hill equations. Figure 4.5 clearly shows a very good agreement between these two reversible Hill equations. At low substrate and high product concentrations these two plots do differ. The logarithmic scale makes this difference more easily visible, and it can be explained by the difference in the two equations.

$$v = \frac{V_f \alpha (1 - \Gamma/K_{eq})(\alpha + \pi)}{1 + (\alpha + \pi)^2} \quad \text{Reversible Hill uni-reactant} \quad (4.1)$$

$$v = \frac{V_f \alpha (\alpha + \pi)}{1 + (\alpha + \pi)^2} \cdot \frac{\beta^2}{(1 + \beta^2)} \quad \text{Reversible Hill bi-substrate} \quad (4.2)$$

Equations 4.1 and 4.2 show the simplified reversible Hill uni-substrate and bi-substrate equations for $[R] = 0$ and $h = 2$. The bi-substrate reversible Hill equation has an extra term that describes binding to site-B. At high concentration of substrate B the term $\beta^2/(1+\beta^2)$ tends towards unity, rendering its contribution to reaction rate negligible. The uni-reactant reversible Hill equation has the thermodynamic term present in its formulation. At high concentration of substrate A the thermodynamic term tends towards 1 as Γ tends towards 0. Therefore, under high substrate A conditions, the difference in rates of the two reversible Hill equations is undetectable as illustrated by Figure 4.5. However, at low concentration of substrate A, the thermodynamic term $(1-\Gamma/K_{eq})$ tends towards 0 as the disequilibrium constant, Γ/K_{eq} , becomes 1 when $[P] = [A]^*K_{eq}$. In contrast the bi-substrate reversible Hill equation has no thermodynamic term as a result of $[R] = 0$ and will therefore give a faster rate under the same conditions. Increasing the K_{eq} from 1000 to 10^5 shows how the contribution of the thermodynamic disequilibrium constant can be negated, essentially rendering the uni-reactant reversible Hill equation irreversible. As expected, with the altered K_{eq} the two equations give indistinguishable surface plots (Figure 4.6).

Figures 4.5 and 4.6 also show at low $[A]$ an increase in product $[P]$ causes an increase in reaction rate for both equations. This product activation (PA) has been shown by Hofmeyr *et al.* [10] and Olivier [70] for the uni-reactant reversible Hill equation. Evidently, the bi-substrate reversible Hill equation shares this behaviour with the uni-reactant case, and will be discussed later.

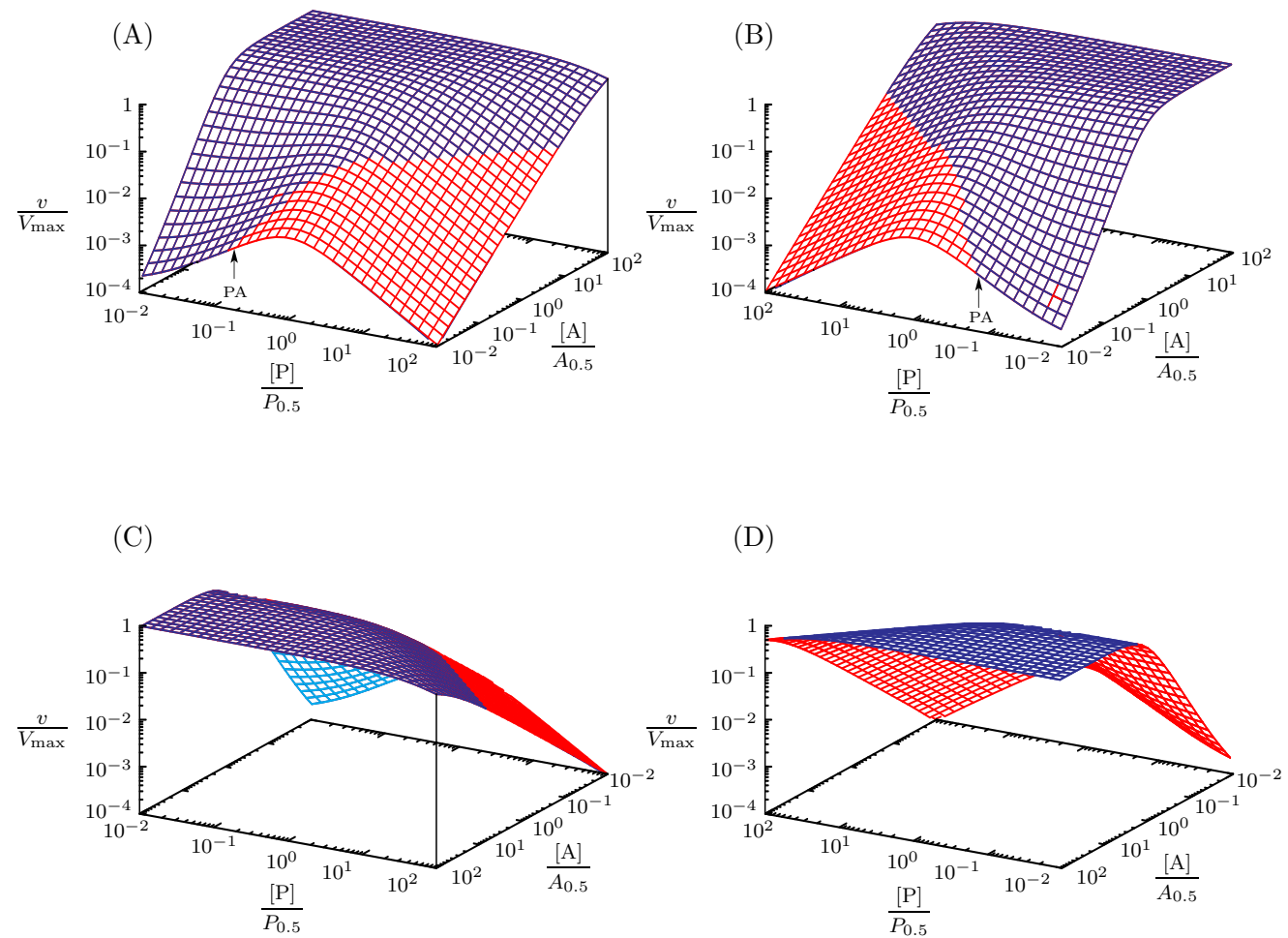


Figure 4.6: Multiple angle views of a surface plot comparing the one substrate reversible Hill and the bi-substrate reversible Hill equations.

Substrate B is saturated at 10^5 , $h = 2$, $K_{eq} = 10^5$ and $[R] = 0$. PA = product activation.

From the comparison to the uni-reactant reversible Hill equation, it is clear that the bi-substrate reversible Hill equation gives a good approximation to uni-substrate cooperative reactions under saturating second substrate (B) conditions.

4.1.3 The Hill equation

The irreversible Hill equation has been shown to fit an overwhelming amount of experimental data very well. The comparison between the bi-substrate reversible Hill equation and irreversible Hill equation is shown in Figure 4.7. Substrate B was increased from low to a saturating concentration with both product concentrations [P] and [R] set to zero. Figure 4.7 confirms that the bi-substrate reversible Hill equation gives identical rates to that of the irreversible Hill equation at saturating conditions of substrate B. We conclude that the derived GRH equation with $n_s = 2$, accurately accounts for cooperativity in bi-substrate reactions.

4.2 Non-cooperative kinetics: GRH equation as a generic rate equation

In the previous section the GRH equation with two substrates was shown to give behaviour indistinguishable from the Adair bi-substrate equation, uni-reactant reversible Hill and irreversible Hill equations. One of the advantages of equations based on Hill kinetics is that they accurately predict conversion rates irrespective of the underlying binding mechanism. The derivation of the bi-substrate reversible Hill equation (3.20) was based upon assuming complete random binding of substrates and products. Comparing eq. 3.20 to the bi-substrate random-order ternary-complex equation will therefore be a good test case for the non-cooperative bi-substrate reversible Hill equation as both equations infer random binding. The equation for the random-order ternary-complex mechanism [2] can be written as:

$$v = \frac{\frac{V_f[A][B]}{K_{iA}K_{mB}} - \frac{V_r[P][R]}{K_{mP}K_{iR}}}{1 + \frac{[A]}{K_{iA}} + \frac{[B]}{K_{iB}} + \frac{[P]}{K_{iP}} + \frac{[R]}{K_{iR}} + \frac{[A][B]}{K_{iA}K_{mB}} + \frac{[P][R]}{K_{mP}K_{iR}}} \quad (4.3)$$

where $K_{mA,mB}$ indicate the Michaelis constants for substrates A and B in the forward reaction and $K_{mP,mQ}$, the Michaelis constants when products P and R are used as substrates of the reverse reaction. Moreover, K_{iA} and K_{iB} indicate the product inhibition constants when substrates A and B are used as product inhibitors of the reverse reaction, and K_{iP} and K_{iR} the product inhibition constants of products P and R in the forward reaction.

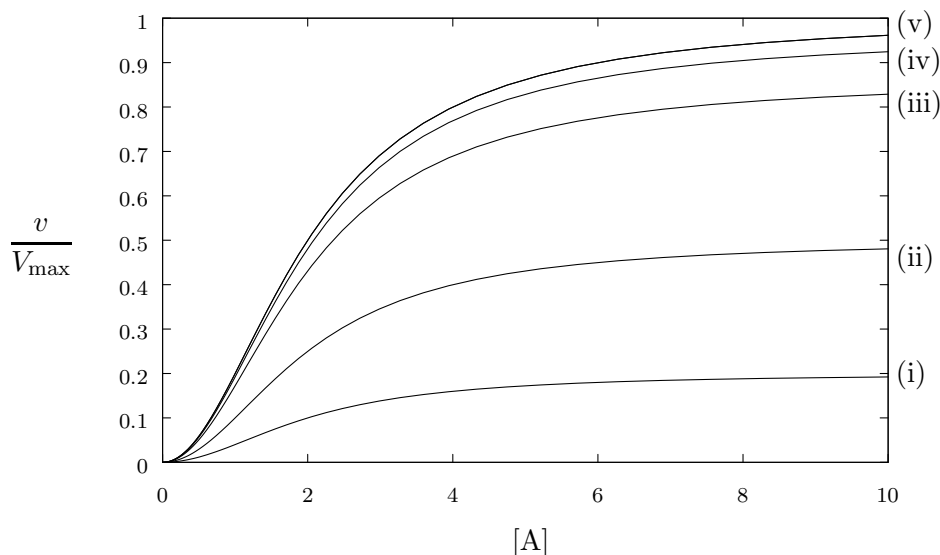
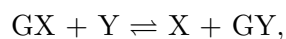


Figure 4.7: An illustration of the behaviour of the bi-substrate reversible Hill equation compared to that of the irreversible Hill equation. The bi-substrate reversible Hill equation substrate B concentrations are i) 1, ii) 2, iii) 5, iv) 10 and v) saturating at 1000 with $h = 2$. The irreversible Hill equation line lies exactly on line (v). $A_{0.5} = 2$ and $B_{0.5} = 2$.

Setting $h = 1$ for eq. 3.20 the equation simplifies to:

$$v = \frac{\frac{V_f[A][B]}{A_{0.5}B_{0.5}} - \frac{V_r[P][R]}{P_{0.5}R_{0.5}}}{1 + \frac{[A]}{A_{0.5}} + \frac{[P]}{P_{0.5}} + \frac{[B]}{B_{0.5}} + \frac{[R]}{R_{0.5}} + \frac{[A][B]}{A_{0.5}B_{0.5}} + \frac{[P][R]}{P_{0.5}R_{0.5}} + \left(\frac{[A][R]}{A_{0.5}R_{0.5}} + \frac{[P][B]}{P_{0.5}B_{0.5}} \right)} \quad (4.4)$$

Note that for $h = 1$, all the substrate $X_{0.5}$ values for eq. 4.4 can be considered as Michaelis constants as no interaction parameters are present. The numerators in eqs. 4.3 and 4.4 are identical. The only discrepancy lies within the denominator where eq. 4.4 has two extra entries shown in parenthesis. This can be explained by first considering the notation of Wong & Hanes [71] for the conversion of $A + B \rightleftharpoons P + R$. As nearly all bi-substrate conversion reactions are a result of a group-transfer reaction, Wong & Hanes proposed the following notation:



where $GX = A$, $Y = B$, $X = P$ and $GY = R$. From their formulation of the random-order ternary mechanism, the species EXY and $EXG \bullet GY$ were excluded from their initial reaction scheme as both these species are non-productive enzyme complexes which will not take part in catalysis, thus their absence in rate eq. 4.3. These two species can be written in single letter notation as EPB and EAR and are the two extra entries visible in eq. 4.4. The existence of these two species can however not be denied as they will most certainly occur as a result of binding

as noted by Cornish-Bowden [2]. Their contribution to catalytic rate is certainly zero, and if they were to be excluded on this basis from eq. 4.4, eq. 4.4 gives the same denominator to eq. 4.3. Experimentally, these two equations will give near indistinguishable rates, whether EAR or EPB is present in the equation formulation or not. Figure 4.8 shows that this is indeed the case as the two surface plots of eqs. 4.3 and 4.4 are almost indistinguishable. This observation prompted the question to what degree the non-cooperative bi-substrate reversible Hill equation can account for other bi-substrate non-cooperative kinetic equations. Figure 4.9 shows this comparison between the bi-substrate reversible Hill equation— $h = 1$ —and other Michaellean equations (see eqs. 8.5–8.7).

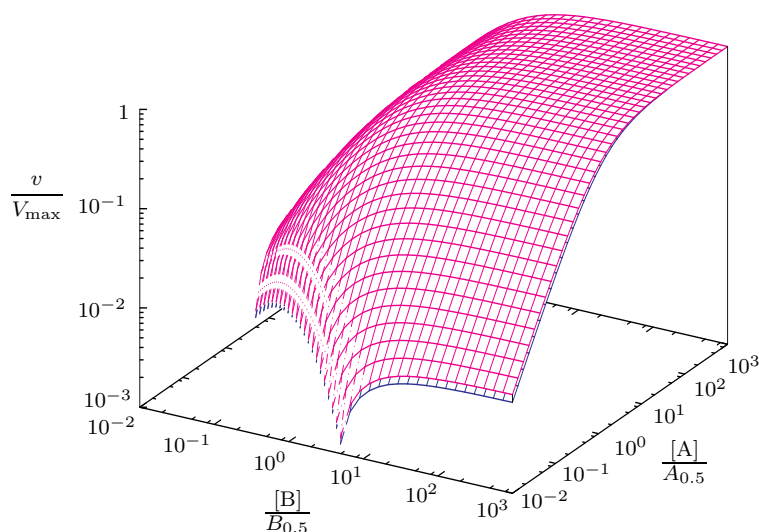


Figure 4.8: 3D plot showing the visual comparison of eq. 4.4 vs the random-order ternary-complex equation (eq. 4.3). The surfaces are obtained by setting all rate constants in the mechanism = 1, plotted in triple logarithmic space with $n = 1$ and product concentrations = 0.5 Km.

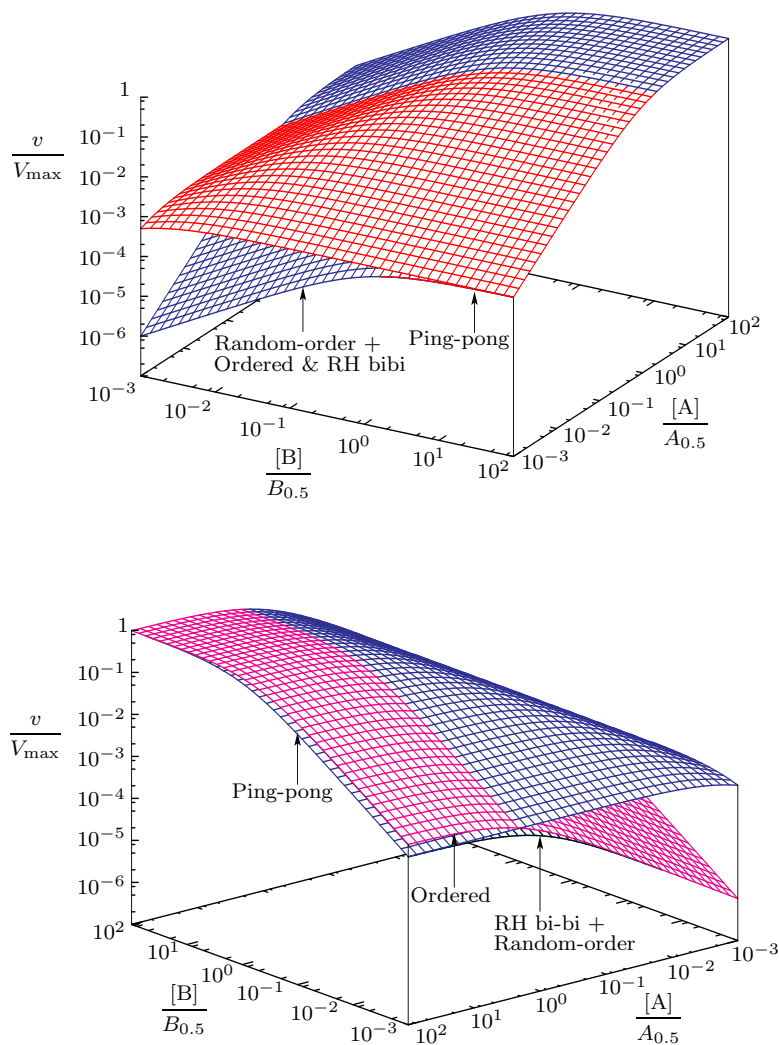


Figure 4.9: Surface plots at two different angles of the bi-substrate reversible Hill equation with a Hill coefficient of 1 plotted against known kinetic models used to describe non-cooperative bi-substrate kinetics. The data were generated with the compulsory-order ternary-complex equation (eq. 8.6), the random-order ternary complex equation (eq. 4.3), the substituted-enzyme mechanism (ping-pong) equation (eq. 8.7) and the bi-substrate reversible Hill equation (eq. 4.4). Hill parameter values are $A_{0.5} = 1.0$, $B_{0.5} = 1.0$, $P_{0.5} = 1.0$, $R_{0.5} = 1.0$ and $h = 1.0$. Ordered parameter values are: all K_i and K_m values = 1.0 with the exception of $K_{mA} = 0.5$, $K_{mQ} = 0.5$, $K_{iB} = 2.0$ and $K_{iP} = 2.0$. Ping-pong parameter values are: all K_i and K_m values = 1.0. Random parameter values are: all K_i and K_m values = 1.0.

From Figure 4.9 it can be seen that the bi-substrate reversible Hill equation gives an excellent approximation to the compulsory-order ternary-complex mechanism (ordered), random-order ternary-complex mechanism and the substituted-enzyme mechanism (Ping-Pong). At the extremes the bi-substrate reversible Hill equation differs significantly from the Ping-Pong equation. However, this difference can be regarded as insignificant when looking at the v/V_{\max} values. These values show that the reversible Hill and Ping-Pong equations will give a difference in reaction rates that will most certainly be undetectable experimentally.

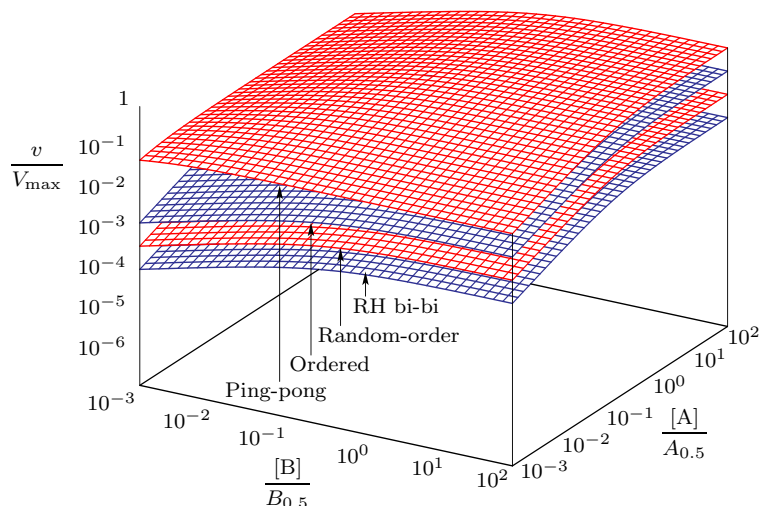


Figure 4.10: 3D plot showing the visual comparison of reversible generic equation vs Random-order ternary-complex, compulsory-order ternary-complex and substituted-enzyme mechanism (Ping-Pong) equations for conversion of $A + B \rightleftharpoons P + R$ at $[P]$ and $[R] = 0$. A value of 0.1, 10 and 100 was multiplied to the V_{\max} values of the bi-substrate reversible Hill, ordered and Ping-Pong rates to separate the surfaces in Figure 4.9. The surfaces are plotted in triple logarithmic space. The data were generated as per the legend of Figure 4.9.

Figure 4.10 shows surface plots drawn from the same equations used in Figure 4.9. A value of 0.1, 10 and 100 was multiplied to the V_{\max} values of the reversible Hill, ordered and Ping-Pong rates. The resultant plots (Figure 4.10) show that the compulsory-order ternary-complex mechanism and random-order ternary-complex mechanism surfaces can be separated from that of the bi-substrate reversible Hill equation. It is clear that the bi-substrate reversible Hill equation with a Hill coefficient of 1 gives behaviour virtually indistinguishable from those of the other models mentioned here. The bi-substrate reversible Hill equation with $h = 1$ can therefore be regarded

as a bi-substrate **generic equation** to describe non-cooperative bi-substrate kinetics. The bi-substrate compulsory-order, random-order and Ping-Pong equations all assume a certain binding mechanism, or a compulsory sequence of events for catalysis, or both. The advantage of the **generic** bi-substrate rate equation is its ability to accurately predict substrate conversion rates irrespective of the underlying binding mechanism.

Equation 3.20 has been shown to account for all the criteria that a bi-substrate reversible Hill equation should, and more. The proposed generic rate equation shows excellent agreement to the non-cooperative testcases. Thus, the proposed GRH equation can be regarded as a generic rate equation for both multisubstrate cooperative and non-cooperative ($h = 1$) kinetics.

To this point, all equations considered have not included the effect of a modifier. In chapter five, the bi-substrate formulation of the GRH equation ($n_s = 2$) and three substrate formulation ($n_s = 3$) will be extended to account for allosteric modifier effects.

5 Adding modifier behaviour to the bi-substrate reversible Hill equation

Inside metabolic pathways, reaction rates can either be inhibited or activated. For enzymes that follow Michaelian kinetics a moderate increase of reaction rate from $0.1 V_f$ to $0.9 V_f$ needs a 81-fold increase in substrate concentration. Comparatively, enzymes that obey cooperative Hill kinetics only need a 9-fold increase in substrate concentration for a Hill coefficient of 2. Cooperative enzymes are therefore sensitive to small changes in substrate concentration and it is important that such cooperative enzymes be regulated with a high degree of precision. Product inhibition is one mechanism that accomplishes such regulation. These products can be either reaction or pathway products, and are often structurally dissimilar to the enzyme substrates. Products responsible for this increase or decrease in conversion rate bind to an alternate site on the enzyme called an allosteric site. Molecules that affect reaction rate by binding to an allosteric site are called allosteric effectors, I shall refer to them from now on as *modifiers*. Many cooperative enzymes are also allosteric proteins; the proposed bi-substrate reversible Hill equation must therefore account for modifier behaviour to be complete.

5.1 One modifier: Equation derivation

To extend the bi-substrate reversible Hill equation to account for modifier effects, the following will be assumed:

1. A dimeric enzyme catalyzing the conversion of $A + B \rightleftharpoons P + R$ is considered.
2. One modifier molecule with concentration $[M]$ can bind to each subunit of the enzyme with dissociation constant K_{mod} .
3. Binding of the first modifier molecule affects binding of the second modifier molecule by increasing or decreasing K_{mod} by δ_{mm} .

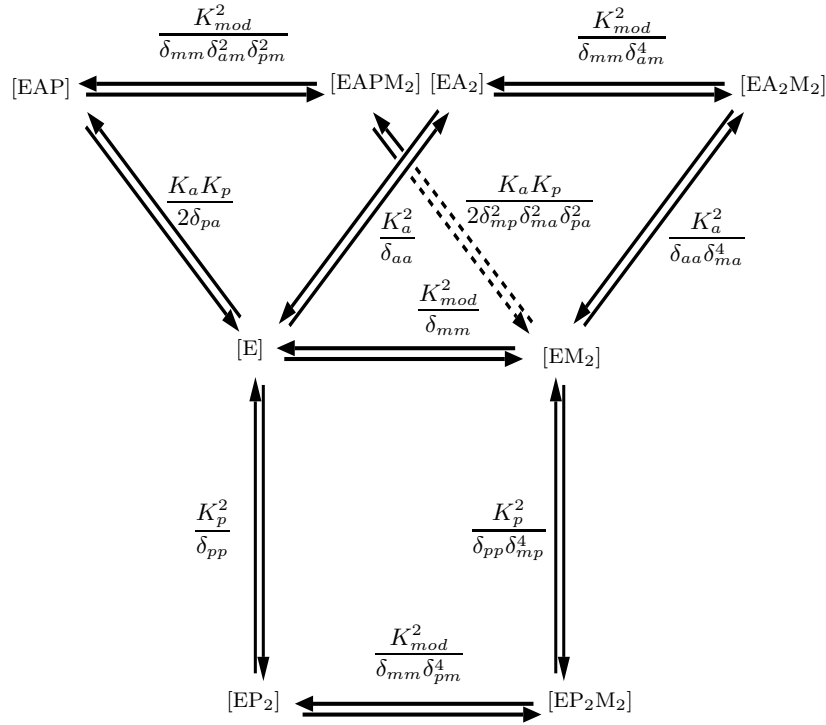


Figure 5.1: Bound enzyme forms for molecules A, P and M binding to free enzyme, assuming the limiting case of cooperativity.

4. Each modifier molecule affects the dissociation constant of each substrate and product binding site on both subunits by δ_{ma} , δ_{mb} , δ_{mp} , δ_{mr} , respectively.
5. A bound molecule of A or P affects the subsequent binding of A or P to site-A by δ_{aa} , δ_{ap} , or δ_{pp} . The same analogy holds for site-B.
6. The limiting case of cooperativity.

From these assumptions, considering only the effect of binding of modifier on site-A, Figure 5.1 can be drawn. Figure 5.1 shows that for microscopic reversibility to hold, the following assignments can be made:

- $\delta_{pm} = \delta_{mp} = \delta_{ma} = \delta_{am}$, (let them = σ)
- $\delta_{ap} = \delta_{pa} = \delta_{aa} = \delta_{pp}$, (let them = ω)
- let $\delta_{mm} = \kappa$

From above relationships, Figure 5.1 can now be redrawn to Figure 5.2.

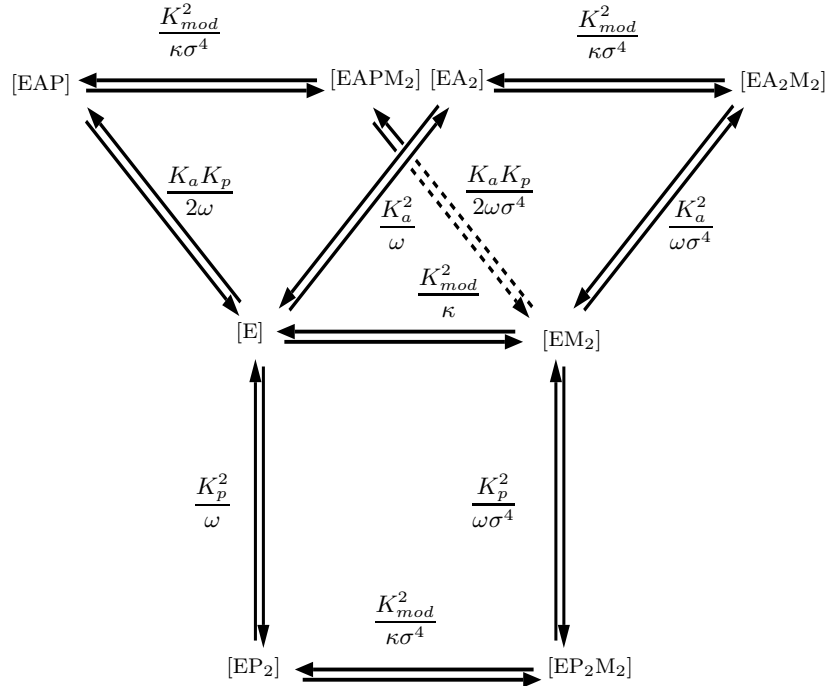


Figure 5.2: Rewritten interaction factors for molecules A, P and M binding to free enzyme.

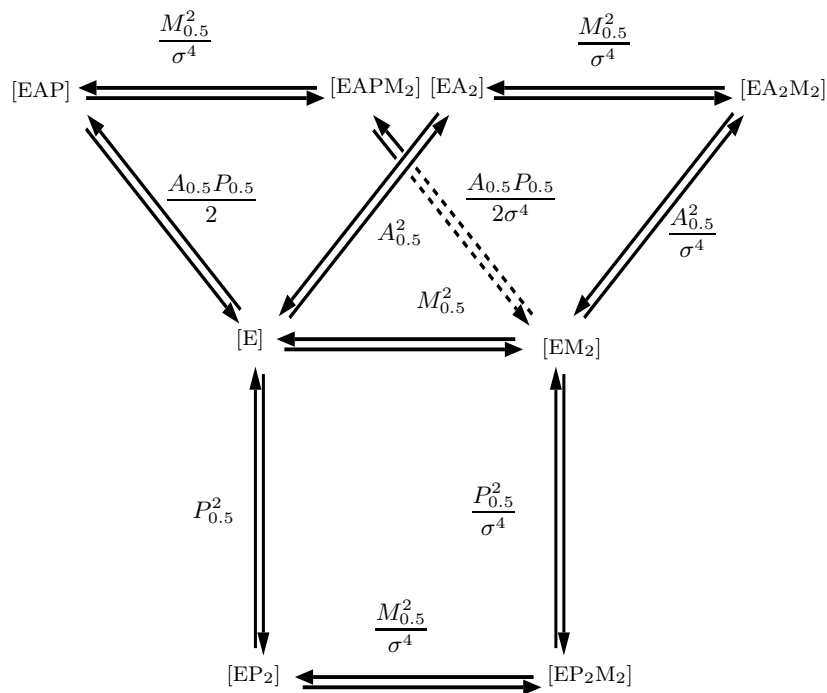


Figure 5.3: Rewritten dissociation constants and interaction parameters for molecules A, P and M binding to free enzyme.

σ is therefore a measure of the influence that the modifier has on site-A where $\sigma > 1$ shows an apparent increase in affinity of site-A for molecules A and P. Alternatively, $\sigma < 1$ will show an apparent decrease in affinity of site-A for molecules A and P. As was the case for the derivation of the Adair- and bi-substrate reversible Hill equations, the dissociation constants (eq. 2.5 and Figures 3.2 and 3.3) from Figure 5.2 can be rewritten in terms of their concentrations needed for half maximal saturation as follows:

- $K_a = \omega^{0.5} A_{0.5}$
- $K_p = \omega^{0.5} P_{0.5}$
- $K_{mod} = \kappa^{0.5} M_{0.5}$

Substituting these dissociation constants for their respective $X_{0.5}$ notations, Figure 5.2 takes on the form of Figure 5.3. Figure 5.3 is now a simplified representation for the limiting case of cooperativity of molecules A, P and modifier M binding to free enzyme. Figures 5.2 and 5.3 can of course also be drawn for binding to site-B, by substituting A for B and P for R. The species that result from binding of molecules A, P, B, R and modifier M to the enzyme can be written in terms of their substrate $X_{0.5}$ notations as shown in Table 5.1. This table also shows the scaled notation of the species with each metabolite concentration scaled to its substrate $X_{0.5}$ concentration, where α , β , π and ρ have the same definitions as before and $\mu = [M]/M_{0.5}$.

The species from Table 5.1 can now be illustrated in Figure 5.4 as a binding model for reaction $A + B \rightleftharpoons P + R$ with modifier M binding to a third, allosteric site on each subunit.

Only fully liganded species were considered for catalysis. For the forward reaction such a fully liganded species must have at least one molecule of A bound to site-A and one molecule of B bound to site-B. For the reverse reaction the fully liganded species' site-A and -B must house at least one P and one R molecule. The species considered for the conversion reaction can be shown in the following catalytic scheme:

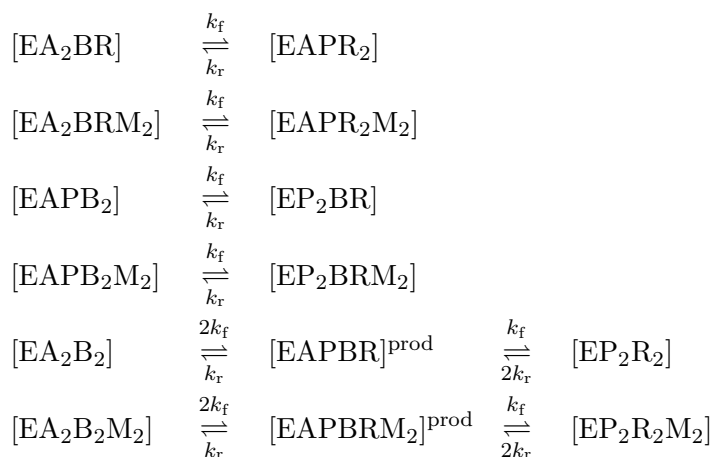


Table 5.1: Summary of the enzyme species considered for E_T , rewritten as an equilibrium expression (Equil. exp.) in terms of free metabolite concentrations and scaled notations ([E] factored out).

Species	Equil. exp.	Scaled notation	Species	Equil. exp.	Scaled notation
[E]	$\frac{[E]}{[E]}$	1	[EP ₂]	$\frac{[P]^2}{P_{0.5}^2}$	π^2
[EA ₂]	$\frac{[A]^2}{A_{0.5}^2}$	α^2	[EAP]	$\frac{2[A][P]}{A_{0.5}P_{0.5}}$	$2\alpha\pi$
[EB ₂]	$\frac{[B]^2}{B_{0.5}^2}$	β^2	[ER ₂]	$\frac{[R]^2}{R_{0.5}^2}$	ρ^2
[EBR]	$\frac{2[BR]}{B_{0.5}R_{0.5}}$	$2\beta\rho$	[EA ₂ B ₂]	$\frac{[A]^2[B]_2}{A_{0.5}^2B_{0.5}^2}$	$\alpha^2\beta^2$
[EP ₂ R ₂]	$\frac{[P]^2[R]_2}{P_{0.5}^2R_{0.5}^2}$	$\pi^2\rho^2$	[EA ₂ R ₂]	$\frac{[A]^2[R]_2}{A_{0.5}^2R_{0.5}^2}$	$\alpha^2\rho^2$
[EP ₂ B ₂]	$\frac{[P]^2[B]_2}{P_{0.5}^2B_{0.5}^2}$	$\pi^2\beta^2$	[EP ₂ BR]	$\frac{2[P]^2[B][R]}{P_{0.5}^2B_{0.5}R_{0.5}}$	$2\pi^2\beta\rho$
[EA ₂ BR]	$\frac{2[A]^2[B][R]}{A_{0.5}^2B_{0.5}R_{0.5}}$	$2\alpha^2\beta\rho$	[EAPB ₂]	$\frac{2[A][P][B]^2}{A_{0.5}P_{0.5}B_{0.5}^2}$	$2\alpha\pi\beta^2$
[EAPR ₂]	$\frac{2[A][P][R]^2}{A_{0.5}P_{0.5}R_{0.5}^2}$	$2\alpha\pi\rho^2$	[EAPBR]	$\frac{4[A][P][B][R]}{A_{0.5}P_{0.5}B_{0.5}R_{0.5}}$	$4\alpha\pi\beta\rho$
[EM ₂]	$\frac{[M]^2}{M_{0.5}^2}$	μ^2	[EA ₂ M ₂]	$\frac{\sigma^4[A]^2[M]_2}{A_{0.5}^2M_{0.5}^2}$	$\sigma^4\alpha^2\mu^2$
[EB ₂ M ₂]	$\frac{\sigma^4[B]^2[M]_2}{B_{0.5}^2M_{0.5}^2}$	$\sigma^4\beta^2\mu^2$	[EP ₂ M ₂]	$\frac{\sigma^4[P]^2[M]_2}{P_{0.5}^2M_{0.5}^2}$	$\sigma^4\pi^2\mu^2$
[ER ₂ M ₂]	$\frac{\sigma^4[R]^2[M]_2}{R_{0.5}^2M_{0.5}^2}$	$\sigma^4\rho^2\mu^2$	[EAPM ₂]	$\frac{2\sigma^4[A][P][M]^2}{A_{0.5}P_{0.5}M_{0.5}^2}$	$2\sigma^4\alpha\pi\mu^2$
[EBRM ₂]	$\frac{2\sigma^4[B][R][M]^2}{B_{0.5}R_{0.5}M_{0.5}^2}$	$2\sigma^4\beta\rho\mu^2$	[EA ₂ B ₂ M ₂]	$\frac{\sigma^8[A]^2[B]^2[M]^2}{A_{0.5}^2B_{0.5}^2M_{0.5}^2}$	$\sigma^8\alpha^2\beta^2\mu^2$
[EP ₂ R ₂ M ₂]	$\frac{\sigma^8[P]^2[R]^2[M]^2}{P_{0.5}^2R_{0.5}^2M_{0.5}^2}$	$\sigma^8\pi^2\rho^2\mu^2$	[EA ₂ R ₂ M ₂]	$\frac{\sigma^8[A]^2[R]^2[M]^2}{A_{0.5}^2R_{0.5}^2M_{0.5}^2}$	$\sigma^8\alpha^2\rho^2\mu^2$
[EP ₂ B ₂ M ₂]	$\frac{\sigma^8[P]^2[B]^2[M]^2}{P_{0.5}^2B_{0.5}^2M_{0.5}^2}$	$\sigma^8\pi^2\beta^2\mu^2$	[EP ₂ BRM ₂]	$\frac{2\sigma^8[P]^2[B][R][M]^2}{P_{0.5}^2B_{0.5}R_{0.5}M_{0.5}^2}$	$2\sigma^8\pi^2\beta\rho\mu^2$
[EA ₂ BRM ₂]	$\frac{2\sigma^8[A]^2[B][R][M]^2}{A_{0.5}^2B_{0.5}R_{0.5}M_{0.5}^2}$	$2\sigma^8\alpha^2\beta\rho\mu^2$	[EAPB ₂ M ₂]	$\frac{2\sigma^8[A][P][B]^2[M]^2}{A_{0.5}P_{0.5}B_{0.5}^2M_{0.5}^2}$	$2\sigma^8\alpha\pi\beta^2\mu^2$
[EAPR ₂ M ₂]	$\frac{2\sigma^8[A][P][R]^2[M]^2}{A_{0.5}P_{0.5}R_{0.5}^2M_{0.5}^2}$	$2\sigma^8\alpha\pi\rho^2\mu^2$	[EAPBRM ₂]	$\frac{4\sigma^8[A][P][B][R][M]^2}{A_{0.5}P_{0.5}B_{0.5}R_{0.5}M_{0.5}^2}$	$4\sigma^8\alpha\pi\beta\rho\mu^2$

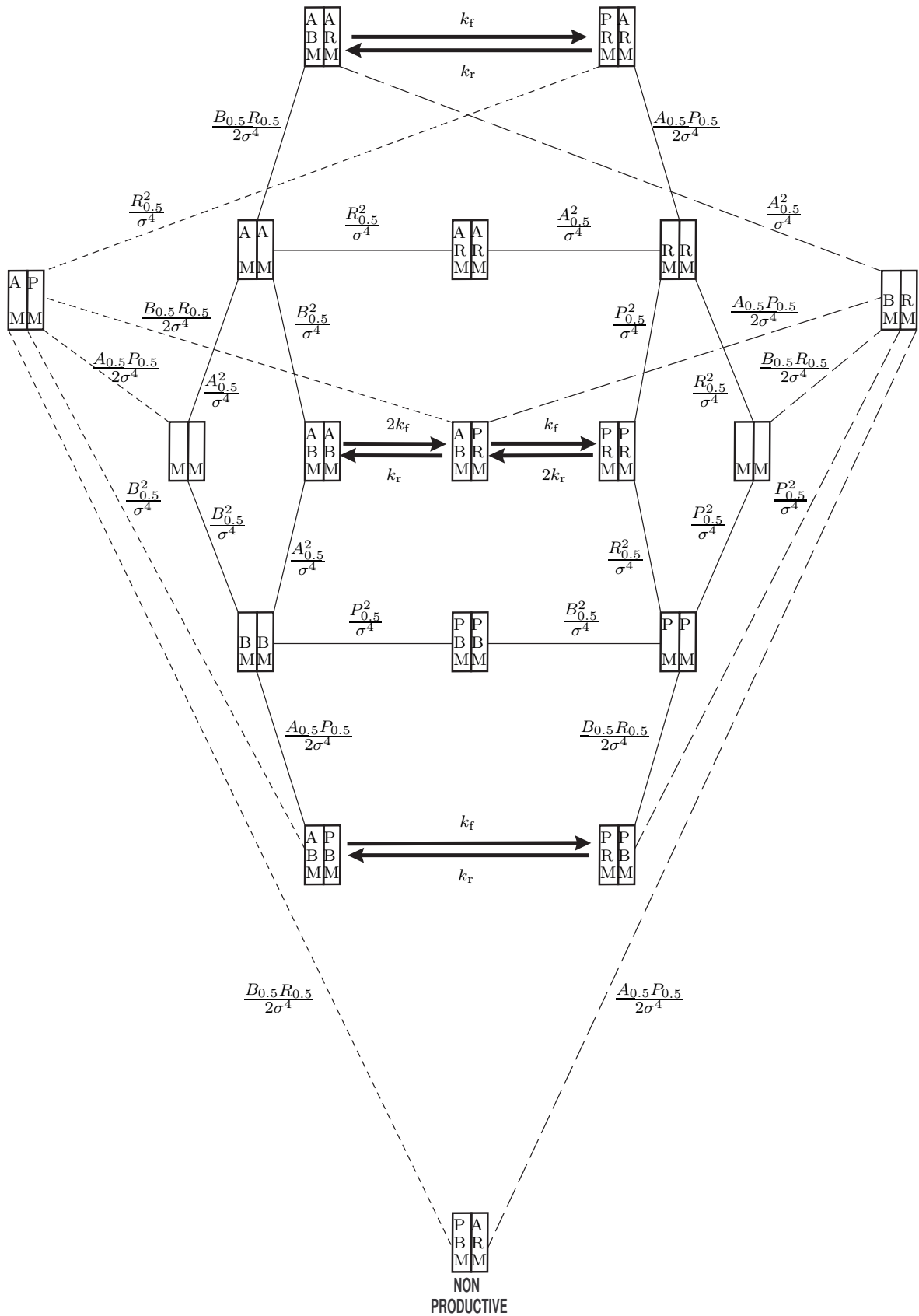


Figure 5.4: Bound enzyme forms for reaction $A + B \rightleftharpoons P + R$ including modifier (M) binding.

The notation for species $[\text{EAPBR}]^{\text{prod}}$ and $[\text{EAPBRM}_2]^{\text{prod}}$ (p. 84) shows that although both species have a statistical binding factor of 4, only 2 of these configurations will be catalytically active with the other 2 configurations being dead-end complexes. From the above catalytic scheme the conversion rate can be formulated as follows:

$$\begin{aligned} \frac{v}{[\text{E}_T]} &= \frac{k_f([\text{EA}_2\text{BR}] + 2[\text{EA}_2\text{B}_2] + [\text{EAPBR}]^{\text{prod}} + [\text{EAPB}_2]) +}{k_f([\text{EA}_2\text{BRM}_2] + 2[\text{EA}_2\text{B}_2\text{M}_2] + [\text{EAPBRM}_2]^{\text{prod}} + [\text{EAPB}_2\text{M}_2]) -} \\ &\quad \frac{k_r([\text{EAPR}_2] + [\text{EAPBR}]^{\text{prod}} + 2[\text{EP}_2\text{R}_2] + [\text{EP}_2\text{BR}]) -}{k_r([\text{EAPR}_2\text{M}_2] + [\text{EAPBRM}_2]^{\text{prod}} + 2[\text{EP}_2\text{R}_2\text{M}_2] + [\text{EP}_2\text{BRM}_2])} \\ &\quad \frac{1}{[\text{E}_T]} \end{aligned}$$

Rewriting these species in terms of their scaled notations found in Table 5.1, the catalytic conversion can be written as:

$$\begin{aligned} \frac{v}{[\text{E}_T]} &= \frac{k_f(2\alpha^2\beta\rho + 2\alpha^2\beta^2 + 2\alpha\pi\beta\rho + 2\alpha\pi\beta^2) +}{k_f(2\sigma^8\alpha^2\beta\rho\mu^2 + 2\sigma^8\alpha^2\beta^2\mu^2 + 2\sigma^8\alpha\pi\beta\rho\mu^2 + 2\sigma^8\alpha\pi\beta^2\mu^2) -} \\ &\quad \frac{k_r(2\alpha\pi\rho^2 + 2\alpha\pi\beta\rho + 2\pi^2\rho^2 + 2\pi^2\beta\rho) -}{k_r(2\sigma^8\alpha\pi\rho^2\mu^2 + 2\sigma^8\alpha\pi\beta\rho\mu^2 + 2\sigma^8\pi^2\rho^2\mu^2 + 2\sigma^8\pi^2\beta\rho\mu^2)} \\ &= \frac{D_M}{2k_f\alpha\beta(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta) + 2k_f\alpha\beta(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta)(\sigma^8\mu^2) -} \\ &\quad \frac{2k_r\pi\rho(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta) - 2k_r\pi\rho(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta)(\sigma^8\mu^2)}{D_M} \\ &= \frac{2k_f\alpha\beta(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta)(1 + \sigma^8\mu^2) - 2k_r\pi\rho(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta)(1 + \sigma^8\mu^2)}{D_M} \\ &= \frac{(2k_f\alpha\beta - 2k_r\pi\rho)(\alpha\rho + \alpha\beta + \pi\rho + \pi\beta)(1 + \sigma^8\mu^2)}{D_M} \end{aligned}$$

where $D_M = [\text{E}_T]/[\text{E}]$. Let $V_f = 2k_f \cdot \text{E}_T$ and $V_r = 2k_r \cdot \text{E}_T$. $v/[\text{E}_T]$ can now be rewritten as:

$$v = \frac{(V_f\alpha\beta - V_r\pi\rho)(\alpha + \pi)(\beta + \rho)(1 + \sigma^8\mu^2)}{D_M} \quad (5.1)$$

Pooling all the species from Table 5.1 together, D_M can now be factorized to give:

$$\begin{aligned}
 D_M &= 1 + \alpha^2 + \beta^2 + \pi^2 + \rho^2 + 2\alpha\pi + 2\beta\rho + \alpha^2\beta^2 + 2\alpha^2\beta\rho + \alpha^2\rho^2 + \\
 &\quad 2\alpha\pi\beta^2 + 4\alpha\pi\beta\rho + 2\alpha\pi\rho^2 + \pi^2\beta^2 + 2\pi^2\beta\rho + \pi^2\rho^2 + \\
 &\quad \mu^2 + \sigma^4\alpha^2\mu^2 + \sigma^4\beta^2\mu^2 + \sigma^4\pi^2\mu^2 + \sigma^4\rho^2\mu^2 + 2\sigma^4\alpha\pi\mu^2 + 2\sigma^4\beta\rho\mu^2 + \\
 &\quad \sigma^8\alpha^2\beta^2\mu^2 + 2\sigma^8\alpha^2\beta\rho\mu^2 + \sigma^8\alpha^2\rho^2\mu^2 + 2\sigma^8\alpha\pi\beta^2\mu^2 + \\
 &\quad 4\sigma^8\alpha\pi\beta\rho\mu^2 + 2\sigma^8\alpha\pi\rho^2\mu^2 + \sigma^8\pi^2\beta^2\mu^2 + 2\sigma^8\pi^2\beta\rho\mu^2 + \sigma^8\pi^2\rho^2\mu^2 \\
 &= (1 + \mu^2) + (\alpha^2 + \beta^2 + \pi^2 + \rho^2 + 2\alpha\pi + 2\beta\rho)(1 + \sigma^4\mu^2) + \\
 &\quad (\alpha^2\beta^2 + 2\alpha^2\beta\rho + \alpha^2\rho^2 + 2\alpha\pi\beta^2 + 4\alpha\pi\beta\rho + 2\alpha\pi\rho^2 + \pi^2\beta^2 + 2\pi^2\beta\rho + \pi^2\rho^2)(1 + \sigma^8\mu^2) \\
 &= (1 + \mu^2) + [(\alpha + \pi)^2 + (\beta + \rho)^2](1 + \sigma^4\mu^2) + (\alpha + \pi)^2(\beta + \rho)^2(1 + \sigma^8\mu^2)
 \end{aligned}$$

Substituting D_M into eq. 5.1 and cancelling the modifier term from the numerator gives the following equation:

$$v = \frac{(V_f\alpha\beta - V_r\pi\rho)(\alpha + \pi)(\beta + \rho)}{\left(\frac{1 + \mu^2}{1 + \sigma^8\mu^2}\right) + \left(\frac{1 + \sigma^4\mu^2}{1 + \sigma^8\mu^2}\right)[(\alpha + \pi)^2 + (\beta + \rho)^2] + (\alpha + \pi)^2(\beta + \rho)^2} \quad (5.2)$$

Using the Haldane relationship and mass action ration as:

$$K_{eq} = \frac{V_f}{V_r} \cdot \frac{P_{0.5}R_{0.5}}{A_{0.5}B_{0.5}} \quad \text{and} \quad \Gamma = \frac{[P][R]}{[A][B]}$$

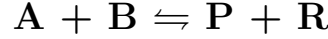
and generalizing from $n = 2$ to $n = h$, eq. 5.2 can now be written to give the **bi-substrate reversible Hill equation including modifier** as follows:

$$v = \frac{V_f\alpha\beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^{h-1}(\beta + \rho)^{h-1}}{\left(\frac{1 + \mu^h}{1 + \sigma^{4h}\mu^h}\right) + \left(\frac{1 + \sigma^{2h}\mu^h}{1 + \sigma^{4h}\mu^h}\right)[(\alpha + \pi)^h + (\beta + \rho)^h] + (\alpha + \pi)^h(\beta + \rho)^h} \quad (5.3)$$

where $\sigma < 1$ will result in inhibition of the reaction rate and $\sigma > 1$ will result in activation. Setting $\mu = 0$ or $\sigma = 1$, eq. 5.3 will simplify to the bi-substrate reversible Hill equation without a modifier (eq. 3.20).

5.2 Extending the bi-substrate reversible Hill equation to incorporate n independent modifiers

Consider the bi-substrate conversion reaction as follows:



As before, a modifier M binds to an allosteric site on each subunit of a dimeric enzyme. M increases or decreases the affinity of substrates for binding site A and B, on both subunits, by a factor σ_1 . A second modifier (M') binds independently of M to a separate allosteric site on each subunit. M' affects the affinity of substrates and products across all subunits by a factor σ_2 , where $\sigma_1 \neq \sigma_2$. If M is bound to both its allosteric sites (fully liganded, M₂), species fully liganded at one substrate binding site (e.g. EA₂) will show four modifier effects, species fully liganded at both substrate binding sites (e.g. EA₂B₂) will show eight modifier effects. The same holds for modifier M'. When all allosteric sites are liganded (EM₂M'₂), species fully liganded at one substrate binding site will show four effects for M and four effects for M'. A species fully liganded at both substrate binding sites will show eight effects for M and eight effects for M'. As a result of simultaneous conversion of substrates, the species considered for catalysis must have fully liganded substrate and product binding sites. These species can either have empty modifier binding sites, sites fully liganded with M, sites fully liganded with M' or fully liganded with both. From this it then becomes clear that the numerator in the rate equation can be written as:

$$V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^{h-1} (\beta + \rho)^{h-1} (1 + \sigma_1^{4h} \mu_1^h) (1 + \sigma_2^{4h} \mu_2^h) \quad (5.4)$$

where $\mu_1 = [M]/M_{0.5}$, $\mu_2 = [M']/M'_{0.5}$, $\alpha = [A]/A_{0.5}$, $\beta = [B]/B_{0.5}$, $\pi = [P]/P_{0.5}$, $\rho = [R]/R_{0.5}$ and $h = 2$. V_f , Γ and K_{eq} are defined as before. The species considered for E_T will have modifier binding sites that are either empty, fully liganded with M, fully liganded with M' or fully liganded with both. The denominator (D_{MM'}) can then be written as follows:

$$D_{MM'} = (1 + \mu_1^h)(1 + \mu_2^h) + (1 + \sigma_1^{2h} \mu_1^h)(1 + \sigma_2^{2h} \mu_2^h) \left[(\alpha + \pi)^h + (\beta + \rho)^h \right] + (\alpha + \pi)^h (\beta + \rho)^h (1 + \sigma_1^{4h} \mu_1^h) (1 + \sigma_2^{4h} \mu_2^h)$$

The rate equation is thus written as follows:

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^{h-1} (\beta + \rho)^{h-1}}{\frac{(1 + \mu_1^h)(1 + \mu_2^h)}{(1 + \sigma_1^{4h} \mu_1^h)(1 + \sigma_2^{4h} \mu_2^h)} + \frac{(1 + \sigma_1^{2h} \mu_1^h)(1 + \sigma_2^{2h} \mu_2^h)}{(1 + \sigma_1^{4h} \mu_1^h)(1 + \sigma_2^{4h} \mu_2^h)} \left[(\alpha + \pi)^h + (\beta + \rho)^h \right] + (\alpha + \pi)^h (\beta + \rho)^h} \quad (5.5)$$

From the bi-substrate reversible Hill equation with one modifier (eq. 5.3) and eq. 5.5 above, the bi-substrate reversible Hill equation including any number of independently binding modifiers (BRH^{mod}), is given by:

$$v = \frac{V_f \alpha \beta \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha + \pi)^{h-1} (\beta + \rho)^{h-1}}{\prod_j^{n_m} \left[\frac{(1 + \mu_j^h)}{(1 + \sigma_j^{4h} \mu_j^h)} \right] + \prod_j^{n_m} \left[\frac{(1 + \sigma_j^{2h} \mu_j^h)}{(1 + \sigma_j^{4h} \mu_j^h)} \right] [(\alpha + \pi)^h + (\beta + \rho)^h] + (\alpha + \pi)^h (\beta + \rho)^h} \quad (5.6)$$

where n_m is the number of modifiers. Eq. 5.6 simplifies to the unmodified bi-substrate reversible Hill equation when modifier(s) are absent. It also simplifies to the non-cooperative bi-substrate generic equation (eq. 3.22, $n_s = 2$, $h = 1$).

5.3 Extending the three substrate reversible Hill equation to incorporate n independent modifiers

Following the same analogy to the derivation of the bi-substrate case with two modifiers, the three-substrate case with two modifiers give the following equation (factorization not shown):

$$v = \frac{V_f \alpha_1 \alpha_2 \alpha_3 \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha_1 + \pi_1)^{h-1} (\alpha_2 + \pi_2)^{h-1} (\alpha_3 + \pi_3)^{h-1}}{\frac{(1 + \mu_1^h)(1 + \mu_2^h)}{(1 + \sigma_1^{6h} \mu_1^h)(1 + \sigma_2^{6h} \mu_2^h)} + \frac{(1 + \sigma_1^{2h} \mu_1^h)(1 + \sigma_2^{2h} \mu_2^h)}{(1 + \sigma_1^{6h} \mu_1^h)(1 + \sigma_2^{6h} \mu_2^h)} [(\alpha_1 + \pi_1)^h + (\alpha_2 + \pi_2)^h + (\alpha_3 + \pi_3)^h] + \frac{(1 + \sigma_1^{4h} \mu_1^h)(1 + \sigma_2^{4h} \mu_2^h)}{(1 + \sigma_1^{6h} \mu_1^h)(1 + \sigma_2^{6h} \mu_2^h)} [(\alpha_1 + \pi_1)^h (\alpha_2 + \pi_2)^h + (\alpha_1 + \pi_1)^h (\alpha_3 + \pi_3)^h + (\alpha_2 + \pi_2)^h (\alpha_3 + \pi_3)^h] + (\alpha_1 + \pi_1)^h (\alpha_2 + \pi_2)^h (\alpha_3 + \pi_3)^h} \quad (5.7)$$

From eq. 5.7, the reversible Hill equation for three substrate to three product reactions with any number of modifiers, can be written as:

$$v = \frac{V_f \alpha_1 \alpha_2 \alpha_3 \left(1 - \frac{\Gamma}{K_{eq}}\right) (\alpha_1 + \pi_1)^{h-1} (\alpha_2 + \pi_2)^{h-1} (\alpha_3 + \pi_3)^{h-1}}{\prod_j^{n_m} \left[\frac{(1 + \mu_j^h)}{(1 + \sigma_j^{6h} \mu_j^h)} \right] + \prod_j^{n_m} \left[\frac{(1 + \sigma_j^{2h} \mu_j^h)}{(1 + \sigma_j^{6h} \mu_j^h)} \right] [(\alpha_1 + \pi_1)^h + (\alpha_2 + \pi_2)^h + (\alpha_3 + \pi_3)^h] + \prod_j^{n_m} \left[\frac{(1 + \sigma_j^{4h} \mu_j^h)}{(1 + \sigma_j^{6h} \mu_j^h)} \right] [(\alpha_1 + \pi_1)^h (\alpha_2 + \pi_2)^h + (\alpha_1 + \pi_1)^h (\alpha_3 + \pi_3)^h + (\alpha_2 + \pi_2)^h (\alpha_3 + \pi_3)^h] + (\alpha_1 + \pi_1)^h (\alpha_2 + \pi_2)^h (\alpha_3 + \pi_3)^h} \quad (5.8)$$

where n_m is the number of modifiers.

5.4 A comparison to the Monod, Wyman and Changeux model

The inability of the MWC model [6] to account for allosteric modifier saturation has been demonstrated previously by Hofmeyr & Cornish-Bowden [3]. A theoretical comparison was made *in silico* between the derived reversible Hill for single substrate reactions and the irreversible MWC model for uni-reactant conversions. Many allosterically regulated cooperative reactions inside metabolic pathways are, however, of a multisubstrate nature. Such reactions have previously been characterized with either the MWC model or more recently, with the reversible Hill uni-substrate equation. The use of the reversible MWC bi-substrate equation (eq. 8.10 in Appendix) has been restricted due to its sheer complexity. The BRH^{mod} equation is independent of underlying mechanism and allows for both allosteric inhibition and activation as a result of the interaction parameter σ . It will be shown to give a realistic account of modifier effects compared to the MWC model.

Figure 5.5 show the BRH^{mod} equation (eq. 5.6) for one modifier ($n_m = 1$) behaviour over a range of substrate and modifier concentrations. The modifier is an inhibitor with a σ value of 0.1. From Figure 5.5 it can be seen how the bi-substrate reversible Hill equation is able to account for substrate-modifier saturation. At high concentrations of substrate—Figures 5.5 (A)→(D)—the effect of the modifier can be nullified. This observation agrees well with the findings of Hofmeyr & Cornish-Bowden for the uni-reactant reversible Hill equation [3]. Figure 5.6 shows the comparative modifier behaviour of the bi-substrate reversible Hill equation and the MWC model (eq. 8.11) over a range of substrate and modifier concentrations.

Figure 5.6 clearly shows the inability of the MWC model to allow for inhibitor (I) saturation. In the MWC model the allosteric inhibitor always has an effect even at saturating conditions for the substrate. This behaviour is analogous to the inhibitor ‘competing’ with the substrate for the same binding site and is a result of the MWC postulate that modifiers bind exclusively to either the T-form (inhibitors) or R-form (activators) of the enzyme. We believe that the behaviour predicted by the BRH^{mod} equation is a more realistic reflection of physiological response to allosteric inhibition than the behaviour predicted by the MWC model.

In the next chapter we shall investigate the *in vitro* behaviour of a 2-site cooperative enzyme at various substrate and inhibitor concentrations. Reaction rate plots similar to Figures 5.6 (A) and (C) and not Figures 5.6 (B) and (D) will serve as validation of the derived BRH^{mod} equation.

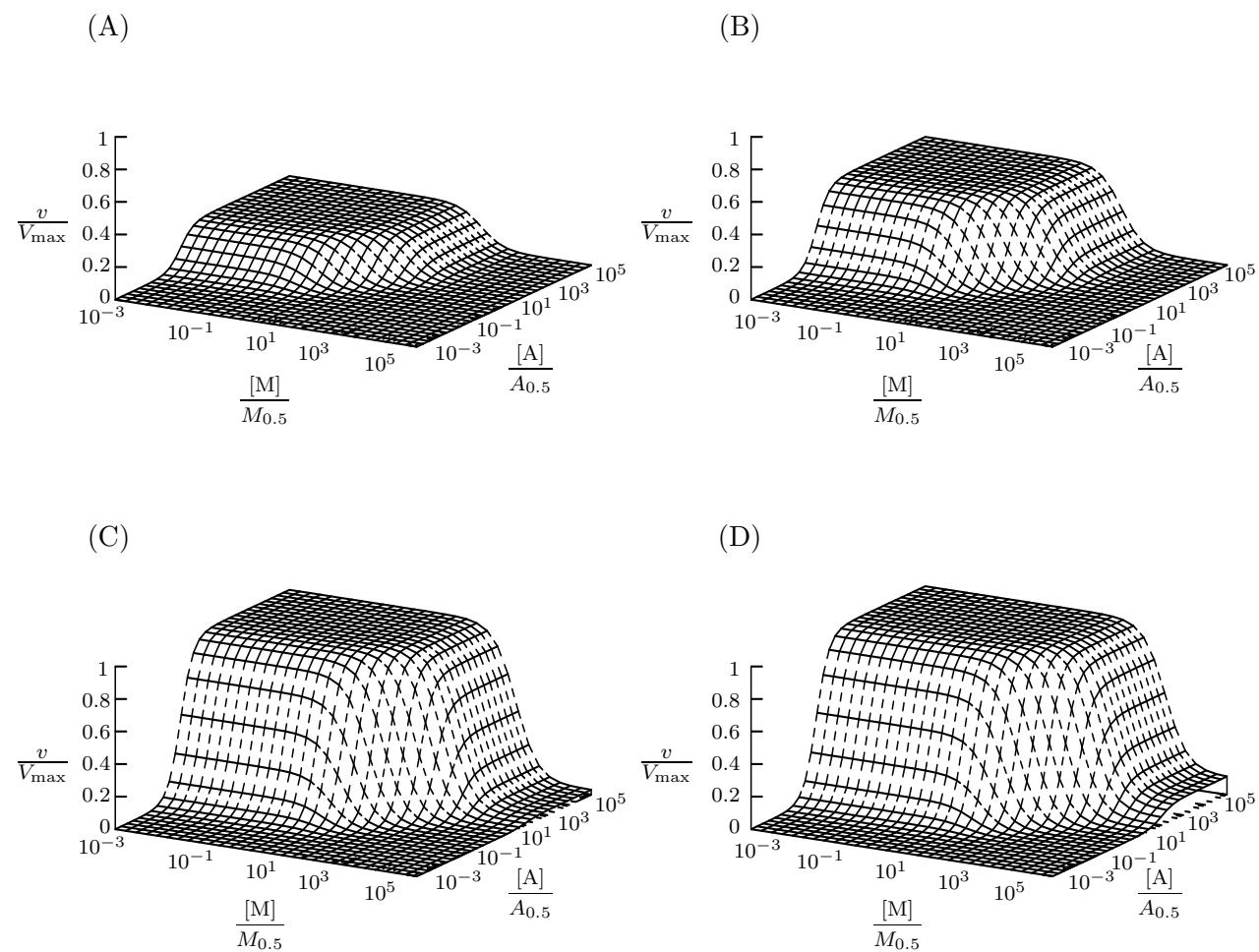


Figure 5.5: Multiplet showing the substrate-modifier effects of the bi-substrate reversible Hill equation at low $[B]$. All plots were drawn with $[P]/P_{0.5}$ and $[R]/R_{0.5} = 0$, $A_{0.5}$ and $M_{0.5} = 1$, $h = 2$ and $\sigma = 0.1$. (A) $[B]=0.5$, (B) $[B] = 1$, (C) $[B]=10$ and (D) $[B]=25$.

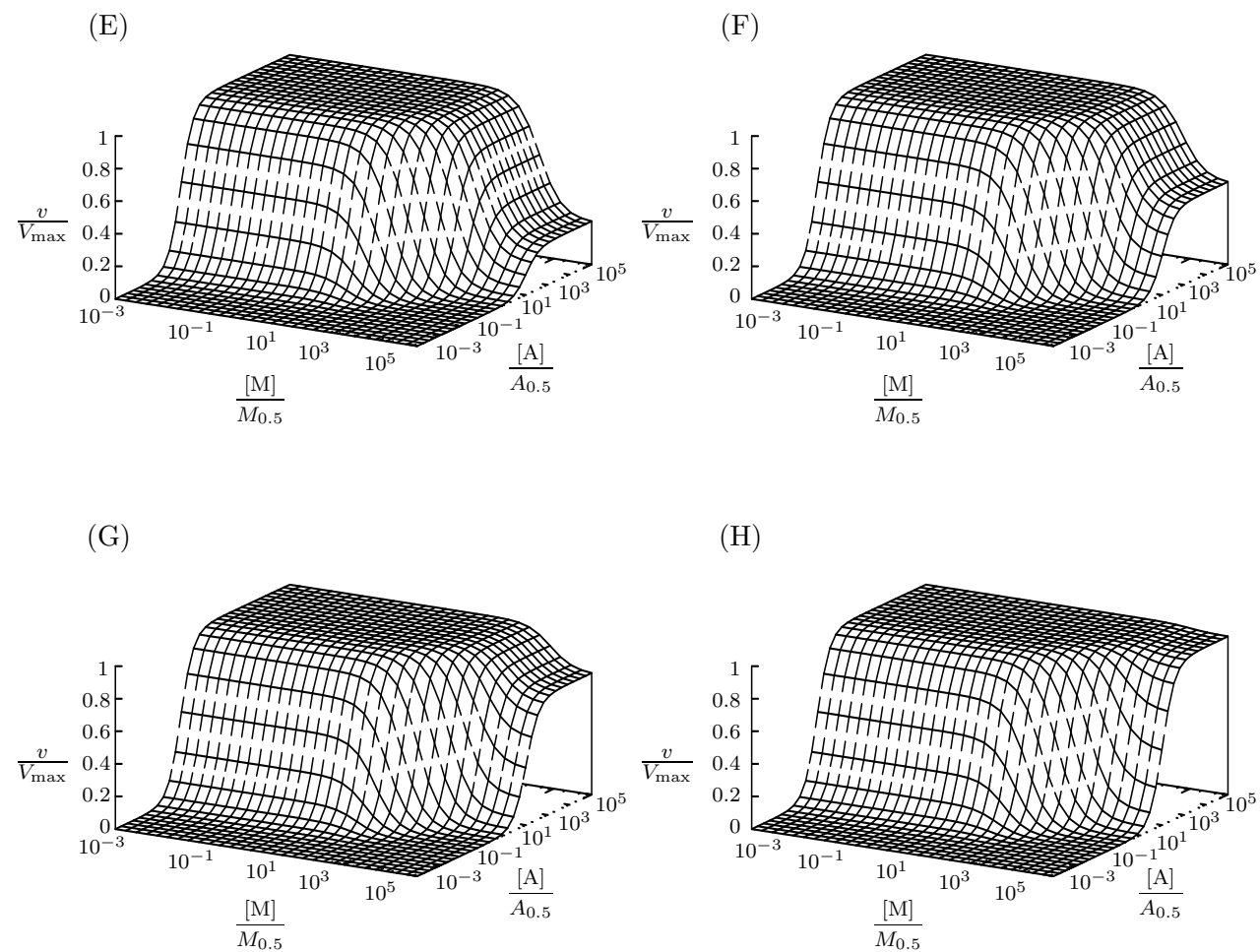


Figure 5.5: (Continued) Multiplot showing the substrate-modifier effects of the bi-substrate reversible Hill equation. All plots were drawn with $[P]/P_{0.5}$ and $[R]/R_{0.5} = 0$, $A_{0.5}$ and $M_{0.5} = 1$, $h = 2$ and $\sigma = 0.1$. (E) $[B]=50$, (F) $[B] = 100$, (G) $[B]=200$ and (H) $[B]=1000$.

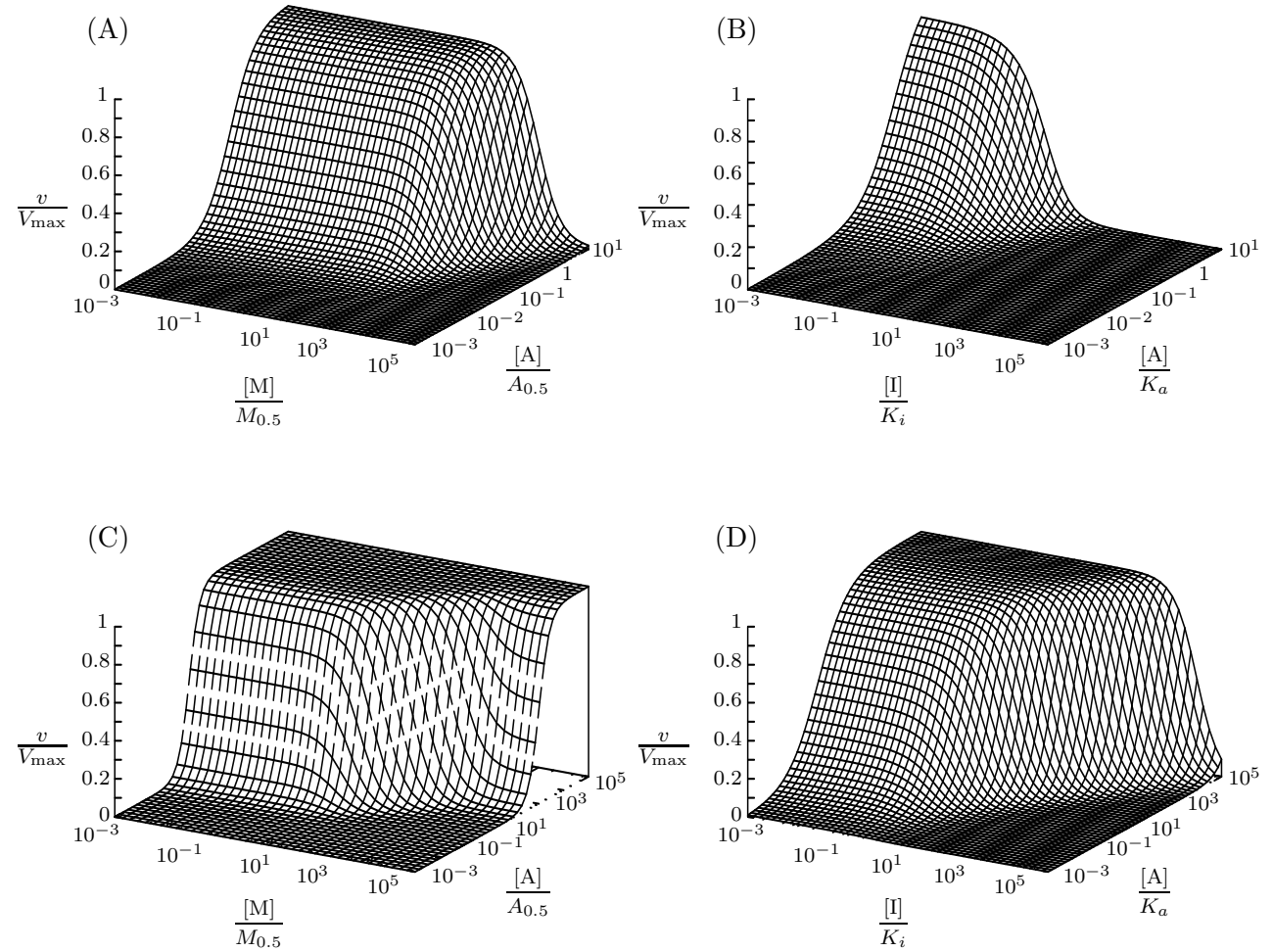


Figure 5.6: Multiplot of bi-substrate reversible Hill equation (RH) and MWC uni-substrate model with product concentrations = 0. For the RH-equation, $[B] = 10^4$, $h=2$, $\sigma = 0.1$, $A_{0.5}$, $B_{0.5}$ and $M_{0.5} = 1$. For the MWC model (eq. 8.11 in Appendix), K_a and $K_i = 1$, $n = 2$ and $L = 10$. (A) RH: low $[A]$, (B) MWC: low $[A]$, (C) RH: high $[A]$, (D) MWC: high $[A]$.

6 Experimental validation of the derived bi-substrate reversible Hill equation with one modifier

6.1 Outline

The aim of this chapter is not to report a detailed kinetic study of a cooperative enzyme, but to investigate whether the *in silico* predicted modifier effect on a cooperative enzyme catalysing a bi-substrate reaction (see Figure 5.5 E to H) is present *in vitro*. The bi-substrate case of the GRH equation predicted increasing substrate concentration will overcome the effect of a single allosteric inhibitor (Figures 5.5 and 5.6 A and C) i.e. the GRH equation shows modifier saturation, as opposed to the MWC model, which does not show this saturating inhibitory effect (see section 5.4 and Figure 5.6 B and D). Should this effect be present in a bi-substrate cooperative enzyme *in vitro*, it will confirm that the bi-substrate case of the GRH with one modifier gives a physiologically relevant account of modifier behaviour *in silico*.

6.2 Materials and Methods

6.2.1 Reagents

Phosphoenolpyruvate sodium salt (PEP), adenosine 5-diphosphate sodium salt (ADP), nicotinamide adenine di-nucleotide reduced form (NADH), imidazole, *Bacillus stearothermophilus* pyruvate kinase (PK) and bovine lactate dehydrogenase (LDH) were obtained from Sigma. Sodium dihydrogenphosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), magnesium chloride (MgCl_2) and potassium chloride (KCl) were purchased from Saarchem. All other chemicals used were reagent grade.

6.2.2 Spectrophotometric enzyme assay

Inhibitor studies—PK activity was assayed according to the method of Bücher & Pfeleiderer at 30 °C [72]. Minor changes were made to this protocol. The reaction mixture contained in 1ml: 50 μmol imidazole-HCl buffer, pH 7.2, 50 μmol KCl, 8 μmol MgCl_2 , 0.15-0.175 μmol NADH, 5 units (U) LDH and various amounts of PEP and ADP. The equimolar concentrations of PEP and ADP were 1, 4, 10 and 20 $\mu\text{mol}/\text{ml}$. For each increase, the reaction velocities were measured over a NaH_2PO_4 concentration range of 0–64 $\mu\text{mol}/\text{ml}$. The pH of the phosphate solution was adjusted to 7.2 prior to addition. MgCl_2 concentration was kept equimolar to ADP concentration. The reaction mixture was incubated at 30 °C for 2 minutes, after which enzyme reaction was initiated by addition of 0.1 U PK. The change in absorbance at 340 nm was monitored for 2 minutes on a Beckman DU-65 spectrophotometer. The spectrophotometric chamber temperature was maintained at 30 °C with a variable temperature unit. One unit of enzyme is defined as the amount of protein required to catalyse formation of 1 μmol of pyruvate per minute. PK activity was 1000 U / 4.9 mg protein. 0.1 U PK = 0.00049 mg protein. Reaction velocity is reported as $\mu\text{mol}/(\text{min}\cdot\text{mg})$.

6.2.3 Method optimisation

In the coupled assay, PK converts PEP to pyruvate, which in turn gets converted to lactate by LDH. The LDH conversion uses NADH as cofactor, with the subsequent production of NAD^+ . The turnover of pyruvate to lactate must be immediate to ensure that accurate reaction velocities are determined. Thus, should LDH be in excess in the reaction mixture, the conversion of PEP to lactate is essentially a one conversion reaction. Therefore, at a fixed concentration of protein, the conversion rate is only dependent on the concentration of PEP. Figure 6.1 shows a linear relationship between fixed concentration of LDH and increasing concentration of PK. This shows that doubling the amount of PK results in a two-fold increase in reaction rate. This linear relationship between increasing amount of PK at a constant amount of LDH shows that reaction velocity is solely dependent on the amount of PK. The LDH : PK ratio required for linear relationship is therefore any ratio larger than 5:1. The LDH : PK ratio in the coupled assay was 50:1 (5 U LDH : 0.1 U PK).

6.3 Results

Bacillus stearothermophilus pyruvate kinase has been proposed to be a model microbial cooperative enzyme, exhibiting cooperativity towards substrate PEP and Michaelian kinetics for substrate ADP [73]. This enzyme was chosen for this study because i) its cooperative behaviour, structure and thermal stability have been well documented [66, 73–79], ii) the purified enzyme can be purchased without necessity for isolation, iii) the enzyme has been shown to have both potent activators and inhibitors and iv) the spectrophotometric assay protocol [72] has been optimised and used in numerous studies [66, 73, 74]. In this study, we focused on the effect of an inhibitor, inorganic phosphate (P_i), on PK reaction rates.

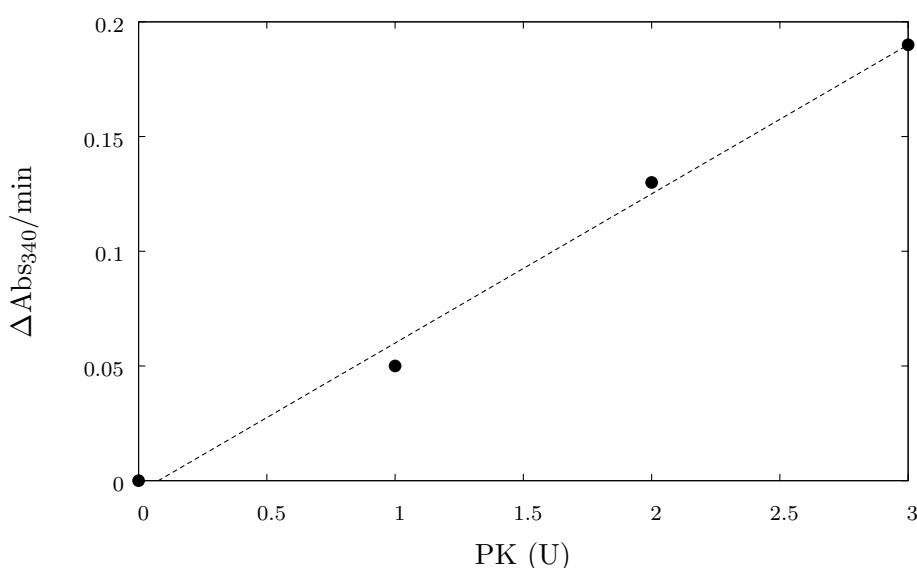


Figure 6.1: Plot of reaction velocity as a function of PK (U). Assay performed as described in text. LDH = 15 U, [PEP] = 4 $\mu\text{mol}/\text{ml}$, [ADP] = 2 $\mu\text{mol}/\text{ml}$ and [NADH] = 0.15 $\mu\text{mol}/\text{ml}$. $R^2 = 0.994$

Inhibition by inorganic phosphate—The effect of inorganic phosphate (P_i) on reaction rate is shown in Figure 6.2 (plotted in double logarithmic space). Reaction rates, given in $\mu\text{mol}/(\text{min}\cdot\text{mg})$, were calculated from ΔA_{340} values using a NADH extinction coefficient of $6.3 \times 10^3 \text{M}^{-1}\cdot\text{cm}^{-1}$. An increase in substrate concentrations overcomes the inhibitory effect of P_i . This observation is consistent with the predicted reaction rates of the bi-substrate reversible Hill equation at saturating substrate concentrations compared to inhibitor concentration (Figures 5.5 and 5.6). Increasing substrate concentrations increase the apparent $M_{0.5}$ of the inhibitor, leading to an apparent decrease in inhibitor affinity, and a resultant diminished effect on reaction rate [3].

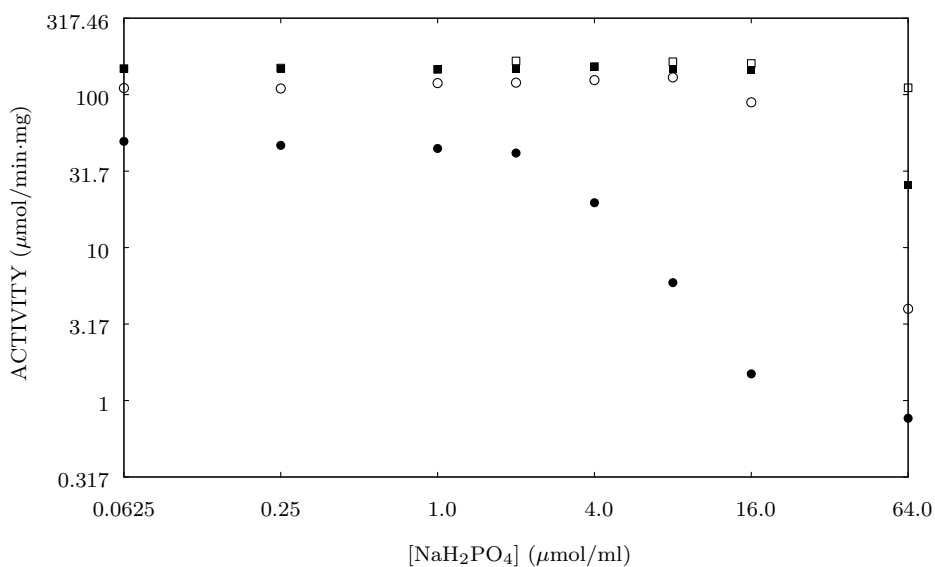


Figure 6.2: Plot of enzyme activity as a function of inhibitor concentration (NaH_2PO_4) at increasing substrate conditions. Data are plotted in double logarithmic space. The assay mixture contained equimolar amounts of PEP and ADP; 1 mM (●), 4 mM (○), 10 mM (■) and 20 mM (□).

6.4 Conclusion

The experimental data presented here clearly show that increasing concentration of substrate leads to an increase in the reaction rate at every concentration of inhibitor tested. Comparing Figure 6.2 with the *in silico* predictions of the bi-substrate reversible Hill equation with one allosteric inhibitor (Figures 5.5 and 5.6), it is evident that the derived equation predicts behaviour synonymous to PK behaviour. The experimental data also confirm the MWC model inability to predict allosteric inhibitor saturation, a phenomenon shown to be present in a model cooperative enzyme. We therefore believe that the strong correlation between predicted and experimental reaction rates serves as validation for the derived bi-substrate reversible Hill equation with one allosteric modifier.

7 Discussion

Many enzymes in metabolic pathways are subject to a high degree of control to ensure cellular conditions far from equilibrium [2]. The ability to accurately predict their behaviour *in silico* is of great use when attempting to understand pathway behaviour at the hands of the individual enzymes. The development of *in silico* models to characterise metabolic pathway kinetics has been a useful tool to predict pathway behaviour. Certain pathway constituent enzymes possess the property of cooperativity, enabling them to respond to minor metabolite changes with a significant increase or decrease in reaction flux. An accurate mathematical description of such enzyme behaviour allows refinement of kinetic pathway models, e.g. in perturbation analysis where fluxes are monitored while small changes are made in metabolite concentrations. In metabolic models, the most commonly used models to describe cooperative behaviour are the irreversible uni-substrate Hill and MWC equations and the reversible uni-substrate Hill equation. The uni- and bi-substrate KNF and reversible MWC models have rarely been used, if at all. The MWC and KNF models differ from Hill equations (irreversible and reversible) in two major respects:

- they depend on prior knowledge of the binding mechanism, and
- the MWC reversible models derived by Popova & Sel'kov contain numerous parameters, many of which cannot be determined empirically.

Moreover, since some of the reversible MWC models' parameters are outside the scope of experimental determination, the investigator eventually has to guess some of their values. The Hill equation, in contrast, does not have these restrictions and has been shown to fit a wide range of experimental data very well. For these reasons it has become the preferred method to determine enzyme properties from sigmoidal kinetic data. The general uni-substrate reversible Hill equation shows separate terms for the kinetic and thermodynamic properties of the enzyme. This reversible Hill equation has been shown by Hofmeyr & Cornish-Bowden [3] to predict correct allosteric behaviour compared to the MWC model, which does not. Moreover, many equations available to characterise enzyme behaviour are mechanistic irreversible uni-reactant equations

since little kinetic data, if any, is available on the reverse conversion rates. The dangers of using irreversible equations in metabolic modelling have been showed by Cornish-Bowden & Cárdenas [1]. An equation that incorporates cooperative reversibility for any number of substrates based on the Hill equation will overcome these limitations.

From Figure 4.1 the derived Adair equation for bi-substrate cooperativity shows perfect correlation to the commonly used uni-substrate Adair equation. Velocity equations based on the uni-substrate Adair equation have been used most commonly to evaluate sigmoidal kinetic data. The uni-substrate MWC model, assuming the ‘perfect- K ’ system, can be written to take the form of the uni-substrate Adair equation [3, 2]. Nearly all authors who applied the MWC model in their cooperative enzyme studies have used the ‘perfect- K ’ form of the MWC model [3]. The Adair bi-substrate equation has been shown to fit very well to the uni-substrate Adair equation. From this, a comparison to the Adair bi-substrate equation is believed to supply the best base for evaluating the bi-substrate case of the proposed general reversible Hill (GRH) equation (eq. 3.22).

Figure 4.2 shows that the bi-substrate reversible Hill equation (eq. 3.20) predicts reaction rates indistinguishable to those of the Adair bi-substrate equation, only failing at the extremes of substrate saturation where the difference in rates will be experimentally undetectable. The bi-substrate Adair equation accounts for independent substrate effects, cooperative or not, at both binding sites. The derived GRH equation shows for both the numerator and denominator the entries in each term represent a specific binding site. This feature is of integral importance when characterising cooperative enzymes catalysing a bi-substrate, or more, conversion reaction. Many multisubstrate cooperative enzymes show cooperativity for the one substrate, and Michaelis-Menten kinetics for the other(s). The separate substrate binding site terms in the GRH equation, thus, separate the binding characteristics of the reaction metabolites.

The reversible Hill bi-substrate equation has been shown to give reaction rates in good correlation to the uni-substrate reversible Hill equation (Figure 4.5), the Hill equation (Figure 4.7) and commonly used non-cooperative bi-substrate equations for $h = 1$ (Figure 4.9). Figures 4.5 and 4.6 show at low $[A]$, an increase in $[P]$ leads to activation of the reaction rate. This behaviour of the reversible Hill bi-substrate equation is a feature shared with the uni-reactant reversible Hill equation and the MWC model. Hofmeyr *et al.* [10] showed this behaviour in a synthetic system subject to pathway end-product inhibition. The competitive inhibitory effect of the direct product P on the reversible Hill bi-substrate equation reaction rate is a result of $[P]$ occurring as a positive term in the denominator. In contrast, the activatory effect of P is a result of $[P]$ being present as a positive term in the numerator. The GRH equation separates the terms for binding

to each substrate site, which results in this effect made possible for any number of substrates. An in-depth analysis of product-activation was done by Olivier [70] who showed the presence of multiple steady states (stable and unstable) or hysteretic pathway behaviour as a direct result of the formulation of the uni-reactant reversible Hill equation.

Setting $h = 1$ in the GRH equation results in the *generic* equation for Michaelian conversion reactions for any number of substrates. The bi-substrate case of the generic equation is independent of binding mechanism and gives good fits to all the non-cooperative test cases in the physiological range of enzyme action. Many of the non-cooperative test cases have parameters that are not always measured or are not available from published data. The generic equation does not have this restriction, all parameters are the half-saturating substrate concentrations that are usually available from the literature or, if not, can easily be determined experimentally. The generic equation can also be used in metabolic models when the enzyme binding mechanism for a certain non-cooperative conversion reaction is unknown.

Comparing the formulations of the bi-substrate reversible Hill equation and the bi-substrate reversible MWC model (eq. 8.10), the bi-substrate reversible Hill equation requires seven parameters to the sixteen parameters of the bi-substrate MWC model. In contrast to the bi-substrate MWC model, reversible Hill equation parameters all have physiological meaning and can be determined empirically. At very low substrate conditions reversible Hill equations fail to predict correct behaviour. Under these circumstances the MWC model is the preferred model. Furthermore, the reversible MWC model accounts for both binding and kinetic cooperativity, compared to reversible Hill equations which only show binding cooperativity. However, reversible Hill equations allow for both positive and negative cooperativity, contrary to the MWC model which can only show positive cooperativity. Metabolic models are concerned with physiological conditions where the Hill equations give excellent fits.

The bi-substrate case ($n_s = 2$) of the derived generalised reversible Hill equation with one ($n_m = 1$) modifier (BRH^{mod}), eq. 5.6, was shown to predict correct allosteric modifier effects *in silico*. The *in vitro* inhibitor studies (Chapter six) show at substrate saturation, the modifier ceases to have any noticeable effect on reaction rate. This is the result of saturating substrate increasing the apparent $M_{0.5}$ of the inhibitor. Similarly, the inhibitor tends to decrease the affinity of PEP, causing an increase in its apparent $A_{0.5}$ value. These effects are shown at the extremes of substrate saturation in Figure 5.5. The experimental data is consistent with *in silico* predicted BRH^{mod} behaviour, which validates the derived BRH^{mod} equation. These substrate saturation effects are not present in the MWC model, as shown by Figures 5.6 (B) and (D). Thus, allosterically, the BRH^{mod} equation succeeds where the MWC model fails.

To conclude, in this work we present a general reversible Hill (GRH) equation (eq. 3.22) for any arbitrary but equal number of substrates and products. The proposed equation separates the thermodynamic and kinetic properties of a cooperative enzyme. The GRH equation can be regarded as a generic rate equation for Michaelian kinetics, with the assignment of $h = 1$. The GRH equation has nine fewer parameters than the equivalent MWC equation, all of which can be determined empirically. The BRH^{mod} equation gives correct modifier behaviour over physiological ranges of modifier concentration. The main advantage of the proposed reversible Hill equations is its ability to accurately predict reaction rates in the 10–90% range of substrate saturation, with the substrate binding mechanism unresolved. It can successfully be applied to any cooperative (GRH) and non-cooperative (generic) enzyme action. The GRH equation is independent of knowledge of binding mechanism and protein subunit composition, which several current models depend on.

The importance of allosteric control of enzymes is known. The BRH^{mod} shows correct inhibitory allosteric effects, contrary to the MWC model, which does not. From this observation, we propose that the reversible Hill equation for three substrates and any number of modifiers (eq. 5.8) will also give correct modifier behaviour. We also propose reversible Hill equations for two substrate to one product and one substrate to two product reactions (eqs. 3.29 and 3.30). These two equations can also be used in non-cooperative kinetics when $h = 1$, independent of the non-cooperative binding mechanism.

Rate equations based on Hill kinetics are derived from a random binding mechanism and contain operational parameter definitions, in contrast to purely mechanistic rate equations. Moreover, these Hill equations retain terms in the isotherm that allow them to encompass the thermodynamic reaction constraints separate from kinetic characteristics, thus incorporating both metabolite saturation and reversibility. This makes it possible for Hill equations to predict reaction rates in good agreement to mechanistic equations, yet requires fewer kinetic parameters to do so. All the kinetic parameters in Hill rate equations are amenable to direct experimental determination. In the field of computational systems biology, Hill equations take a place between purely mechanistic equations and purely empirical formulations. They incorporate the main kinetic information (which can either be found in the literature or determined directly) needed to predict cooperative enzyme behaviour in a simple mathematical expression that is able to give a good approximation to more complex mechanistic approaches, yet are based on mechanism in their derivation which clearly sets them apart from an arbitrary empirical mathematical model. In a sense, Hill equations thus provide a level of description of enzymatic reactions that incorporates the “best of both worlds”.

The proposed reversible Hill equations are attractive possibilities when constructing detailed *in silico* kinetic models. We foresee that the GRH equation and the bi-uni, uni-bi reversible Hill equations can successfully be applied to any unmodified cooperative and non-cooperative reaction. Moreover, the one, two and three substrate reversible Hill equations with any number of modifiers can also be applied to any allosterically regulated reaction of the same order in the physiological substrate concentration range. All proposed equations are summarised in Table 7.1.

7.1 Future prospects and perspectives

The equations summarised in Table 7.1 provide a collection of formulae that will account for reactions of almost all orders. However, there are enzymes that catalyse three substrates to two products, two substrate to three products, three substrates to one product and one substrate to three products. Many of these reactions are allosterically regulated which will necessitate the extension of their reversible Hill formulations to account for modifier behaviour. Moreover, the proposed bi-uni and uni-bi reversible Hill equations (eqs. 3.29 and 3.30) have not been extended to incorporate modifier effects. The validity of these two equations as well as the reversible Hill equation for three substrates and any number of modifiers can also be validated experimentally.

We hope that the equations derived in this thesis will lay the groundwork for a ‘new’ enzyme kinetics for computational modelling in systems biology. We foresee that application of the proposed equations in kinetic modelling will contribute significantly to the construction of simpler and better kinetic models. The improved models result from the proposed cooperative and non-cooperative equations giving accurate modifier behaviour and having fewer parameters than previously used equations. The few parameters present have clear operational meaning and can either be found in the literature or determined from fitting to experimental data. Moreover, the proposed equations can be applied to cooperative and non-cooperative kinetics of nearly all orders of enzyme reactions. The use of these equations will enable the modeller to construct kinetic models that give more realistically accurate accounts of physiological enzyme behaviour than was possible before, and as a result, will undeniably contribute to our understanding and knowledge of how networks and their constituent enzymes and proteins function inside the cell.

Table 7.1: Summary of available reversible Hill equations. This includes the five proposed equations from this thesis as well as the uni-uni reversible Hill equation with any number of modifiers (rewritten from Hofmeyr & Cornish-Bowden [3], eq. 17 therein). Setting $h = 1$ in any of the equations, will result in that equation simplifying to the its non-cooperative case. For reasons of consistency, all substrates are denoted as α ($[A]/A_{0.5}$) and all products are denoted as π ($[P]/P_{0.5}$). n_s = the number of substrates and n_m = the number of modifiers.

Reversible Hill equations without modifier effects
<p>GRH equation: n Substrates $\rightleftharpoons n$ Products</p> $v = V_f \prod_{i=1}^{n_s} \alpha_i \left(1 - \frac{\Gamma}{K_{eq}} \right) \prod_{i=1}^{n_s} \left(\frac{(\alpha_i + \pi_i)^{h-1}}{1 + (\alpha_i + \pi_i)^h} \right)$
<p>Bi-uni reversible Hill equation: $A_1 + A_2 \rightleftharpoons P$</p> $v = \frac{V_f \alpha_1 \alpha_2 \left(1 - \frac{\Gamma}{K_{eq}} \right) (\alpha_1 \alpha_2 + \pi)^{h-1}}{1 + (\alpha_1 + \pi)^h + (\alpha_2 + \pi)^h + (\alpha_1 \alpha_2 + \pi)^h - 2\pi^h}$
<p>Uni-bi reversible Hill equation: $A \rightleftharpoons P_1 + P_2$</p> $v = \frac{V_f \alpha \left(1 - \frac{\Gamma}{K_{eq}} \right) (\alpha + \pi_1 \pi_2)^{h-1}}{1 + (\alpha + \pi_1)^h + (\alpha + \pi_2)^h + (\alpha + \pi_1 \pi_2)^h - 2\alpha^h}$
Reversible Hill equations with independent modifier effects
<p>Uni-uni reversible Hill equation: $A \rightleftharpoons P$</p> $v = \frac{V_f \alpha \left(1 - \frac{\Gamma}{K_{eq}} \right) (\alpha + \pi)^{h-1}}{\prod_j^{n_m} \left[\frac{(1 + \mu_j^h)}{(1 + \sigma_j^{2h} \mu_j^h)} \right] + (\alpha + \pi)^h}$
<p>Bi-bi reversible Hill equation: $A_1 + A_2 \rightleftharpoons P_1 + P_2$</p> $v = \frac{V_f \alpha_1 \alpha_2 \left(1 - \frac{\Gamma}{K_{eq}} \right) (\alpha_1 + \pi_1)^{h-1} (\alpha_2 + \pi_2)^{h-1}}{\prod_j^{n_m} \left[\frac{(1 + \mu_j^h)}{(1 + \sigma_j^{4h} \mu_j^h)} \right] + \prod_j^{n_m} \left[\frac{(1 + \sigma_j^{2h} \mu_j^h)}{(1 + \sigma_j^{4h} \mu_j^h)} \right] [(\alpha_1 + \pi_1)^h + (\alpha_2 + \pi_2)^h]^{n-1} + (\alpha_1 + \pi_1)^h (\alpha_2 + \pi_2)^h}$
<p>Three substrate reversible Hill equation: $A_1 + A_2 + A_3 \rightleftharpoons P_1 + P_2 + P_3$</p> $v = \frac{V_f \alpha_1 \alpha_2 \alpha_3 \left(1 - \frac{\Gamma}{K_{eq}} \right) (\alpha_1 + \pi_1)^{h-1} (\alpha_2 + \pi_2)^{h-1} (\alpha_3 + \pi_3)^{h-1}}{\prod_j^{n_m} \left[\frac{(1 + \mu_j^h)}{(1 + \sigma_j^{6h} \mu_j^h)} \right] + \prod_j^{n_m} \left[\frac{(1 + \sigma_j^{2h} \mu_j^h)}{(1 + \sigma_j^{6h} \mu_j^h)} \right] [(\alpha_1 + \pi_1)^h + (\alpha_2 + \pi_2)^h + (\alpha_3 + \pi_3)^h] + \prod_j^{n_m} \left[\frac{(1 + \sigma_j^{4h} \mu_j^h)}{(1 + \sigma_j^{6h} \mu_j^h)} \right] [(\alpha_1 + \pi_1)^h (\alpha_2 + \pi_2)^h + (\alpha_1 + \pi_1)^h (\alpha_3 + \pi_3)^h + (\alpha_2 + \pi_2)^h (\alpha_3 + \pi_3)^h] + (\alpha_1 + \pi_1)^h (\alpha_2 + \pi_2)^h (\alpha_3 + \pi_3)^h}$

8 Appendix

8.1 K-series vs V-series enzymes

K-series enzymes are proteins where the bound effector changes the binding affinity of the enzyme for its substrate, but not the maximum velocity V_f of the reaction it catalyzes. When referring to cooperative enzymes, the terminology $A_{0.5}$ ($[A]$ required to saturate enzyme 50%) is used instead of K_m . For K-series enzymes the $A_{0.5}$ varies in direct relationship to the increase or decrease of effector concentration. The MWC model postulates that these enzymes' substrates bind preferentially to the R -form resulting in a sigmoidal binding curve. It then follows that allosteric inhibitors increase the equilibrium constant L , leading to an increase in the sigmoidal nature of the substrate saturation curve (see Figure 2.3) resulting in a decrease in fractional saturation. This effect is seen inside the extremes of saturation, $[A]_0 > i < A_\infty$ and leads to a direct decrease in reaction velocity v . From Figure.2.3 it can be seen that the allosteric activator has the exact opposite effect, the point being that the V_{\max} does not get affected.

V-series enzymes are proteins where the bound effector changes the V_f of the reaction, but it does not affect the $A_{0.5}$ for the substrate. The MWC model explains this as the result of the substrate having equal affinity to both the R - and T -forms, but the reaction catalyzed by the R -form is faster than that of the T -form. For the case where the R - and T -forms have different affinities for the ligand and catalyze the reaction at different rates, allosteric effectors will have an effect on both the V_f and $A_{0.5}$ parameters. A mechanism of distinguishing between K, V and K-V enzyme systems was proposed by comparing Hill plots for binding and kinetic cooperativity [80].

Adair equation for 4-site haemoglobin

$$\bar{Y} = \frac{\frac{a}{K_1} + \frac{3a^2}{K_1K_2} + \frac{3a^3}{K_1K_2K_3} + \frac{a^4}{K_1K_2K_3K_4}}{1 + \frac{4a}{K_1} + \frac{6a^2}{K_1K_2} + \frac{4a^3}{K_1K_2K_3} + \frac{a^4}{K_1K_2K_3K_4}} \quad (8.1)$$

Michaelis-Menten equation

$$\frac{v}{V_f} = \frac{\frac{[A]}{K_m}}{1 + \frac{[A]}{K_m}} \quad (8.2)$$

Kurganov's equation

$$v = \frac{\frac{V_f[A]}{K_0 + (K_{lim} - K_0)(v/V_f)}}{1 + \frac{[A]}{K_0 + (K_{lim} - K_0)(v/V_{max})}}$$

This equation can be rewritten to show only the substrate concentrations on the right hand side as:

$$\frac{v}{V_f} = \frac{\sqrt{\left(\frac{2 + (1 + \varrho)[A]}{[A_{0.5}]}\right)^2 + \frac{8(\varrho^2 - 1)[A]}{[A_{0.5}]} - 2} - \frac{(1 + \varrho)[A]}{[A_{0.5}]}}{4(\varrho - 1)} \quad (8.3)$$

Equations used in comparison study–Chapter 3

Adair uni-reactant equation

$$v = \frac{\frac{V_f[A]}{A_{0.5}} \left(1 - \frac{\Gamma}{K_{eq}}\right) \left(\gamma^{0.5} + \frac{[A]}{A_{0.5}} + \frac{[P]}{P_{0.5}}\right)}{1 + \left(\frac{[A]}{A_{0.5}} + \frac{[P]}{P_{0.5}}\right) \left(2\gamma^{0.5} + \frac{[A]}{A_{0.5}} + \frac{[P]}{P_{0.5}}\right)} \quad (8.4)$$

Random-order ternary-complex mechanism

$$v = \frac{\frac{V_f[A][B]}{K_{iA}K_{mB}} - \frac{V_r[P][R]}{K_{mP}K_{iR}}}{1 + \frac{[A]}{K_{iA}} + \frac{[B]}{K_{iB}} + \frac{[P]}{K_{iP}} + \frac{[R]}{K_{iR}} + \frac{[A][B]}{K_{iA}K_{mB}} + \frac{[P][R]}{K_{mP}K_{iR}}} \quad (8.5)$$

Compulsory-order ternary-complex mechanism

$$v = \frac{\frac{V_f[A][B]}{K_{iA}K_{mB}} - \frac{V_r[P][R]}{K_{mP}K_{iR}}}{1 + \frac{[A]}{K_{iA}} + \frac{[B]K_{mA}}{K_{iA}K_{mB}} + \frac{[P]K_{mR}}{K_{mP}K_{iR}} + \frac{[R]}{K_{iR}} + \frac{[A][B]}{K_{iA}K_{mB}} + \frac{[A][P]K_{mR}}{K_{iA}K_{mP}K_{iR}} + \frac{[B][R]K_{mA}}{K_{iA}K_{mB}K_{iR}} + \frac{[P][R]}{K_{mP}K_{iR}} + \frac{[A][B][R]}{K_{iA}K_{mB}K_{iP}} + \frac{[B][P][R]}{K_{iB}K_{mP}K_{iR}}} \quad (8.6)$$

Substituted-enzyme mechanism (Ping-Pong)

$$v = \frac{\frac{V_f[A][B]}{K_{iA}K_{mB}} - \frac{V_r[P][R]}{K_{iP}K_{mR}}}{1 + \frac{[A]}{K_{iA}} + \frac{[B]K_{mA}}{K_{iA}K_{mB}} + \frac{[P]}{K_{iP}} + \frac{[R]K_{mP}}{K_{iP}K_{mR}} + \frac{[A][B]}{K_{iA}K_{mB}} + \frac{[A][P]}{K_{iA}K_{iP}} + \frac{[B][R]K_{mA}}{K_{iA}K_{mB}K_{iR}} + \frac{[P][R]}{K_{iP}K_{mR}}} \quad (8.7)$$

Irreversible Hill equation

$$v = \frac{\frac{V_f[A]^h}{A_{0.5}^h}}{1 + \frac{[A]^h}{A_{0.5}^h}} \quad (8.8)$$

Irreversible one-substrate MWC model

$$v = \frac{\frac{V_f[A]}{K_a} \left(1 + \frac{[A]}{K_a}\right)^{n-1}}{L \left(1 + \frac{[I]}{K_i}\right)^n + \left(1 + \frac{[A]}{K_a}\right)^n} \quad (8.9)$$

MWC bi-substrate equation for $S_1 + S_2 \rightleftharpoons S_3 + S_4$

$$v = \frac{V_f \frac{S_1 S_2}{K_{m1} K_{m2}} - \frac{\chi S_3 S_4}{K_{m3} K_{m4}}}{\Delta} \cdot \frac{1 + aL(\Delta'/\Delta)^{n-1}}{1 + L(\Delta'/\Delta)^n}$$

$$\Delta = \left(1 + \frac{S_1}{K_{m1}}\right) \left(1 + \frac{S_2}{K_{m2}}\right) + \frac{S_3}{K_{m3}} + \frac{S_4}{K_{m4}} \left(1 + \frac{S_3}{K_{m3}}\right) \quad (8.10)$$

$$\Delta' = \left(1 + \frac{S_1}{K'_{m1}}\right) \left(1 + \frac{S_2}{K'_{m2}}\right) + \frac{S_3}{K'_{m3}} + \frac{S_4}{K'_{m4}} \left(1 + \frac{S_3}{K'_{m3}}\right)$$

$$\chi = V_f / V_r \quad a = V'_f K_{m1} K_{m2} / V'_r K'_{m1} K'_{m2}$$

Uni-reactant MWC equation with modifier

$$v_{mwc} = \frac{\frac{[A]}{K_a} \left(1 + \frac{[A]}{K_a}\right)^{n-1}}{\left(1 + \frac{[A]}{K_a}\right)^n + L \left(1 + \frac{[I]}{K_i}\right)^n} \quad (8.11)$$

where K_a is the dissociation constant for substrate A, $[I]$ is the concentration of inhibitor, K_i is the dissociation constant for inhibitor I, n = number of subunits and L is the equilibrium ratio of T_n/R_n . For this comparison, it was assumed that the inhibitor binds only to the T-form of a dimeric enzyme with $L = 10$.

9 Bibliography

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