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S. A. Bernhard and H. Gutfreund

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Steps in the reactions of proteolytic enzymes with their substrates

By S. A. Bernhard† and H. Gutfreund‡
† *Institute of Molecular Biology, University of Oregon, U.S.A.*
‡ *Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol*

Kinetic experiments should be designed to answer specific questions about a reaction mechanism. The present paper is intended to show how a number of specific questions have been answered. Chymotrypsin and trypsin are mainly used to illustrate the different approaches, but many of the arguments used are equally applicable to the reactions of other hydrolytic enzymes with serine-OH or cysteine-SH at the active site.

The recognition of serine-OH and cysteine-SH as essential groups at the active sites of different hydrolytic enzymes did not rest on kinetic evidence. This was deduced from the correlation of enzyme activity with the extent of modification of specially reactive groups.

The investigation of proton dissociation equilibria and the assignment of dissociation constants to groups with specified functions in substrate binding, catalysis or protein conformation was the first objective of serious kinetic studies of enzyme reactions. Steady state rate measurements over a wide range of pH showed that groups with pK 6.25 and 6.85 respectively are involved in the catalytic activity of trypsin and chymotrypsin with certain specific substrates (Hammond & Gutfreund 1955). In the case of chymotrypsin it was also shown by Hammond & Gutfreund (1955) that a group with a more alkaline pK is involved in substrate binding. This latter group was subsequently identified and its function was elucidated through the elegant experiments of Oppenheimer, Labouresse & Hess (1966).

The identification of histidine as the group with pK_A near neutrality, involved in the catalytic mechanism of trypsin and chymotrypsin, was subsequently confirmed by direct chemical methods by Schoelmann & Shaw (1963). Only kinetic analysis can demonstrate the involvement of proton donors or acceptors with specific properties in enzyme–substrate interaction or in catalysis. The clear identification of chemical groups capable of performing such functions is coming from the crystallographic analysis of the three-dimensional structure at the site of enzyme–substrate interaction, as illustrated in other papers presented in this discussion. Very interesting chemical information is obtained when the effect of structure on reactivity is synthesized from the composite of crystallographic and kinetic data.

Transient techniques involving observations of the active site

The use of nitrophenyl acetate by Hartley & Kilby (1952) as a reagent for the study of the active site of chymotrypsin produced information which had a decisive influence on much of the work carried out on hydrolytic enzymes in subsequent years. They found that during the first turnover of the reaction of chymotrypsin with nitrophenyl acetate, approximately one mole of nitrophenol per mole of enzyme is liberated very rapidly, while the subsequent slower release of acetate from its enzyme complex is rate determining for the steady state rate of the hydrolysis

of nitrophenyl acetate. Investigations with rapid spectrophotometric techniques by Gutfreund & Sturtevant (1956) and Gutfreund & Hammond (1959) produced evidence for two distinct steps during chemical catalysis subsequent to substrate binding. It was concluded that an imidazole group of histidine was involved in both the formation and decomposition of an acyl enzyme. The acylation did not involve a transient intermediate with the imidazole but affected its ionization constant.

The observation of a select portion of a reaction sequence via the formation of a chromophoric product is a well established technique. It was precisely by such a technique that Hartley & Kilby first inferred the formation of an acyl enzyme intermediate in the reaction of α-chymotrypsin with *p*-nitrophenyl acetate. Two types of information are obtainable from this type of experiment: (1) the transient kinetics of nitrophenol release due to stoichiometric reaction of substrate with the active site serine hydroxyl and, in the presence of excess pseudo-substrate; (2) the steady state turnover of substrate determined by various slow steps in the mechanism of hydrolysis.

Further studies were directed towards the elucidation of the reaction intermediates and the nature of the substrate binding site through the use of chromophoric substrates and inhibitors. Greater details of the transient mechanism can be obtained from any 'signal' of reaction which is relevant to the state of the enzyme site rather than to that of the substrate or the products. Such signals will undergo a set of transient transformations dependent on the variety of steps in the mechanism. A good example is in the stoichiometric reaction of the α-chymotrypsin site with β-2-furylacryloylimidazole. The reaction can be followed spectrophotometrically in two ways. The effect of substrate on the non-covalent complex between a chromophoric inhibitor, proflavin, and the enzyme site can be observed (Bernhard & Gutfreund 1965). Proflavin is a competitive inhibitor of chymotrypsin and trypsin, and only one molecule of dye complexes with an enzyme molecule at moderate concentrations of dye ($0.1\ \mathrm{mmol\ l^{-1}}$). The visible absorption spectrum of the dye is different in the complex than it is in aqueous solvent, as would be anticipated if the polarity of the environment in the region of the adsorption site differs from that of water. Competitive and time dependent reactions of enzyme with substrate in the presence of dye leads to corresponding time dependent changes in the observable dye spectrum. The reaction involves an enzyme–substrate intermediate which is formed slowly (compared with diffusion controlled rates) and is still more slowly converted to product. The overall reaction pathway is suggestive of the acyl-enzyme mechanism. In the case of furylacryloylimidazole this mechanism can be shown to operate by a second method: the unique and distinct spectrophotometric identifications of reactant, acyl-enzyme and product during the course of the stoichiometric reaction (Charney & Bernhard 1967). The reaction can be followed near the isosbestic point (315 nm) between substrate and product to give a maximum signal for the formation and decomposition of acyl-enzyme. The rate of acyl enzyme appearance and subsequent (slower) disappearance observed in this way is indistinguishable from the disappearance and subsequent (slower) re-emergence of enzyme–dye complex. It follows, therefore, that the sequence of events, $\mathrm{E} + \mathrm{S} \rightarrow$ acyl enzyme $\rightarrow \mathrm{E} + \mathrm{P}$, includes all stoichiometrically significant enzyme-contained species which occur during the course of the catalysed reaction. Finally, the acylation–deacylation mechanism can be substantiated by the simultaneous measurement of the rate of appearance and disappearance of acyl enzyme and the rate of formation of *p*-nitrophenol from the stoichiometric reaction of enzyme with the reagent β-2-furylacryloyl-*p*-nitrophenylate. The overall rates of hydrolysis of the imidazole and nitrophenol derivatives are identical and are

determined by the rate of decomposition of furylacryloyl-chymotrypsin. The observation of identical rates of reaction for a set of homologous derivatives has often been invoked in substantiating mechanisms involving a common intermediate. A common acyl-enzyme intermediate has been proposed for the reaction of two acyl derivatives, the rate of deacylation being rate controlling and common to the two reactions. With other substrates and other enzyme systems this argument has proved to be very misleading (Gutfreund 1968) and it must not be used without direct evidence to support it. For the hydrolytic reactions of β-2-furylacryloyl acylating reagents with α-chymotrypsin, the acyl-enzyme pathway has been substantiated. All methods for observation, namely via chromophoric substrate and products, via enzyme dependent chromophoric substrates, and via chromophoric competitive inhibitors are mutually kinetically consistent with the same, unique mechanism.

The maximal specific rate of hydrolysis of β-2-furylacryloyl-chymotrypsin is only 5×10^{-3} s^{-1} at 25 °C. Although this is a uniquely rapid hydrolysis for an *O*-aroyl serine ester at neutral pH, it is to be compared with first order rate constants of approximately 100 s^{-1} for the turnover of optimal ester substrates of chymotrypsin. It is entirely conceivable that with these much faster reacting substrates, different rate controlling processes (and, consequently, different reaction intermediates) may modify or complicate the reaction scheme established for furylacryloyl derivatives. Our aim in the experiments which follow is to utilize the techniques described above for the elucidation of the mechanism of chymotrypsin and trypsin catalysis of rapidly hydrolysed specific substrates.

Stoichiometric transient reactions with specific substrates

The substrate configuration necessary for rapid chymotryptic and tryptic catalysis involves a tetrahedral α-carbon. It is not possible to prepare chromophoric acyl derivatives in which the acyl chromophore might be expected to interact directly (covalently) with the active site serine as was the case with the furylacryloyl (pseudo) substrates. Nevertheless, intensely chromophoric residues can be introduced into substrates without loss of reactivity or specificity by acylation at the amino group, for example, with furylacrylate. Intense chromophores, such as the furylacrylamido group, are sensitive in their electronic vibrational spectra to the influence of the solvent environment. Thus it was anticipated that transient perturbations in this spectrum would accompany the binding of substrate to the active site. The kinetically determined dissociation constant from steady state data, K_m, is very much smaller than the equilibrium dissociation constant of competitive inhibitors (K_I) with equivalent van der Waal structures (e.g. a methyl ester and an *N*-methyl amide of the same acyl group). K_m is a direct measure of the total dissociability of all stoichiometrically significant enzyme–substrate intermediates. The difference in magnitude between K_m and K_I must reflect more tightly bound substrate species in the transient case, and is presumably due to the formation of transient covalent intermediates in stoichiometrically significant quantities. From these facts one would anticipate that stoichiometric reactions of excess enzyme with substrate built up from a specific amino acid (for instance lysine in the case of trypsin or tryptophan in the case of chymotrypsin) *N*-acylated with furylacryloate, could lead to the following set of events:

(1) A very rapid adsorption of a fraction of the total substrate determined by the excessive enzyme concentration: since the rate of such interactions are usually diffusion controlled

($k_{\text{association}} \simeq 10^8$ mol^{-1} l s^{-1}, $k_{\text{dissociation}} \simeq 10^4$ s^{-1}), the kinetic measurement of the interaction is beyond the limits of time resolution of stopped-flow techniques. We would anticipate a 'blue shift' in the absorption spectrum at the end of the mixing time (1 ms) due to the binding of this chromophore in a less polar environment.

(2) A slower process leading to tighter binding of substrate via the formation of covalent intermediate(s): if the enzyme site remains identical (rigid) in conformation during catalysis, a further time dependent blue shift in spectrum should accompany this chemical process.

(3) The transformation (via hydrolysis) of the covalent intermediate(s) to weakly bound carboxylate product: again, assuming a constant site environment, the liberation of furylacrylamido chromophore into the aqueous environment should lead to a 'red shift' relative to the enzyme bound covalent intermediates.

Bernhard (1968) reported studies with furylacryloyl derivatives of specific substrates for trypsin and chymotrypsin. Stopped-flow kinetic measurements were made at 335 nm, a wavelength significantly to the 'red' of the $\lambda_{\max}$ of the furylacrylamido chromophore in water (304 nm). The binding of chromophore to the site should therefore lead to a decrease in absorption at the observed wavelength. This is, in fact, observed at the earliest measurable time. However, chemical transformation of this earliest E–S complex leads to a very large (unanticipated) red shift with a consequent very large increase in absorption at 335 nm. The subsequent transformation to products necessarily produces a large decrease in absorption at 335 nm. The magnitude of both the wavelength shift and the extinction change in the overall transient reaction indicate that 'chemical' changes (changes in the types of bonds at the chromophoric acyl linkage) occur during the transient period, although the normal furylacrylamido spectrum (and bond types) are restored in the product.

From the transient data it is possible to compute two specific rate parameters and one binding parameter: These parameters and the differences in extinction among reactant, intermediate(s) and product can be computed on the basis of the model:

$$\mathrm{E} + \mathrm{S} \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_1} \mathrm{EP} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \qquad (1)$$

so that a complete fit of parameters to a transient curve involves the assignment of the two specific rates k_1 and k_2, the dissociation constant $K_S = k_{-s}/k_s$ and the molar extinction coefficients. The resultant differential equations are readily integrable for the conditions $E_0 \gg S_0$ and $k_{-s} \gg k_1$, although the computed solution is obtainable regardless of conditions.

It is important to ascertain whether the kinetically observable steps describable by the two kinetic constants (k_1 and k_2) are specific to the method of observation (the change of absorbance at 335 nm). These rapid specific reactions can be followed by the same proflavin displacement technique described above. At 335 nm proflavin is virtually transparent, whereas at 470 nm (near the maximum $\Delta\epsilon$ between dye and enzyme–dye complex) the furylacryloyl chromophore is transparent. Bernhard (1968) has provided clear evidence that identical transient reactions measured at each of these wavelengths correspond to the furylacryloyl chromophore and proflavin displacement respectively. A minimum of two reaction steps is indicated at each of the two wavelengths. A either wavelength, two specific rate parameters suffice to fit the transient kinetic data, and the computed specific rates (k_1 and k_2) are found to be independent of wavelength utilized. In the light of these findings, it is suggestive that the simple acyl enzyme model is valid for *specific* substrates as well as for pseudo-substrates and that the two kinetic parameters, k_1 and k_2, can be correlated with the acylation and deacylation of the enzyme. The

independently determinable steady state parameter, K_m, can be calculated on the basis of the model (1) or of the acyl enzyme model which is merely a special case, from the relation:

$$K_m = \frac{k_2}{k_1 + k_2} K_S. \tag{2}$$

The correspondence between calculated and observed constants is well within experimental limits of error, and clearly establishes the validity of the model of equation (1). In order to examine the validity of the acyl enzyme model specifically, however, it is obligatory to measure the rate of appearance of the leaving group from the substrate. The unique feature of the acyl enzyme model is that the leaving group must be generated from the initial E–S complex with a specific rate identical to k_1. The experiments described below have been designed to test this prediction.

Comparison of a methyl and a nitrophenyl ester

For reasons of solubility, availability and binding affinity we selected two esters of the same trypsin specific acyl group, *N*-α-carbobenzyloxyl-L-lysine. The enormously different electron withdrawing properties of methoxyl and *p*-nitrophenoxyl present good test conditions for the acyl-enzyme hypothesis. According to this model we would make the following straightforward predictions:

(1) The appearance of nitrophenol from the nitrophenyl ester (which is independently measureable by its unique spectrum) should occur with the same specific rate (k_1) as the appearance of acyl enzyme. In this particular case, $k_1 \gg k_2$, and K_S is small (about 0.1 mmol l^{-1}). For this reason, the rate of the first step of the biphasic proflavin displacement curve, if it is correlated with acylation, should be the same as the monophasic rate of nitrophenol release.

(2) Since the model involves the formation of a common acyl enzyme intermediate via either ester substrate, the slower specific rate constant should be independent of the leaving group and equal in magnitude to the turnover number.

(3) The faster specific rate should be dependent on the particular ester substrate; the better the leaving group, the greater k_1.

(4) In a large excess of enzyme ($E_0 \gg 10\ S_0$), the observed specific rate of acylation or the equivalent rate of nitrophenol release is given by equation (3):

$$k_{obs} = k_{acylation} \frac{E_0}{K_S + E_0}. \tag{3}$$

Pertinent experimental results are summarized as follows:

(1) The first order rate of nitrophenol release is markedly slower than the initial rate of proflavin displacement, contrary to the prediction based on the acyl enzyme hypothesis.

(2) The slower specific rate (k_2) is, however, independent of the leaving group and equal to the steady state turnover number.

(3) The methylester is much more poorly bound to the enzyme than the nitrophenyl ester as indicated by the extent of the initial proflavin displacement, the enzyme concentration dependence of the rate of the first step of the biphasic reaction, and the steady state rate–concentration dependence. When saturation is reached, however, the specific rate of the initial step of proflavin displacement (k_1) is a constant independent of the particular leaving group; a result contrary to expectations on the basis of the acyl-enzyme mechanism.

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(4) The dependence of the first order rate of nitrophenol formation on enzyme concentration is different from that of the initial rate of proflavin displacement by the nitrophenyl ester. This indicates multiple steps in the reaction pathway for nitrophenol formation, and is contrary to the simple model of acylation–deacylation. Since our results with a variety of ester substrates indicate that the steady state data can be quantitatively accounted for by the two-step mechanism, it must follow that liberation of the leaving group is not concomitant on the formation of an intermediate designated as ES′. The acyl enzyme model demands this relation and hence it has to be modified to fit the reactions of specific substrates:

$$E + S \rightleftharpoons ES \xrightarrow{k_1} ES' \xrightarrow{k_2} EP \longrightarrow E + P \qquad (4)$$

More complex mechanisms involving an obligatory acyl enzyme intermediate, can be proposed. In such models the initial dye displacement kinetics need not follow that observed for nitrophenol liberation, nor is there necessarily a common concentration dependence for the two processes. It is of interest to note the common specific rate (k_1) with a nitrophenyl and a methyl ester. The use of the argument that common rates give evidence for common *chemical* intermediates fails in the present system. The actual results suggest that an enzyme–substrate complex undergoes a relatively slow (k_1) transition in which changes in the structure of the enzyme–substrate complex are rate limiting. Similar conclusions were drawn by Barman & Gutfreund (1966) from measurements of the rate of liberation of ethanol from specific ester substrates during, single turnover reactions of trypsin and chymotrypsin.

Reactions between enzymes and substrates involve (1) complex formation and the response of the enzyme to form a reactive intermediate, (2) chemical catalysis. In this paper we are mainly concerned with the former problem, which is more specifically an enzymological one. The very much larger reaction rates are a clear indication that more favourable compounds are formed in response to specific substrates compared with the reagents which simulate the reaction. The very large spectral changes of chromophores attached to the amino group of the specific substrate amino acid provide strong evidence that the rearrangement involved in the formation of ES′, the reactive enzyme–substrate compound, will be influenced by other amino acids attached in a natural substrate.

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