Optical and Chemical Identification of Kinetic Steps in Trypsin- and Chymotrypsin-Catalysed Reactions

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Previous interpretations of the mechanism of trypsin- and chymotrypsin-catalysed reactions in terms of two intermediates, the Michaelis complex and an acyl-enzyme, were based on steady-state studies and on the observation of individual steps under sub-optimum conditions. In the present paper new methods for the rapid analysis of chemical events and for the spectrophotometric detection of individual steps are applied to these two enzymes. These methods can be used to study reactions with specific amino acid ester substrates. It can be shown that there are at least three distinct steps between the Michaelis complex and the release of ethanol; the latter is likely to correspond to acyl-enzyme formation. The relative rates of these three steps are measured by rapid-flow techniques from observations of the displacement of chromophoric inhibitors and reactions with specific substrates containing chromophores, as well as from ethanol analyses during a single turnover of the enzyme reactions. It is concluded that the reactions of trypsin and chymotrypsin with their specific substrates involve the formation of a specially reactive conformation of the enzyme-substrate complex and that the rate constants involved in this rearrangement are at least as important for the overall reaction as those of the subsequent formation and decomposition of the acyl-enzyme.

Trypsin and chymotrypsin belong to a large group of enzymes that have one especially reactive serine hydroxyl group among the 20–30 serine residues of the protein molecules. The enzymes of this group all catalyse acyl or phosphoryl transfer reactions, hydrolysis being a special case of transfer to water as the acceptor. The first suggestion that *O*-serine-linked acyl or phosphoryl compounds of the enzymes occur as reaction intermediates came from Wilson, Bergmann & Nachmansohn (1950) as an interpretation of the reactions of acetylcholinesterase with specific inhibitors that react with the one reactive serine residue of the enzyme. Hartley & Kilby (1954) have shown that the reaction of chymotrypsin with nitrophenyl acetate involves the rapid formation of acetylchymotrypsin and nitrophenol and the relatively slow hydrolysis of the acetyl-enzyme to form acetate and free enzyme. This discovery encouraged Gutfreund & Sturtevant (1956) and Bender (for references see Zerner & Bender, 1964) to characterize the acylation and deacylation reactions of

chymotrypsin by detailed kinetic studies with pseudo-substrates that gave signals in the form of spectral changes during the two steps. These substrates are hydrolysed much more slowly by chymotrypsin than the specific substrates for this enzyme and they also are usually good acylating reagents. The simplest mechanism that could be formulated to describe the results obtained was as follows:

EAB is the Michaelis complex, EA the acyl-enzyme and C is water or some other nucleophilic acceptor. The kinetic parameters of this mechanism are: $K_s = k_{-1}/k_{+1}$ and $K_m = K_s k_{+3}/(k_{+2} + k_{+3})$, assuming $k_{-1} \gg k_{+2}$; or for the more general case $K_m = k_{+3}(k_{-1} + k_{+2})/k_{+1}(k_{+2} + k_{+3})$; the turnover, k_0 , of the enzyme is defined by $k_0 = V/[E]_0 = k_{+2}k_{+3}/(k_{+2} + k_{+3})$, where $[E]_0$ is the molar concentration of enzyme active sites. The reversal of the second and third steps can be neglected for low concentrations of products.

Gutfreund & Hammond (1959) and Zerner,

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Bond & Bender (1964) have shown that the Michaelis parameters (V and K_m) evaluated from steady-state rate measurements of the chymotrypsin-catalysed hydrolysis of a range of specific substrates are consistent with the hypothesis that acyl-enzymes are formed. This consistency, which is also observed with trypsin (Baines, Baird & Elmore, 1964), is based on an argument that can be illustrated by the following example. A series of widely different esters of *N*-acetyl-L-tryptophan are hydrolysed by chymotrypsin at the same maximum velocity but with K_m inversely proportional to the rate of base-catalysed hydrolysis of the ester. From this it is argued that: (1) there must be a common intermediate, acetyltryptophanyl-chymotrypsin, for all the different esters; (2) k_{+3} , the hydrolysis of this intermediate, is rate-determining; (3) k_{+2} varies at least qualitatively in the same way as the base catalysis of the hydrolysis of the esters and the changing ratio $k_{+3}/(k_{+2} + k_{+3})$ causes the change in K_m referred to above. In addition to this kinetic consistency with the acyl-enzyme hypothesis, Kezdy, Clement & Bender (1964) have given spectral evidence for the transient formation of an acetyltryptophanyl-chymotrypsin compound in acidic (pH 2–4) solution. Further, Gutfreund & Hammond (1959), as well as Kezdy *et al.* (1964), have shown that during the enzymic hydrolysis of nitrophenyl esters of specific amino acid substrates the initial liberation of nitrophenol is faster than the steady-state liberation of acid.

Zerner & Bender (1964) discuss the difficulties of observing the individual steps in the reactions of specific substrates with hydrolytic enzymes. It is clearly necessary to devise methods that allow one to follow the exceedingly fast steps in the hydrolysis of amides or alkyl esters of specific amino acids at a pH near neutrality. Bernhard & Gutfreund (1965) and Barman & Gutfreund (1965) have given a preliminary account of two new approaches to this problem and have illustrated it with some results obtained with the system trypsin-*N*-benzoyl-L-arginine ethyl ester. The competitive inhibitor proflavine changes its spectrum when it combines with trypsin or chymotrypsin. The rates of enzyme-substrate reactions can be followed by monitoring the extinction change at a fixed wavelength, as the inhibitor is displaced by the substrate and recombines with enzyme again as the products dissociate. Barman & Gutfreund (1965) demonstrated how the rapid-quenching technique can be used to determine the rate of liberation of ethanol during the first turnover of ester hydrolysis catalysed by trypsin. The conclusion reached by Barman & Gutfreund (1965) was that the rate of liberation of ethanol, which should reflect the rate of acyl-enzyme formation, is lower than that of the formation of an intermediate indicated by dye

displacement and that another intermediate, rather than the acyl-enzyme, is the one described by the steady-state kinetic argument developed above.

In the present paper this comparison of the rates of dye displacement and recombination with the rates of ethanol liberation are extended to various conditions for the hydrolysis of specific substrates catalysed by trypsin and chymotrypsin. Further, the rates of formation and decomposition of intermediates in reactions of chymotrypsin are followed with substrates containing a chromophore that is not linked by resonance to the reactive carbonyl group (this idea was developed by Dr S. A. Bernhard).

The results presented below are intended to show that methods are now available that should make it possible to obtain more insight into the sequence of events occurring during the reactions catalysed by this group of enzymes. It is shown that the arguments leading to consistency with the acyl-enzyme hypothesis are too simple. The new information obtained allows one to propose some details of the reaction pathway, but it would be premature at this time to propose a complete mechanism for reactions catalysed by trypsin and chymotrypsin.

METHODS AND MATERIALS

The stopped-flow technique (Gibson, 1954) was used to record the rapid extinction changes. The particular model used was the one described by Gutfreund (1965) with a 1 cm. optical path and a hand barrier for moving the syringes; a mixing chamber with eight jets was inserted in place of the system of two jets. Interference filters (Balzers A.-G., Liechtenstein) and a 12 v, 24 w Mazda projector lamp were used as a light-source at the appropriate wavelengths. A Transtab (model H) power supply (Joyce, Loeb and Co. Ltd., Gateshead-upon-Tyne) was used for the tungsten lamp and a type D4140 high-voltage supply (Labgear, Cambridge) for the photomultiplier (type 6256B; E.M.I. Electronics Ltd., Ruislip, Middlesex). The current output of the photomultiplier was fed into a differential amplifier (CX1442; Solatron Ltd., Farnborough, Hants.) across a 100 k Ω resistance and a 0.01 μ F condenser. The other channel of the differential amplifier was used to feed in a calibrated offset. The change in percentage transmission during experiments was recorded on a CD1400 oscilloscope (Solatron Ltd.) and photographed with a Polaroid camera. The response of the whole system to a sudden change in current was found to have a half-time of approx. 0.7 msec., as expected from the circuit. The experiments recorded below involved less than 20% change in transmission and the amplifier output was assumed to be directly proportional to the concentration of the absorbing reaction intermediate.

The experiments involving sampling of enzyme-substrate mixtures over the time-scale of milliseconds were performed by the method described by Barman & Gutfreund (1964). Equal amounts of enzyme and substrate solutions are injected through a mixing chamber with two jets into a

capillary tube (0.1 cm. diam.) and the mixture flowed through this tube at a rate of 8.7 m./sec. (7 ml./sec.). The end of the capillary tube dipped into a solution of suitable composition to stop the reaction. Different lengths of tubing were inserted into the apparatus to vary the time between mixing enzyme with substrate and quenching the reaction. The volume of reaction mixture delivered into the quenching solution was determined in the way described by Barman & Gutfreund (1964).

The quenched reaction mixture (about 6.5 ml.) was washed into a 500 ml. flask with about 2 ml. of HClO_4 (3.3*N*) and distilled *in vacuo* into a U-tube immersed in a mixture of solid CO_2 and acetone. In this way a neutral solution of the enzyme, which was free from ethanol and which would be partly reactivated when the acid is removed, was obtained without causing any acid hydrolysis of the substrate. The distillates were analysed for ethanol essentially by the alcohol-dehydrogenase method of Lundquist (1959). Overall alcohol blanks were obtained by analysis of reaction mixtures with the quenching acid added to the substrate or enzyme solution before mixing.

The optical stopped-flow measurements as well as the rapid-flow sampling experiments were carried out at room temperatures of $22 \pm 1^\circ$.

Salt-free freeze-dried bovine α -chymotrypsin and trypsin were obtained from Seravac Laboratories (Hollyport, Maidenhead, Berks.) and enzyme solutions were made up in the appropriate buffers not more than 10 min. before use for kinetic experiments. The molarities of active sites were estimated by titration with nitrophenyl esters (see e.g. Bender, Killheffer & Roeske, 1965). This determination of active-site concentrations gives an operational rather than an absolute definition since it depends on the assumption of the simple acyl-enzyme pathway, which is not proven even for the reaction of nitrophenyl esters.

BAEE* was obtained from Seravac Laboratories, proflavine was from British Drug Houses Ltd., Poole, Dorset, and FATEE was provided by Dr S. A. Bernhard.

RESULTS AND DISCUSSION

Reactions between trypsin and benzoyl-L-arginine ethyl ester. Barman & Gutfreund (1965) have shown that at pH 7.2 the rate of liberation of ethanol in a single-turnover experiment, when enzyme and substrate concentrations are approximately equal (about 0.1 mM), is about the same as the steady-state turnover with k_0 15 sec.⁻¹ at 25°. In such experiments a small proportion of the ethanol, about 10%, is liberated much more rapidly, i.e. within 4 msec., whereas the rest of the ethanol is liberated with a half-time of 50 msec. The turnover rate of the trypsin-BAEE reaction, under steady-state conditions, does not change significantly in the range pH 7.2–8.0 (Gutfreund, 1955). Fig. 1 gives the results of the analysis of ethanol liberation during a single turnover of the reaction of trypsin and BAEE at pH 7.9 in the form of a first-order plot. It is clear that under these conditions there

Fig. 1. First-order plot of the rate of liberation of ethanol during the reaction of trypsin (0.2 mM) with BAEE (0.18 mM) in 0.08 M-sodium phosphate buffer, pH 7.9. The reaction was quenched by injecting enzyme-substrate mixture of the age indicated into an approximately equal volume of 0.36 N - H_2SO_4 .

occurs a much larger 'burst' of very rapid ethanol release, whereas about 50% of the ethanol is released with a half-time of 16 msec., giving a first-order rate constant 43.5 sec.⁻¹, nearly three times the steady-state liberation of ethanol. Identical results were obtained in sodium phosphate and in tris buffers (0.1 M, pH 7.9).

As pointed out by Bernhard & Gutfreund (1965), if the rate of ethanol liberation during the first turnover of the reaction of trypsin with BAEE were characterized by k'_{+2} (eqn. 1) the apparent rate k'_{+2} at a given substrate concentration should be $k_{+2}\{[S]/([S] + K_s)\}$. They also estimated from the K_s/K_m ratio that the k_{+2}/k_0 ratio should be about 1000, i.e. k_{+2} should be 1.5×10^4 sec.⁻¹ and k'_{+2} at $[S]$ 0.2 mM should be about 700 sec.⁻¹. Clearly this calculation is only correct for the case $[S] \gg [E]_0$, which does not hold for the single-turnover experiments reported here. It is, however, evident that the rate of ethanol liberation is not rapid enough to correspond to the rate of enzyme acylation required for the simple scheme. The point that the first-order kinetics for the second half of the ethanol liberation is also not consistent with such a simple model is discussed again below.

Bernhard & Gutfreund's (1965) studies of the rate of displacement, on addition of BAEE, of proflavine bound by trypsin, as well as the rate of recombination of the dye with enzyme at the end of the enzyme-substrate reaction, were extended over the range pH 4.8–7.9. The rate of recombination of proflavine and trypsin is easier to interpret than the displacement of dye, especially over the range pH 4.8–7.2, when the rate of recombination

* Abbreviations: BAEE, *N*-benzoyl-L-arginine ethyl ester; FATEE, *N*-β-(2-furyl)acryloyl-L-tyrosine ethyl ester.

Fig. 2. Record of the change in transmission at 470 $m\mu$ during the reaction of trypsin (0.2 mM), proflavine (0.2 mM) and BAEE (0.2 mM) in 0.1 M-acetate buffer, pH 5.3.

Fig. 3. Record of the change in transmission at 470 $m\mu$ during the reaction of trypsin (0.1 mM), proflavine (0.2 mM) and BAEE (0.1 mM) in 0.1 M-tris buffer, pH 7.9.

is identical with that observed by Gutfreund (1955) for the overall turnover rate for the trypsin-catalysed hydrolysis of BAEE. However, when the pH is raised from 7.2 to 7.9, though there is no significant change in turnover number, the rate of dye recombination is increased nearly threefold and corresponds to the rate (k 43.5 sec.⁻¹) of the first-order phase of ethanol liberation. Fig. 2 gives the record of dye displacement and recombination at pH 5.3. There are several difficulties about the interpretation of the kinetics and pH-dependence of the dye-displacement reaction, which is more complex than that interpreted by Bernhard & Gutfreund (1965). It is, however, clear that the displacement of dye is characteristic of a step that precedes the labilization of ethanol (Bernhard & Gutfreund, 1965; Bernhard, Lee & Tashjian, 1966). It has also been shown (S. A. Bernhard, personal communication) that, during the single-turnover reaction of trypsin with the *p*-nitrophenyl ester of α -N-benzoyloxycarbonyl-L-lysine in the presence of proflavine, the liberation of *p*-nitrophenol is accurately first-order and slower than the displacement of proflavine from the enzyme. As pointed out by Bernhard & Gutfreund (1965) the rate of displacement of proflavine from enzyme, at enzyme and substrate concentrations about 0.1 mM, does not reflect the formation of the small amount of initial enzyme-substrate complex, but it is caused by the subsequent formation of some other intermediate.

Fig. 3 shows the record of proflavine displacement and recombination during the reaction of trypsin with BAEE at pH 7.9. The displacement at this pH is so fast that the time-scale of this experiment only resolves its final portion. There is, however, a distinct lag phase between the rapid dye displacement and the relatively slower first-order recombination. This indicates yet another kinetically distinct step between those responsible for dye displacement and dye recombination. The correlation between those steps and the chemical

Fig. 4. Record of the change in transmission at 340 $m\mu$ during the reaction of chymotrypsin (0.27 mM) with FATEE (0.22 mM) in 0.08 M-phosphate buffer, pH 6.6.

events of alcohol and acid liberation are discussed below together with the results obtained from studies with chymotrypsin.

Reaction between chymotrypsin and N- β -(2-furyl)-acryloyl-L-tyrosine ethyl ester. Preliminary studies in Dr S. A. Bernhard's Laboratory showed that in acidic solutions a single turnover of chymotrypsin with FATEE could be followed in a conventional spectrophotometer. The extinction changes during the reaction, which are maximal at 325 $m\mu$, involve first a rapid increase and then a somewhat slower decrease. Even in the stopped-flow apparatus the highest pH at which both phases of the reaction could be followed was 6.6. Fig. 4 shows a record of such an observation at 340 $m\mu$, which is a more convenient wavelength to use in our equipment. From such records one can calculate a number of important parameters of the reaction. The rapid increase in extinction has already started before the reaction mixture has reached the observation chamber of the stopped-flow apparatus. A reasonable estimate of the rate of this reaction can be made from the half-time of 2 msec. of the rest of the rise. This would give a rate constant of 350 sec.⁻¹, which must be corrected for the response

Fig. 5. First-order plot of the rate of liberation of ethanol during the reaction of chymotrypsin (0.27 mM) with FATEE (0.22 mM) in 0.08 M-phosphate buffer, pH 6.6. The reaction was quenched by injecting enzyme-substrate mixture of the age indicated into an approximately equal volume of 0.36 N- H_2SO_4 .

time of 1 msec. for the circuit of the instrument. Such a correction gives k 540 sec. $^{-1}$ as an approximate value for the reaction. The subsequent decrease in extinction has a half-time of 25 msec., which corresponds to a rate constant of 28 sec. $^{-1}$, which is within experimental error the same as the steady-state turnover of chymotrypsin with FATEE at pH 6.6.

Exactly the same reaction mixture of chymotrypsin and FATEE was studied in the rapid-flow quenching apparatus and a first-order plot of the rate of ethanol liberation is given in Fig. 5. It is especially noteworthy that the reaction follows first-order kinetics (k 147 sec. $^{-1}$) over at least 95% of its course. This point has already been raised in connexion with the reactions of trypsin. If the rate of liberation of ethanol or nitrophenol (with nitrophenyl esters) characterizes the rate of formation of the acyl-enzyme as described by eqn. (1), under conditions of the present experiments, when $[\text{E}]_0 \gg [\text{S}]_0 \ll K_m$, the formation of EA would have to deviate from first-order kinetics. However, in the chymotrypsin-FATEE reaction it has been shown that some other intermediate is formed about four times as fast as the liberation of ethanol. The rate of liberation of ethanol corresponds to the first-order transformation of one intermediate into another. The complexity of the situation is increased even further by the finding that displacement of proflavine from chymotrypsin by FATEE occurs somewhat faster than the increase in

extinction at 340 m μ . In summary, we have the following steps during the chymotrypsin-FATEE reaction: (1) formation of Michaelis complex; (2) displacement of proflavine; (3) increase in extinction at 340 m μ ; (4) liberation of ethanol; (5) decrease in extinction at 340 m μ .

General discussion. The formation of acyl-enzymes during the reaction of chymotrypsin or trypsin with nitrophenyl esters is demonstrated readily by observing the relative rate of liberation of nitrophenol and acid during the first turnover of the enzyme. The same type of experiment cannot be carried out to investigate the reactions of alkyl esters. The liberation of ethanol cannot be followed directly in the stopped-flow apparatus. The direct observation of the reduction of NAD^+ in the presence of alcohol dehydrogenase is not a practical proposition for monitoring the rate of appearance of ethanol under the conditions used for the study of the first turnover of proteolytic enzymes. The rapid-sampling method employed here to obtain the rate of liberation of ethanol on a millisecond time-scale has one fundamental weakness. To stop the reaction at the chosen time-interval after mixing enzyme with substrate, the enzyme has to be reversibly or irreversibly inactivated. This inactivation could itself produce some reaction intermediate that is not on the normal pathway. Fig. 5 indicates that the rate of liberation of ethanol was first-order, but this may be the result of two distinct causes. An acyl intermediate of chymotrypsin may be formed with concomitant liberation of ethanol at pH 6.6 at the rate indicated, or some other intermediate may be formed at this rate but this intermediate is converted into the acyl-enzyme when the reaction is quenched with acid. This uncertainty of interpretation of the quenching experiments is not resolved at present and it is only possible to say that some intermediate is formed at the rate indicated. This intermediate might be an acyl-enzyme, but in any case the acyl-enzyme is not formed more rapidly than the rate constant of ethanol liberation. It is possible that the diphasic rate of liberation of ethanol during the reaction between trypsin and BAEE (Fig. 1) is due to two intermediates' being responsible for the amounts of free ethanol found after quenching. The first of these intermediates is formed very rapidly and is converted into the acyl-enzyme on quenching, whereas the second intermediate, the acyl-enzyme, is formed more slowly from the first. The first intermediate would have to be in equilibrium with the Michaelis complex. There is some evidence for this from experiments at pH 7.2. It has been stated by Barman & Gutfreund (1965) that a small amount of ethanol is liberated more rapidly than the rest. When the size of this 'burst' of ethanol was determined over a range of BAEE

concentrations, the following results were obtained. The molar ratio of 'burst' to trypsin concentration (constant at 0.2mm) at 0.2mm-, 1.0mm- and 10mm-BAEE was 0.1, 0.2 and 0.5 respectively.

The methods described in the present paper were developed to attempt the examination of individual steps of some enzyme reactions, at or near optimum pH, with specific substrates that have reactivities similar to the natural ones. The study of the reaction mechanisms of enzymes at sub-optimum pH or with pseudo-substrates has yielded much useful information but obviously has its pitfalls. The changing balance of different steps, as well as the appearance of side reactions and conformation changes in the protein because of change in pH, can give a wrong picture of the relative importance of intermediates of the reaction. The conformation of the active site of an enzyme is likely to be critically dependent on the composition of the solution and on the structure of the substrate combined with it. The use of pseudo-substrates, like the study of the reactivities of essential groups on the active site with inhibitors and reagents, is not likely to give information about the true reactivity of such groups within the enzyme-substrate compound.

The conclusion to be drawn from the experiments described in the present paper is that there are other important intermediates in reactions catalysed by trypsin and chymotrypsin besides the Michaelis complex and the acyl-enzyme. Detailed kinetic studies of the dependence of the individual steps on reagent concentrations and pH should eventually allow one to improve any proposed model of the reaction mechanism. The existence of a number of steps contributing to the overall rate during the steady state account for superficial agreement with the simple acyl-enzyme model, which fails in detail.

Bernhard *et al.* (1966) have proposed that the substrates and competitive inhibitors of trypsin and chymotrypsin are bound in a hydrophobic crevasse of the enzyme molecule. One or more of the steps observed may be controlled by the formation or unfolding of the proper conformation of the enzyme-substrate complex to give it optimum reactivity with specific substrates. The reactive conformation would be induced (Koshland, 1958) after the formation of the Michaelis complex and its stability would depend on the reactivity of the bond to be hydrolysed. Such a phenomenon would make it possible that the transfer of the substrate and the groups on the catalytic site of the enzyme, from an aqueous into a non-polar medium, might have a considerable effect on the reactivity of the resulting enzyme-substrate intermediate. It would be unwise to make too close a comparison of the trypsin-BAEE and chymotrypsin-FATEE reactions. Although there are considerable similarities

in the qualitative behaviour of the two systems, the quantitative relation between the individual steps is sufficiently different for the two enzymes for alternative steps to be rate-determining. It will be of importance to determine the rate of acid liberation during single-turnover experiments. This could probably be best resolved in a rapid-flow calorimeter. The resulting information would enable one to build up a more complete picture of the relative rates of different chemical events and of the conformation changes during the overall reactions of these two enzymes with their specific substrates. The pH-dependence of conformation changes of chymotrypsin has been studied by Oppenheimer, Labouesse & Hess (1966).

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