KINETICS: THE GRAMMAR OF ENZYMOLOGY

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There are two different and important reasons why rate measurements on enzyme catalysed reactions have been pursued with ever increasing vigour and sophistication during the last hundred years. Firstly, kinetics are the bases of most tools for the elucidation of chemical mechanisms. Secondly, in biology a rate measurement is often not just a means to an end but an end in itself, answering a specific question. One wants to know how fast a given amount of enzyme can convert a given amount of substrate into product or how rapidly a transient intermediate forms and decays. In more complex processes such as muscle contraction or a protein-mediated transport process, the rates of individual steps describe the macroscopic events of a biological phenomenon. Part of the kinetic description is the identification of the stoichiometric composition of the intermediates. The rates of formation and decomposition of identified intermediates can be correlated with biophysical measurements such as changes in tension, conductivity or chemical potential. In this way a kinetic description of a system must go side by side as an equal partner with the elucidation of its detailed structure.

The development of ideas and techniques for the study of enzyme kinetics has proved important for investigations into the mechanisms of operation of many other functional proteins. Conversely the methods developed for the study of other proteins, haemoglobin for instance, have had applications in the study of enzymes. In this way kinetic analysis has become one of the most important biophysical techniques providing a description of the behaviour of biological systems and processes. The present survey of the development of ideas in enzyme kinetics is intended for biologists in general and not for the expert enzymologist.

From Kühne to Haldane (1876 to 1930)

Segal [1] gives an excellent account of the early history of the development of ideas in the kinetic analysis of chemical catalysis and enzyme reactions. The first reports of kinetic evidence for the occurrence of enzyme–substrate complexes or compounds prior to catalysis were those of Brown [2] and others at the turn of the century. It was found that the rate of the invertase reaction was proportional to substrate only at low sucrose concentrations. It was concluded that substrate binding at high concentration became faster than formation of free product and that enzyme sites were saturated. I have worded this carefully so as not to specify the nature of the complex, since we now know that saturation can occur in the form of an enzyme–substrate, enzyme–product or enzyme–intermediate complex. It showed remarkable judgement on the part of several investigators who, at that time, drew correct conclusions about first-order rates at low substrate and zero-order rate at high substrate, when one considers the methods available and impurity and complexity of the systems studied. One need only mention that, prior to Sörensen's [3] reports in 1909, it was not realised that the rates of enzyme reactions varied considerably with pH and should be studied in buffered solutions.

While it is correct to say that Brown, Henri and others proposed equations to describe the progress of enzyme reactions which were of the form of what is now known as the Michaelis equation, convincing evidence came first from Michaelis and Menten [4]. In their report of a careful re-investigation of the kinetics of invertase action, under rigorously controlled conditions, Michaelis and Menten state that they

extended and put on a firmer basis earlier proposals for the relation:

$$v = \frac{k_3\,(E_o)\,(S)}{K_s + (S)} \qquad (1)$$

between the reaction velocity (v), substrate concentration (S) and enzyme concentration (E_o). This is based on the simple scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P \qquad (2)$$

$$K_s = k_2/k_1$$

The basic assumptions were that the enzyme–substrate complex and the free enzyme and substrate were in equilibrium and that the interconversion of enzyme-bound substrate to product was thought to be rate-limiting for the overall process. Van Slyke [5,6] described a scheme for enzyme reactions in terms of two consecutive steps:

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P \qquad (3)$$

For such a process K_s in equation (1) would be k_2/k_1. To save the inevitable confusion with different numbers as subscripts for the rate constants for the forward and reverse direction of the same step I shall use the nomenclature:

$$\underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \quad \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \quad \text{etc.}$$

for the rest of this article. In honour of Michaelis, equation (1) is now called the Michaelis equation and K_s of this equation is called K_M or the Michaelis constant. The term K_s is reserved for the dissociation constant of the enzyme–substrate complex and its definition in terms of rate constants will be discussed below.

The foundation to modern steady-state kinetics was layed by Briggs and Haldane and this was elaborated by Haldane in 1930 in a book [7] which still today deserves careful study by every serious student of enzyme kinetics. In Haldane's survey of the kinetic investigations on enzymes during the second and third decade of this century, one can see the development of ideas about enzyme–substrate and –inhibitor complexes as well as about the pH-dependence of the reaction rates. Not until 1958 was another book published which gave such a clear view of enzymology at the time of writing [8].

Haldane's treatment of the steady-state approximation depends on the rate equations for the net formation of intermediates

$$\frac{d(ES)}{dt} = k_1(E)\,(S) - (k_{-1} + k_2)\,(ES) \qquad (4)$$

$$= k_1(S)\,\left[(E_o) - (ES)\right] - (k_{-1} + k_2)\,(ES)$$

$$= k_1(S)\,(E_o) - \left[k_1(S) + k_{-1} + k_2\right]\,(ES)$$

$$(ES) = \frac{k_1(S)\,(E_o) - \dfrac{d(ES)}{dt}}{k_1(S) + k_{-1} + k_2} \qquad (5)$$

The steady-state is reached within a fraction of a second after mixing enzyme and substrate (see later for a discussion of the rate of approach to the steady-state) and is defined by

$$k_1(S)\,(E_o) \gg \frac{d(ES)}{dt} \qquad (6)$$

In general the steady-state approximation is valid if the rate of formation of intermediates is much faster than the rate of change in intermediate concentration. For the simple mechanism with one intermediate the velocity $v = d(P)dt = k_2(ES)$ and substituting in equation (5) we get

$$\frac{v}{(E_o)} = \frac{k_2(S)}{(S) + \dfrac{k_{-1} + k_2}{k_1}} \qquad (7)$$

The above treatment can easily be expanded to describe schemes which include enzyme–substrate and –product complexes

$$E + S \rightleftarrows ES \rightleftarrows EP \rightleftarrows E + P \qquad (8)$$

where ES and EP can occur in several forms. However complex equations for reactions with single substrates are, they can always be written in the form of equation (1) as long as all substrate binding sites are identical and there is no interaction between them. These conditions are designated as Michaelis behaviour

and yield hyperbolic saturation curves. As the detail of the description of the mechanism increases, K_M and V_m become functions of more rate constants and K_s can be equal to, smaller than or larger than K_M.

Another contribution by Haldane was his derivation of the relation between the overall equilibrium constant K, between free substrate and product

$$S \longleftrightarrow P \qquad K = \frac{(\bar{P})}{(\bar{S})}$$

where $(\bar{P})$ and $(\bar{S})$ are equilibrium concentrations and the Michaelis parameters. From the steady-state expression for the scheme (8) in the presence of substrate and product one obtains

$$\frac{v}{(E_o)} = \frac{V^s K^p{}_M (S) - V^p K^s{}_M (P)}{K^s{}_M K^p{}_M + K^p{}_M (S) + K^s{}_M (P)} \qquad (9)$$

where $K^s{}_M$, V^s and $K^p{}_M$, V^p are the Michaelis constant and maximum velocity for the forward reaction in the absence of substrate respectively. At equilibrium $v = o$ and the enumerator of equation (9) is zero. Hence,

$$V^s K^p{}_M (\bar{S}) = V^p K^s{}_M (\bar{P})$$

and it follows that,

$$\frac{(\bar{P})}{(\bar{S})} = \frac{V^s K^p{}_M}{V^p K^s{}_M} = \frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}} = K \qquad (10)$$

Expression (10) has recently received attention since it can be used to relate the substrate and product dissociation constants to the difference between the overall equilibrium and the equilibrium of the chemical reaction of the enzyme-bound substrate and product. If the substrate binds much more tightly than the product,

$$\text{i.e. } \frac{k_{-1}}{k_1} > \frac{k_3}{k_{-3}}$$

then the equilibrium k_2/k_{-2} of the 'on enzyme' chemical reaction can be near unity even if the overall reaction is significantly in favour of product formation. The direct techniques of transient kinetics discussed below, permit evaluation of the individual equilibria and with that the energetic contribution of binding to the catalytic process [9].

From steady-states to transients

After Haldane put steady-state kinetics on a sound basis, only limited attention was paid to this field until the explosive developments in the 1950s of ever more complex derivations of rate equation for multi-substrate enzymes. The various useful methods of obtaining linear representations of the dependence of reaction velocity on substrate and inhibitor concentrations were described during that period [8]. Specificity rather than detailed reaction mechanisms were the principal topics of enzyme research between Haldane and the early 1950s. Of other notable events in the field one should mention the identification of covalent enzyme–substrate intermediates (for a review see [10]). This had consequences for the development of kinetic investigation.

During the late '30s and early '40s Chance obtained the first exciting results from kinetic studies of enzyme–substrate complex formation. Although these studies of the formation and decomposition of intermediates during the reactions of peroxidase and catalase were special cases, they provided much inspiration for the general application of the rapid reaction techniques discussed below. Chance's experiments showed, for the first time, the time course of enzyme–substrate combination (on a millisecond time scale) as well as the steady-state concentrations of enzyme intermediates and their decay as the supply of substrate runs out.

Research on nerve gases and insecticides paved the way for many fundamental investigations into the identification of active sites on enzymes and the availability of stable and radioactive isotopes provided the tools for investigations into the chemical pathways during enzyme–substrate reactions. The earlier views on enzyme catalysis tended to be centred on purely physical rather than covalent interactions. While non-covalent interactions are of great interest and importance in specificity and conformational re-arrangements, by 1955 it was established that covalent compounds of enzymes with their substrates and products occurred in addition to the physical complexes which continue to be studied in kinetic investigations. The study of the ionic and chemical properties of the amino acid side chains of enzyme proteins revealed that these

were very different from those of the free amino acids in solution. The following problems presented themselves to those interested in the role of the protein molecule during any one enzymic reaction. How many distinct intermediates occur during the reaction between enzyme and substrate? What is the composition of these intermediates? Which amino acid side chains of the protein are involved in each of these steps? What are the functions and reactivities of the amino acid side chains in each step?

During the last twenty years a variety of different kinetic methods were developed which helped to answer the above questions. These techniques can be divided into two types. First those carried out with catalytic amounts of enzyme (substrate concentrations $\gg$ enzyme concentrations). They involve the measurements of initial rates of product formation or isotope exchange rates between substrates and products. Velocity measurements are carried out over a wide range of substrate and product concentration and the results are examined for compatability with rate equations derived for different mechanisms. A brief inspection of two recent books [11] and [12] will show that the diversity and complexity of the equations has led many authors into a wilderness in which new names for mechanisms take over from physical reality. This is a pity since, in spite of its limitations, steady-state methods can provide much useful information. For one thing they provide essential data for the interpretation of metabolic processes, for another they have proved capable of detailed elucidation of physical events when practiced in proper perspective. Dalziel's [13] investigations of horse liver alcohol dehydrogenase and Schwert's [14] studies of heart lactate dehydrogenase show what can be achieved. Knowles [15] gives other examples. In many of the other investigations the authors would have produced more interesting results if they had directed their attention towards metabolic rather than mechanistic questions.

Another approach to enzyme kinetics involves the study of enzymes at concentrations comparable with those of substrates and sufficiently high to detect and study the characteristic properties of intermediates. This approach was inspired by the work of Chance [16] referred to above. He used his technical ingenuity to devise rapid mixing spectrophotometers, which were sufficiently economical in the use of reagents for the study of enzymes. Chance directed his attention to the study of enzymes with chromophoric prosthetic groups. In collaboration with Theorell he also carried out the first transient kinetic experiments on NAD-linked dehydrogenases [17].

The essence of transient kinetic investigations is that during the first few milliseconds after enzyme and substrate are mixed, one can follow the formation and interconversion of intermediates before they reach their steady-state concentrations. In some cases, when an enzyme concentration higher than the Michaelis constant can be attained, one can follow the formation and decomposition of intermediates during a single turnover by mixing enzyme in excess of substrate. Improved and more easily available electronic equipment, together with the simple design of Gibson's [18] stopped-flow apparatus, encouraged the wider development of the transient kinetic approach. The desire to study a wide range of enzymes without chromophoric prosthetic groups led to the development of new approaches using changes in optical properties of the protein and/or substrate (absorption and fluorescence).

Two complementary types of kinetic analyses can be carried out to identify the number of intermediates and evaluate the rate constants of interconversion during transients.

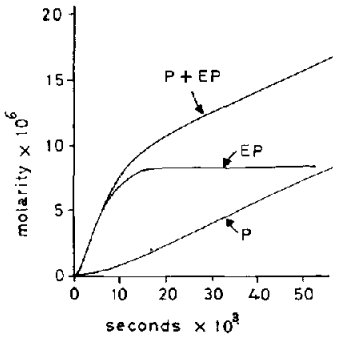

Fig.1. Computer simulation of transient and steady state formation of product (free and enzyme-bound) during the reaction $E + S \rightleftharpoons ES \rightarrow EP \rightarrow E + P$. $K_s = 1\ \mu M$, $k_1 = 2 \times 10^7\ M^{-1}\ sec^{-1}$, $k_2 = 200\ sec^{-1}$, $k_3 = 20\ sec^{-1}$ and the initial concentrations of enzyme and substrate are 10 μM and 25 μM respectively.

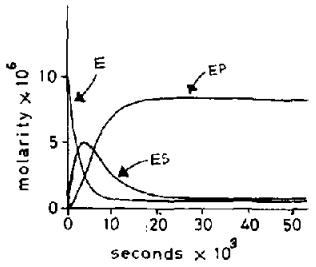

Fig.2. Computer simulation of the interconversion of enzyme to enzyme–substrate and enzyme–product complexes during the approach to the steady state of the reaction $E + S \rightleftharpoons ES \rightarrow EP \rightarrow E + P$. $K_S = 1\ \mu M$, $k_1 = 2 \times 10^7\ M^{-1}\ sec^{-1}$, $k_2 = 200\ sec^{-1}$, $k_3 = 20\ sec^{-1}$ and the initial concentrations of enzyme and substrate are 10 μM and 25 μM respectively.

The observation of the transient formation of product is illustrated (fig.1) in a simulation of the time course of the formation of product (enzyme-bound and free) during the transient (pre-steady-state) and steady-state phase of an enzyme reaction. Algebraic analysis and practical application of this approach was first used for the study of proteolytic enzymes [19] and subsequently adapted for other systems [20].

The observation and analysis of transients of intermediates during approach to the steady state (fig.2) or during a single turnover (fig.3) depends on a change of optical characteristics (due to the protein, substrate or their interaction) during interconversion.

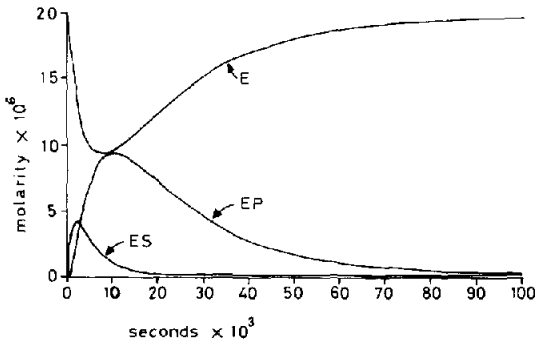

Fig.3. Computer simulation of the interconversion of enzyme to enzyme–substrate and enzyme–product complexes during a single turnover of the reaction $E + S \rightleftharpoons ES \rightarrow EP \rightarrow E + P$. $K_S = 1\ \mu M$, $k_1 = 2 \times 10^7\ M^{-1}\ sec^{-1}$, $k_2 = 500\ sec^{-1}$, $k_3 = 50\ sec^{-1}$ and the initial concentrations of enzyme and substrate are 15 μM and 10 μM respectively.

This method is the only one which can give direct evidence for the occurrence of an intermediate, with its particular characteristics and composition, on the kinetic pathway.

Transient-kinetic techniques have also given a new dimension to the interpretation of the pH-dependence of enzyme reactions. Dissociation constants determined from the pH-dependence of overall rates are of doubtful significance, while those obtained from the characteristics of indiviual steps can give useful information about mechanisms, if treated with caution.

In the ideal case transient-kinetic and steady-state investigations complement each other. A good example is described by Dalziel [13] who summarised his steady-state investigations together with the transient-kinetic studies of Shore and Gutfreund on liver alcohol dehydrogenase.

When one considers what information one can get about mechanisms one is asking an open-ended question, since the word mechanism with reference to enzymes, means different things to different people. A more fruitful way to go about the study of such a complex system is to ask specific questions. As stated above, kinetic investigations are uniquely suited to answering the question: how many intermediates occur at significant concentrations during the course of the reaction and what are their compositions? As part of the information about the composition of intermediates one learns about the uptake and release of protons and with that about pK changes during the interconversion of intermediates [21,22]. Chemical and structural information is required for the identification of the amino acid residues responsible for changes in ionisation. Kinetics must be supplemented by a wide range of monitors to answer questions about details of conformation changes during transformations. Here again it is important to use kinetics as an equal partner with structural investigations. For example if structural studies show that substrate binding causes a change which takes a fluorescent group into a different environment, the time course of the transient fluorescence change will answer the question: during which step does the conformation change occur? The studies on alcohol dehydrogenase [23] and on lysozyme [24] clearly complement the crystallographic investigations.

Spectroscopic techniques and especially

magnetic resonance, provide both kinetic and structural information. This is especially useful when crystallographic information is hard to come by. Recent studies of ^{31}P n.m.r. of the intermediates during the reactions of *E. coli* alkaline phosphatase have settled two long disputed questions about the mechanism of this enzyme [25]. First the equivalence of phosphate binding has eliminated the hypothesis of half-of-sites reactivity. This potentially interesting phenomenon, which may well be significant in some functional proteins, has frequently been claimed to occur in enzymes as a result of misinterpretations of experimental data [20].

The second interesting finding from ^{31}P n.m.r. studies on phosphatase is that they confirmed [26] that the decomposition of the phosphoryl-enzyme intermediate to enzyme and phosphate is a two step process and showed that the rate limiting step is the dissociation of non-covalently bound phosphate, which occurs after the hydrolysis of the phosphoryl-enzyme. It is worth mentioning that slow product release or conformational re-arrangement on substrate binding, rather than the chemical step, are often rate limiting. These specific non-covalent processes appear to control enzyme action and they are being studied by relaxation kinetics.

Relaxation and ligand binding in catalysis and control

In the 1960s the subject of substrate and effector specificity developed in a number of new directions. The 'lock and key' hypothesis of enzyme specificity proposed by Emil Fischer [27] pictured rigid molecules with geometric complementary. Not withstanding lively discussions about the difference of the structure of protein molecules in the crystal and in solution, there is now general agreement that the interactions between proteins and ligands result in considerable conformation changes. The sequence of events during the complex processes of binding and conformation change of protein and/or substrate has been subject to many kinetic investigations. The induced fit hypothesis of Koshland [28] is behind present ideas which postulate that the correct reactive configuration of the catalytic site of enzymes is dependent upon the presence of bound substrate.

Kinetic investigations of reactions of proteins with ligands had applications to the elucidation of many other phenomena in addition to enzyme catalysis. The feed back inhibition of oligomeric enzymes through cooperative structure changes, reactions of muscle proteins, transport ATPases and neuroreceptor proteins are only a few of the many phenomena to which similar thinking has been applied. Enzyme systems have proved particularly suitable for the development of techniques for the study of the physico-chemical changes in proteins, since the catalytic reaction provides an additional monitor.

The relaxation techniques developed by Eigen and deMaeyer [29] complemented the flow techniques for the observations of rapid reactions. The flow techniques are the methods of choice for reactions which go essentially to completion and have elementary steps with halftimes not shorter than a millisecond. The relaxation techniques, which involve the perturbation of a system at equilibrium have a time resolution of about a microsecond. They prove most suitable for the analysis of two step mechanisms (second-order ligand binding followed by a first order re-arrangement) [30,31]. At this stage it is still easy to generalise because the amount of detailed information about substrate binding is very small. There are about as many reviews as original papers.

Apart from their time resolution relaxation techniques provide additional information about protein–protein and protein–ligand interaction and associated conformation changes. When a range of perturbation methods (temperature, pressure or electric field jump) is applied the physical characteristics of these non-covalent phenomena can be effectively analysed. Applications of these approaches to the study of protein–protein and protein–ligand interaction, as well as of cooperative transitions which control many biological phenomena, is leading to a greater understanding of information transfer in macromolecular complexes.

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