Biophysical Chemistry 26 (1987) 117–121
Elsevier

BPC 01132

Reflections on the kinetics of substrate binding

H. Gutfreund

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

Accepted 27 February 1987

Collision complex formation; Conformation change; Enzyme kinetics

The principle questions which are still being asked about enzyme-substrate complex formation are examined. Data for the maximum rates of collision complex formation are reviewed. The limitations of past experimental procedures and the potentialities of new approaches are discussed together with methods that allow the distinction between collision complexes and subsequent events.

1. Introduction

Although this is intended to be a tribute to Manfred Eigen through the presentation of original papers, as one of the oldest contributors I claim the privilege to be allowed to present some problems with an historical perspective. It so happened that we first met at the Faraday Society Discussion on Rapid Reactions in Birmingham in April 1954. At that meeting we both presented new approaches to kinetic problems. Eigen's chemical relaxation technique had, of course, much wider and more fundamental applications than my ideas on the direct determination of individual rate constants of enzyme reactions through the observation of transients in product formation. It could, however, be claimed that after that meeting the use of both relaxation techniques and transient kinetic methods received much wider attention. The latter approach rested, of course, on the technical achievements of Hartridge, Roughton, Chance and Gibson [1].

Further applications of relaxation as well as transient kinetic studies to many enzyme systems had two related aims. These were the elucidation of the kinetics and mechanisms of substrate recognition and of the chemical mechanism of catalysis. One justification for the separation of the two phenomena, which are likely to be intimately connected in enzyme function, is the ubiquitous role of substrate recognition in every biological phenomenon, not just in enzymology. A principal feature of immune response, the transmission of signals in nerve and through other cell membranes, the control of transcription and translation, to name just a few examples apart from the control of enzyme action, is the recognition of a substrate by a specific binding site. The rates and mechanisms of these recognition processes are ill defined because they are often difficult to distinguish from the response to the recognition. The claim that many of these processes are diffusion controlled would, on first impression, make them uninteresting. I should like to suggest that one should not only ask whether substrate binding is diffusion controlled, but also formulate the question: what happens during the diffusion-controlled formation of the collision complex?

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

Correspondence address: H. Gutfreund, Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.
Present address (until 30 June 1987): Fogarty International Center, National Institutes of Health, Building 16, Room 309, Bethesda, MD 20892, U.S.A.

Since Koshland's [2] suggestion of 'induced fit' has been more extensively defined in kinetic and structure studies, it is now generally accepted that, in substrate binding, the formation of a collision complex is followed by conformation changes, which are a specific response. Thus, in many cases the observed binding step is the conformation change, which is preceded by a rapid equilibrium of collision complex formation. It is the kinetics and mechanism of the formation of the collision complex and its significance in the recognition process which require better definition than has so far been achieved.

It is important to make a distinction between substrate-binding steps which are diffusion controlled in absolute terms and processes in which the overall sequence of reactions is controlled by the rate of diffusion of the substrate at its prevailing concentration. I shall return to this point when discussing conclusions about rates of binding from steady-state rates of enzyme reactions.

2. The fastest interactions observed

A frequently quoted dictum of those engaged in the field of kinetic analysis is that whenever either the time or signal resolution for a particular reaction is improved, a new step is discovered. A similar situation arises in the discovery of faster binding steps. New methods are developed and new reactions with faster steps can be resolved. For a long time enzymologists used to be satisfied with the following argument. If the diffusion constants for substrates and protein molecules are substituted into the Smoluchowski [3] equation for collision frequencies, one obtains a value of about 5×10^9 M^{-1} s^{-1}. The surface of a protein molecule is about 100-times the area of the specific binding site; therefore, rate constants for binding steps in the region of 5×10^7 M^{-1} s^{-1} can be considered to apply to diffusion-controlled collision complex formation. The experimental results summarized in a review [4] are still often used in the biochemical and physiological literature to support this. However, Riggs and co-workers [5] determined the association rate constant for protein binding to specific sites on DNA to be up to 10^{10} M^{-1} s^{-1}. Since that time a large number of papers appeared which considered special mechanisms of attractive charge interaction, the reduction in dimensionality, surface diffusion and the rotational diffusion of the protein (see, for instance, ref. 6). It is not my purpose to add to these theoretical discussions, but I shall summarize some experimental results to illustrate the methods which might be used to obtain more information about collision complexes. I shall omit the extensive literature on protein-DNA interaction (see the paper by Von Hippel et al. [7] for a detailed discussion of this area) and restrict myself to rates of interaction of small substrates at specific sites on protein molecules.

An interesting comparison is achieved if one lists the observed rate constants for the interaction of molecular oxygen with the haem pocket of different proteins:

oxygen binding to myoglobin 1.4×10^7 M^{-1} s^{-1}

oxygen binding to haemoglobin from symbiotic organisms 1.5×10^8 M^{-1} s^{-1} [8]

quenching of porphyrin fluorescence of des-Fe-myoglobin by oxygen 4.0×10^8 M^{-1} s^{-1}

quenching of fluorescence of free porphyrin by oxygen 1.0×10^9 M^{-1} s^{-1}

References to the investigations on porphyrin fluorescence quenching are given by Jameson et al. [9] in a paper describing the methods used. Oxygen is of course a small substrate and interaction with a prosthetic group on a protein is a special case, but then in biology nearly everything is a special case! Jameson et al. [9] suggested yet another mechanism, the acceleration of binding to a specific site by diffusion through the protein molecule. This is connected with the prevailing idea that the mobility of protein structures can give rise to very rapid expansion and contraction of spaces within the molecule (volume fluctuations, breathing). In connection with later discussions it is of interest to note that a 4-fold increase in viscosity had no significant effect on the above rates.

Stopped-flow and relaxation experiments on nucleotide (NADH) binding to various dehydrogenases gave second-order rate constants up to 10^8 M^{-1} s^{-1} (for instance, ref. 10). This is similar to the fastest rates observed for oxygen binding to intact haem-proteins, as illustrated above. More rapid binding to the active sites of proteins has only been observed in systems where charge interaction could be demonstrated. The temperature jump studies, carried out by Neumann's group (see ref. 11) on the rate of interaction between fluorescent analogues and acetylcholinesterase gave rate constants of up to 10^{10} M^{-1} s^{-1} as the ionic strength was reduced to zero. Unfortunately, there is no systematic study in the literature of the ionic strength dependence of NADH binding to a dehydrogenase.

3. Indirect information about collision complex formation

As mentioned above, substrate binding to specific sites is always associated with a subsequent conformation change. This means that in enzymes the steps preceding catalysis are regarded as the binding step. The complexity of this overall binding step depends to a large extent on the resolution of the kinetic technique, with respect to both time and signal. The question which arises is whether one can resolve and characterize the 'productive collision complex' and estimate its rate of formation. In the simplest case of a two-step reaction

$$P + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P\text{–}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PS$$

representing the binding of a substrate to the functional site of a protein, the following argument is frequently applied when, as is usual, only the rate of appearance of PS can be monitored. The observed rate constant for the formation of PS is given by

$$k_{obs} = k_{-2} + k_2 \cdot \frac{1}{1 + 1/K[S]} \qquad (1)$$

where $K = k_1/k_{-1}$. The initial slope of the plot of k_{obs} against [S] is $k_2 K$ and although the different values are obtained from exponential records, as long as $[S] \gg [P]$, k_{obs} has the dimensions of a second-order rate constant. This is analogous to the common use in enzymology of k_{cat}/K_m, which corresponds to the initial slope of the plot of velocity against substrate concentration in an enzyme reaction. In eq. 1 K is in the form of an association constant and K_m has the dimensions of a dissociation constant. Eq. 1 is only valid if the first step equilibrates rapidly compared with the second. If this does not hold, then the steady-state approximation has to be applied:

$$d[PS]/dt = [k_1 k_2/(k_{-1} + k_2)][P][S]$$

$$k_{obs} = k_1 k_2/(k_{-1} + k_2)$$

Since the terminology of enzyme kinetics has permeated aspects of biophysics which are generally much more sophisticated in kinetic analysis (see, for instance, the treatment of the kinetics of channel opening by Hille on p. 203 of ref. 12), I shall discuss this problem in relation to substrate binding to enzymes. Reference to a text on enzyme kinetics (see, for instance, p. 134 of ref. 13) will show that k_{cat}/K_m (k_{cat} corresponds to maximum velocity at substrate saturation per mole of active sites) is necessarily smaller than k_1. This has to be taken into account when experimental results from steady-state experiments are used to draw conclusions about the formation of the collision complex.

4. The method dependence of past and future progress

It is not easy to define what is meant by 'the first step' in enzyme-substrate complex formation. A reasonable definition is that it corresponds to the productive collision complex. This would be a diffusion-controlled collision in the correct orientation which is succeeded by a specific response from the binding site. As pointed out before, the difficulty is to ascertain whether one is observing the first step, i.e., k_1, or the product of k_1 with several rate constants, which is obtained from the initial slope of the plot of rate vs. substrate con-

centration. This has, in many cases led to the circular argument that if the observed rate constant is as high as 10^8 M^{-1} s^{-1} then it is a diffusion-controlled step and this must be the formation of the first complex. It is my contention that this problem will not be solved by the large number of theoretical papers alone; these may or may not help in the design or interpretation of the experiments necessary to characterize the productive collision complex. In this concluding section of my survey I shall concern myself with a brief discussion of the kind of experiments which may throw further light on this problem.

One can divide experimental approaches between the extensive photochemical studies on haem-proteins (see, for instance, refs. 14 and 15), which have given valuable information on the internal motions of protein molecules, and studies of reactions in which a substrate binds directly to a site constructed from amino acid residues. I shall concern myself here with the latter problem. The first attempts to determine directly k_1 values for proteins without prosthetic groups were described by Gutfreund [16]. The use of the stopped-flow method for the observation of the rate of approach to the steady state gave only minimum values of $k_1 = 10^7$ M^{-1} s^{-1}. Even at that time it was already concluded that one might be looking at the rate of transformation of the collision complex to a reactive enzyme-substrate complex rather than at the initial complex formation. Relaxation experiments, in Eigen's laboratory, in the mid 60's, began to give results on nucleotide binding to enzymes. The two approaches had different limitations: the flow method on time resolution and the relaxation method on resolving the small signals depending on the equilibrium conditions. While the techniques have certainly improved over the last 20 years, remarkably little information has been obtained about diffusion-controlled collision complexes, although both approaches have taught us a great deal about elementary steps in the reactions of enzymes and other functional proteins (see, for instance, the review by Fersht [17]).

Various techniques depending on the intrinsic fluorescence of protein molecules or on the combination of fluorescence observation with relaxation or flow methods promise the time and signal resolution for more insight into binding phenomena. For instance, the quenching of tryptophan fluorescence by energy transfer to a substrate is a very good monitor for rate processes and the signal is very sensitive to the distance between donor and acceptor. It seems evident that further development of this technique could resolve the time course of the formation of the collision complex from subsequent more intimate attachment of the substrate. This approach, combined with placing tryptophans in the structure by 'protein engineering', could be a powerful tool for such investigations [18]. The extension to and combination with the methods developed by the Urbana group [9] might give information about the movement within the protein molecules of substrates larger than oxygen.

A number of investigations are described in the literature, which were designed to test whether an observed rate constant characterizes a diffusion-controlled step. The rate of translational diffusion is inversely proportional to solvent viscosity and has a characteristically low energy of activation. The effects of viscosity on substrate binding and the associated conformation changes have received particular attention in recent years. Although there is a need for considerably more systematic studies, it is clear that the observations of the perturbations caused by changes in viscosity will add to the information obtained from the range of physical techniques discussed above.

Knowles and his colleagues (see, for instance, ref. 19) have been interested in the consequences of diffusion control for the evolution of perfection in enzyme catalysis. This argument has also been used in discussions of substrate interaction with ionic channels (p. 203 of ref. 12). Albery and Knowles [19] suggest that the perfect enzyme has, at the prevailing substrate concentration, a diffusion-controlled binding step limiting the overall reaction rate and can, therefore, not be improved.

A recent paper by Raines et al. [20] is a paradigm for an investigation into the control of the substrate-binding step of an enzyme reaction. Both the experimental and theoretical pitfalls are well documented in this study of the viscosity dependence of k_{cat}/K_m of the reaction catalysed by

triosephosphate isomerase. They obtain a value of 4×10^8 M^{-1} s^{-1} in aqueous solutions, which is directly proportional to the reciprocal of the viscosity as ethylene glycol is added. Suitable controls were carried out to eliminate other effects of changes in solvent composition. A nice control was presented by their study of a mutant of this enzyme which has a k_{cat} decreased by a factor of 1000 and k_{cat}/K_m independent of viscosity.

Raines et al. [20] point out that one has to distinguish between microviscosity changes, such as those occurring on addition of small molecules, and macroviscosity changes when, for instance, poly(ethylene glycol) is added. It was shown by Graham [21] that the diffusion of ions through gelatine was not significantly retarded.

It must also be noted that not only the translational diffusion of the substrate is slowed down by an increase in viscosity. Structure fluctuations responsible for the stepwise formation of the major substrate-induced conformation changes are also likely to be viscosity dependent (see refs. 22 and 23). The relation between these pico- to nanosecond local fluctuations and the micro- to millisecond major structure changes, observed during the formation of reactive enzyme-substrate complexes, are another interesting subject. An important question is to establish the role of the formation of the productive collision complex in these subsequent events. A major problem is that the concentrations of collision complexes are only likely to be of observable magnitude when the magnitude of the substrate concentration results in a very small relaxation time for its formation.

I am fully aware of the fact that I have only asked questions and tried to document them. I hope I have formulated the questions in a way which helps biochemists and molecular physiologists to think about them more clearly in the future.

References

1 B. Chance and F.J.W. Roughton (1963) in: Techniques in organic chemistry, eds. S.L. Friess, E.S. Lewis and A. Weissberger (Wiley-Interscience, New York, 1963) vol. VIII, ch. XIII.
2 D.E. Koshland, J. Cell. Comp. Physiol. 47, Suppl. 1 (1956) 217.
3 M. Von Smoluchowski, Phys. Z. 17 (1916) 557.
4 H. Gutfreund, Annu. Rev. Biochem. 40 (1971) 315.
5 A.D. Riggs, S. Bourgeois and M. Cohn, J. Mol. Biol. 53 (1970) 40.
6 R.D. Astumian and P.B. Chock, J. Phys. Chem. 89 (1985) 3477.
7 P.H. von Hippel, D.G. Bear, W.D. Morgan and J.A. McSwiggen, Annu. Rev. Biochem. 53 (1984) 389.
8 J.B. Wittenberg, B.A. Wittenberg, Q.H. Gibson, M. Trinick, A.I. Fleming and C.A. Appleby, Biophys. J. 49 (1986) 242.
9 D.M. Jameson, E. Gratton, G. Weber and B. Alpert, Biophys. J. 45 (1984) 795.
10 J.J. Holbrook, M.J. Hardman, D.M. Parker, D.W. Yates and H. Gutfreund, FEBS Lett. 78 (1977) 46.
11 H.-J. Nolte, T.L. Rosenberry and E. Neumann, Biochemistry, 19 (1980) 3705.
12 B. Hille, Ionic channels and excitable membranes (Sinauer Associates, Sunderland, MA, 1985).
13 H. Gutfreund, Enzymes: physical principles (Wiley & Son, London, 1975).
14 R.H. Austin, K.W. Benson, L. Eisenstein, H. Frauenfelder and I.C. Gunsalus, Biochemistry 14 (1975) 5355.
15 E.R. Henry, J.H. Sommer, J. Hofrichter and W.A. Eaton, J. Mol. Biol. 116 (1983) 443.
16 H. Gutfreund, Disc. Faraday Soc. 27 (1955) 229.
17 A.R. Fersht, Enzymes, structure and mechanisms, 2nd edn. (W.H. Freeman, New York, 1985).
18 A.R. Clarke, D.B. Wigley, W.N. Chia, D. Bastow, T. Atkinson and J.J. Holbrook, Nature 324 (1986) 699.
19 W.J. Albery and J.R. Knowles, Biochemistry 15 (1976) 5631.
20 R.T. Raines, S.C. Blacklow, W.A. Lim and J.R. Knowles, submitted for publication.
21 T. Graham, Ann. Chem. 121 (1862) 1.
22 W. Doster, Biophys. Chem. 17 (1983) 97.
23 M. Karplus and J.A. McCammon, Annu. Rev. Biochem. 53 (1984) 263.