Review Letter

RECENT ADVANCES IN THE STUDY OF BIOLOGICAL SYSTEMS WITH RAPID REACTION TECHNIQUES

K.KIRSCHNER

Max-Planck-Institut für physikalische Chemie, 3400 Göttingen, Germany

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1. Introduction

Ever since the advent of rapid mixing and chemical relaxation techniques (46 and 15 years ago, respectively) information on the dynamic function of enzymes and nucleic acids in terms of elementary steps has been increasing steadily. Currently, there seems to be a transition in the growth of the number of papers dealing with the application of rapid reaction techniques from the lag to the logarithmic phase. This development is due to recent advances on several lines. Firstly, various types of equipment for the study of fast reactions is now available commercially. Secondly, several systems of more than common interest (e.g. repressors, DNA- and RNA-polymerase, allosteric enzymes, etc.) can now be obtained in a pure and stable form and in sufficient quantity. Thus the situation of the rare investigator possessing the necessary equipment and in search for suitable objects of study has changed. Many important biochemical problems have developed to a stage where kinetic studies will provide new and valuable information on the mechanism of action and can be readily performed. Thirdly, the number of proteins whose structure (as a result of X-ray crystallography and other methods) is highly resolved is also rapidly increasing. Knowledge of structure provides the complementary information necessary for a complete interpretation of kinetic data. Thus the kinetic investigation of such systems is particularly attractive.

A number of excellent and comprehensive reviews cover the principles and the practical aspects of the various techniques for the study of fast reactions (e.g. Eigen, Quart. Revs. Biophys. 1 (1968) 1; Roughton and Chance, Techniques in org. Chem. 8 (1963) 703). In view of the forthcoming appearance of a new volume of Methods in Enzymology dealing with the recent technical refinement of the various methods, this aspect is purposely neglected in the following survey. The results which have been obtained by the use of rapid mixing and chemical relaxation techniques have also been reviewed extensively (e.g. Hammes, Ann. Revs. Biochem. 37 (1968) 1; and Gibson, Ann. Revs. Biochem. 35 (1966) 435), covering the literature to about 1966. Rather than attempting to survey comprehensively the large number of significant papers which have appeared since, the following examples have been selected to illustrate the different basic problems which are being investigated (with encouraging success) with the aid of rapid reaction techniques.

2. Ligand binding to and conformational changes of enzymes

The kinetic investigation of the binding of ligands to enzymes is currently rather popular for several reasons. First of all, the reversible binding of a ligand (e.g. substrate, inhibitor or effector) represents the simplest aspect of enzyme function since no covalent changes occur and only a restricted number of intermediates need to be considered. Furthermore, beyond measuring absolute rates, an attempt is made to answer the following interesting questions: What is the dynamic basis of specificity, i.e. of the non-covalent interactions between a small molecule and its complementary binding site? What is the functional role of conformational changes (induced or stabilized by the

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bound ligand) in bringing about specific binding and catalytic activity? Finally, how are indirect interactions between distinct binding sites for substrates and effectors mediated by the protein structure and what inferences can one draw about the regulation of enzyme specificity and catalytic activity?

The binding of *N*-acetylglucosamine oligomers (inhibitors) to lysozyme has been investigated with the temperature-jump method (Chipman and Schimmel, J. Biol. Chem. 243 (1968) 3771). Only a single second order process has been observed. Moreover, the increase of affinity of lysozyme for oligomers with increasing chain length is almost entirely due to the increase of dynamic stability (i.e., the half life) of the corresponding complexes. The absence of additional steps related to the conformational change in the active site cleft of the enzyme is possibly due to the small degree of this effect (as established by difference Fourier synthesis).

The binding of proflavin (a competitive inhibitor) to chymotrypsin clearly involves two distinct elementary steps. The results of temperature-jump studies (Havsteen, J. Biol. Chem. 242 (1967) 769) can be interpreted in terms of a rapid binding step coupled to a slower isomerization of the complex. This direct demonstration of an independent first order step is in qualitative agreement with indirect evidence on the obligatory participation of an isomerization in the overall catalytic process (Bernhard et al., J. Mol. Biol. 18 (1966) 405). Disregarding the real differences between the true substrates of chymotrypsin and proflavin, this type of kinetic experiment can lead to a detailed description of the *mechanism* of "fit induction" as a complementary supplement to the more structural concept of "induced fit".

The dangers of interpreting complicated progress curves on the basis of slow conformational changes are illustrated by the finding that liver alcohol dehydrogenase binds NADH (in solution) in a one-step second order process after all (Geraci and Gibson, J. Biol. Chem. 242 (1967) 4275). An interesting variation of the theme of rapid mixing is the study of binding of ligands to enzymes in the crystalline state (Chance, Theorell et al., J. Mol. Biol. 17 (1967) 513, 525). The rate of binding of NADH to liver alcohol dehydrogenase crystals, though not limited by diffusion, is slowed down by a factor of 10^3 compared to the reaction in solution. A tempting (though not unique) hypothesis suggests that the crystal lattice imposes constraints on the conformational mobility of the enzyme. Thus the question at least of *rapid* conformational changes coupled to the binding of NADH to the enzyme is still open.

Discrete conformational changes of the enzyme are the basis of almost every mechanism proposed for the topical phenomenon of cooperative binding ("allosteric effects", see the recent review of Koshland and Neet, Ann. Rev. Biochem. 37 (1968) 359). The majority of papers on regulatory enzymes involved in feedback control deal with steady-state kinetics and, far more rarely, with direct binding studies. These two lines of approach are valuable in establishing the phenomenon of cooperative interactions and in drawing certain limits to the reaction rates and possible mechanisms involved. The effect of allosteric effectors together with specific tests occasionally go a long way in delineating the underlying mechanism (e.g. *E. coli* phosphofructokinase, Blaugy and Buc, J. Mol. Biol. 31 (1968) 13). However, it is gradually being realised that kinetic studies at high (i.e. stoichiometric) enzyme concentrations are far better suited to distinguish between the various possible mechanisms. Paradoxically, it seems as if the very lack of pertinent informational content contributed by kinetics at catalytic enzyme concentrations has encouraged a sometimes rather fruitless controversy between the supporters of sequential versus concerted conformational changes. It must be admitted that steady-state kinetics are often dictated by the scarcity of material. However, an increasing number of laboratories are now turning to rapid reaction techniques as tools for the investigation of cooperative effects. The regulatory enzymes currently under study are, among others, phosphorylase b, aspartokinase-homoserine dehydrogenase, pyruvatekinase, phosphofructokinase and aspartate transcarbamylase. This development bears witness of a healthy trend away from *a priori* dogmaticism and towards more rigorous experimental evidence. The following examples serve to illustrate this statement.

From the beginning, there was no choice but to use rapid mixing as a method for the kinetic investigation of ligand binding to hemoglobin. Surprisingly enough, although the situation with hemoglobin is almost ideal in that the structures of both oxygenated and de-oxy

hemoglobin are known in ever increasing detail, the exact mechanism of the heme-heme interaction is still largely unknown. It would be out of place here to give an account of the wealthy recent literature on the rates and equilibria involving hemoglobin-ligand interactions. Two recent papers reporting the results of rapid reaction studies illustrate the current status of the problem.

With an ingenious combination of rapid mixing and flash photolysis Gibson and Parkhurst (J. Biol. Chem. 243 (1968) 5521) have measured the appearance of "rapidly reacting" hemoglobin as a function of time and saturation with carbon monoxide. The results seem to indicate that the functional unit of hemoglobin is a tetramer and that a second-order step is rate-limiting.

Interestingly, current opinion favours the opposite (i.e., the functional dimer) hypothesis (Antonini et al., J. Biol. Chem. 243 (1968) 1816). The conformational change known to occur when hemoglobin binds ligands cooperatively (Perutz et al., Nature 219 (1968) 131) is thought to be rapid (Berger et al., J. Biol. Chem. 242 (1967) 4841) but it is not yet possible to accommodate this step into the framework of a specific mechanism.

Difficulties are also encountered in the interpretation of the kinetics of oxygen binding to hemoglobin as investigated with temperature-jump and electrical field-jump methods (Schuster and Ilgenfritz, 11. Nobel Symposium (1968)). Three distinct relaxation processes have been discerned. The slowest step is clearly related to the dissociation of the hemoglobin tetramer to dimers, whereas the other two reflect the binding of oxygen to two different states of the (heme) binding site. Taking into account recent EPR and NMR measurements on various kinds of labelled hemoglobin, the odds are in favour of a sequential type of mechanism.

The situation with yeast glyceraldehyde-3-phosphate dehydrogenase is more clear. Temperature-jump studies on the cooperative binding of NAD to this enzyme (Kirschner et al., Proc. Nat. Acad. Sci. 56 (1966) 1661) rule out the sequential mechanism and are in complete accord with the predictions of the concerted model. The slowest of the three distinct relaxation processes can be unequivocally assigned to an all-or-none isomerization of the tetrameric enzyme, coupled to two fast binding steps. Stopped-flow studies (Kirschner, Regulation of Enzyme Activity (1968) 39) and independent measurements of hydrodynamic and optical rotation parameters (Jaenicke and Gratzer, Biochem. (1969) in press) are in quantitative agreement with the concerted model, in showing that the degree of structural change precedes the degree of saturation. On the other hand, the interesting stopped-flow experiments of Chance and Park (J. Biol. Chem. 242 (1967) 5093) show that the situation becomes more complicated when the interaction of the enzyme-NAD complex with substrates is investigated.

It is interesting that rabbit muscle glyceraldehyde-3-phosphate dehydrogenase shows the opposite behaviour at equilibrium, i.e., negative cooperativity in the binding of NAD (Conway and Koshland, Biochem. 7 (1968) 4011). The binding of NAD after rapid mixing occurs in two phases (De Vijlder and Slater, Biochim. Biophys. Acta 167 (1968) 23). This may reflect either a heterogeneity of sites or the presence of a comparatively slow conformational change leading to a decrease of affinity of the remaining free sites with increasing saturation. Unfortunately, temperature-jump experiments are not easily feasible with either rabbit muscle glyceraldehyde-3-phosphate dehydrogenase or the enzyme from lobster (the X-ray crystallography of which is well under way). The reason is the relatively high affinity of the latter enzyme species for NAD.

The example of glyceraldehyde-3-phosphate dehydrogenase clearly shows that *a priori* generalizations about mechanisms of cooperative phenomena are dangerous. On the other hand, if hemoglobin eventually turns out to follow the sequential mechanism (i.e. existence of conformational hybrids) a more pragmatic generalization becomes attractive (Eigen, 1969). It is based on the criteria of the free energy changes between the low and the high-affinity conformations and the activation energies of the transition. Allosteric enzymes with small equilibrium constants and slow transitions are likely to show all-or-none behaviour (i.e. negligible amounts of hybrids, e.g. yeast glyceraldehyde-3-phosphate dehydrogenase). In contrast, extreme conformational equilibria and fast transitions increase the probability of hybrids (e.g. hemoglobin). Exciting clues have already been provided by stopped-flow experiments with a number of regulatory enzymes. The slow transients observed with aspartokinase-homoserine dehydrogenase of *E. coli* (Barber and Bright, Proc. Nat. Acad. Sci. (Wash.) 60 (1968) 1363), threonine deaminase

(Hatfield and Umbarger, Biochem. Biophys. Res. Comm. 33 (1968) 397) and yeast pyruvate kinase (Hess, Studia Biophysica 2 (1966) 41) indicate that slow conformational changes might be a widespread phenomenon among regulatory enzymes.

3. Mechanism of enzymatic catalysis

It is obvious that the mechanism of an enzyme-catalyzed reaction must involve more intermediates than occur in the course of the preceding processes of substrate binding. In order to raise their concentrations to such levels that they become directly observable it is necessary to work at high enzyme concentrations. The patterns of reactions to be expected under these conditions are necessarily very complex and inherently difficult to interpret. It is therefore not surprising that to date only a few catalytic mechanisms have been investigated by rapid reaction techniques.

The extensive kinetic studies of Hammes and coworkers on aspartate amidotransferase and ribonuclease have recently been reviewed (Hammes, Ann. Rev. Biochem. 37 (1968) 1). With the latter enzyme, the findings can be correlated with the structure of ribonuclease as determined by X-ray crystallography. The inclusion of the rapid protolytic equilibria involved makes the picture rather complicated (although more realistic) by the inclusion of parallel pathways.

One of the first problems to be tackled with any enzyme is the question of rate-determining steps (possibly representing a control point of the enzyme's functional behaviour). Particular attention is being paid to the occurrence of conformational changes during the reaction. More specifically the question is whether they are essential to catalytic activity, possibly in the sense of Koshland's "induced fit" hypothesis.

Glutamate dehydrogenase has been studied by stopped-flow kinetics (Iwatsubo and Pantaloni, Bull. Soc. Chim. Biol. 49 (1967) 1563). Depending on the particular substrate, either the off-constant of reduced coenzyme or the on-constant of the substrate appears to be rate-limiting. The study of one of the isozymes of pigheart lactate dehydrogenase has progressed even further in as much as independent investigations on the kinetics of binding of single substrates (or analogs) have been carried out (Heck et al., Biochem. J. 108 (1968) 793). Single turnover (or so-called "burst"-) experiments have subsequently revealed that some step other than the dissociation of enzyme-product complexes (i.e. a first-order process = isomerization) must be rate-limiting (Criddle et al., Nature 220 (1968) 1091). This provides a rationale for the fact that there is an obligatory order of binding of the substrates to this enzyme. The inferences are rather indirect, however, and the existence of on-line conformational changes still awaits a direct demonstration.

Analogous experiments with alkaline phosphatase from *E. coli* (Trentham and Gutfreund, Biochem. J. 106 (1968) 455) lead to similar conclusions. Here again the existence of a rate-limiting isomerization of the enzyme-substrate complex is inferred indirectly from the fact that phosphate esters with widely different leaving groups phosphorylate the enzyme at the same rate.

It is to be expected that this general type of approach will increasingly augment the results obtained by classical steady-state kinetics, thus adding a wealth of new information about the structure-function relationships of enzymes.

4. Model systems

An entirely different approach to the problem of functional conformational changes in enzymes involves the use of models. The reversible thermal transition of trypsin, chymotrypsin and other pancreatic enzymes can be taken as a model for large-scale isomerization of the entire tertiary structure of proteins.

The kinetic studies of the process by a temperature-jump method (Pohl, FEBS letters 3 (1969) 60) have provided strong support for the hypothesis that the transition occurs between two states only. The rate of isomerization is comparatively slow (seconds to minutes). It reflects the high degree of cooperativity of the various interactions stabilizing the two different states. The availability of detailed information on the structure of chymotrypsin provides a sound basis for the interpretation of the effects of pH and solvent composition on the rates and equilibria of the process.

A novel application of dielectric relaxation techniques has finally permitted the direct measurement of the rate of the helix-coil transition of poly-benzyl-glutamate (Schwarz and Seelig, Biopolymers 6 (1968) 1263). This system is a model for changes in the secondary structure of proteins. The results show that the elementary processes of helix-nucleation and propagation are extremely rapid (half lives of 10^{-6} and 10^{-10} sec respectively) indicating that analogous processes in enzymes can be very fast also. Furthermore, the helix-coil equilibrium itself can be perturbed by an applied electric field. This finding is potentially significant with respect to the electric excitability of membranes.

5. Nucleic acids

Considerable advances have been made in the understanding of the mechanism of cooperative base pair formation in nucleic acids. The kinetics of the formation of short helices of oligo-uridylic and oligo-adenylic acid have been measured by temperature-jump and stopped-flow methods (Poerschke, thesis (1968), Göttingen). These investigations do not only provide information on the rate of the elementary steps of nucleation and helix propagation in the course of DNA unwinding (e.g. in replication). They also give an attractive answer to the question of why there are three and only three bases in the codon.

The formation of short helices is found to be very rapid once a "nucleus" of three base pairs has been formed. This leads essentially to an "all-or-none" process of helix formation of oligo nucleotides.

The dynamic stability of a helix of three base pairs evidently strikes an optimal balance between the requirements of specificity of recognition (half-life long enough) on the one hand and of rapidity of "scanning" (half-life short enough so as not to be rate-limiting) on the other. This fact is obviously of great significance for understanding why evolution has selected just this number of base-pairs for the efficient translation of the genetic message.

The kinetics of DNA unwinding have been re-investigated by the temperature-jump method (Spatz and Crothers, J. Mol. Biol. (1969) in press) augmenting the previous results from pH-jump measurements. Three distinct relaxation processes are observed which can now be assigned to various phases of the unwinding process. The rate of the intermediate one appears to be limited by the viscous resistance to rotation of the unwinding DNA helix (speedometer cable model). The new results do away with the previously favoured notion that *in vivo* replication of DNA is limited by viscous drag.

The binding of actinomycin (Müller and Crothers, J. Mol. Biol. 35 (1968) 251) and proflavin (Li and Crothers, J. Mol. Biol. 39 (1968) 461) to DNA is obviously important for understanding the inhibitory and mutagenic action of these dyes *in vivo*. The kinetics have been thoroughly studied with the aid of rapid reaction techniques. Several relaxation processes can be clearly discerned. One seems to be related to the insertion (intercalation) of the chromophores between stacked base pairs with double helical arrangement. This is possibly the primary event leading to misreading of the genetic message.

6. Outlook

From the brief and necessarily incomplete survey above it is clear that the use of rapid reaction techniques is no longer limited to the elucidation of a few singular systems of predominantly physico-chemical interest. The rapid advance is closely coupled to the accelerated progress of technology in several fields (e.g. lasers, integrated circuits, data processing by on-line computers etc.). One can foresee that the flux of new information will soon no longer be limited either by the time resolution and sensitivity of the instruments or evaluation of data, but rather by the isolation of biological systems in sufficient quantity and purity. The problem of finding suitable "probes" for conveniently following the rapid chemical processes is actively being studied in experiments aimed at the chemical modification of substrates and enzymes. Finally the parallel rapid development of structure analysis by X-ray crystallography, EPR and NMR will provide the necessary complementary information for a realistic interpretation of the results obtained from kinetics in terms of molecular interactions in biological systems.