KINETIC INVESTIGATIONS OF THE ALCOHOL RELEASE STEPS IN THE CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SPECIFIC SUBSTRATE ESTERS[1]

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The pre-steady state kinetics of the chymotrypsin-catalyzed hydrolysis of three specific substrate esters has been investigated at selected pH values. The reactions were observed by measuring the displacement of the competitive inhibitor proflavin from the enzyme by substrate (Wallace et al., 1963; Bernhard and Gutfreund, 1965; Weiner and Koshland, 1965; Brandt and Hess, 1966), and the liberation of p-nitrophenol.

The data were analyzed (Brandt and Hess, 1966) in terms of Equation 1 (Hartley and Kilby, 1954; Gutfreund and Sturtevant, 1956; Faller and Sturtevant, 1966):

$$E + S \underset{}{\overset{K'_S}{\rightleftharpoons}} \ldots ES \xrightarrow[k_{23}]{} \overset{P_1}{\nearrow} \quad EP_2 \xrightarrow{k_{34}} E + P_2 \qquad (1)$$

in which S is an ester derivative of an aromatic amino acid, P_1 an alcohol, and P_2 the acid. The experiments presented were designed to test a recently advanced hypothesis (Barman and Gutfreund, 1966) that P_1 in this equation is

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not liberated concomitantly with the formation of EP_2 and that the proflavin displacement method is not a measure of the liberation of P_1. This work is part of a continuing study of the individual steps in chymotrypsin-catalyzed hydrolysis of specific substrates; the first determination of individual rate and enzyme-substrate dissociation constants pertaining to these reactions has been reported (Brandt and Hess, 1966).

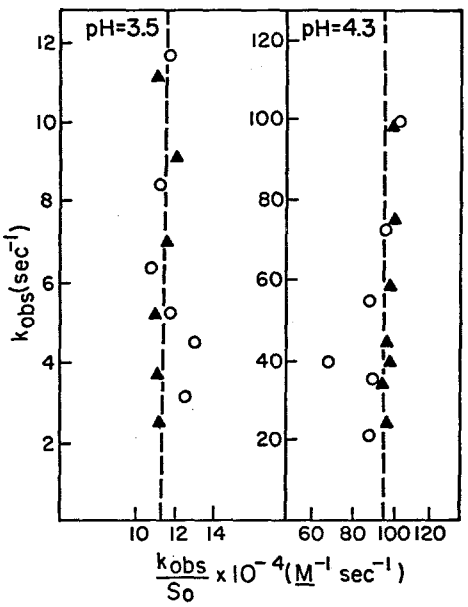

Fig. 1

A plot, according to Equation 2, of data obtained in stopped flow investigations of the pre-steady state portion of the chymotrypsin-catalyzed hydrolysis of ATN, with observation of p-nitrophenol release at 348 mμ (○) and of proflavin displacement at 480 mμ (▲). Temperature = 26.5 ± 0.5°; pH 3.5 and 4.3, with 0.05 acetate buffer and KCl to give a final ionic strength of 0.05; 1.6% acetonitrile present. All solutions contained 4 μM E_o and 50 μM F_o; S_o varied from 25 to 100 μM. K_{EF} (pH 3.5) = 21 μM, and K_{EF} (pH 4.0) = 14 μM. ATN was prepared according to a previously published procedure (Ingles, Knowles, and Tomlinson, 1966). Three times crystallized, salt-free α-chymotrypsin (Lots CDI 6164 and CDI 6168-9) was obtained from Worthington; active site titration indicated that the preparation was 90% active. Proflavin sulfate (Lot G 2200) was obtained from Mann.

Fig. 1 shows the results of pre-steady state kinetic measurements of the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan p-nitrophenyl

ester (ATN). The progress of the reaction was observed by measuring either p-nitrophenol release at 348 mu (O in Fig. 1) or proflavin displacement at 480 mu (▲ in Fig. 1). From such measurements, values of k_{obs} can be determined as previously described (Brandt and Hess, 1966), and values of k_{obs} at various substrate concentrations can be used to evaluate, by digital computer, K'_S, k_{23}, and k_{34} in accordance with the relation (Brandt and Hess, 1966):

$$k_{obs} = k_{23} S_o (S_o + K'_S Z)^{-1} + k_{34} \qquad (2)$$

where $Z = 1 + F_o(K_{EF})^{-1}$, F is molar concentration of proflavin, K_{EF} is the enzyme-substrate dissociation constant, and the subscript zero denotes initial concentration. Although the substrate is not sufficiently soluble to permit evaluation of K'_S and k_{23} separately, their ratio was determined by both methods at pH 3.5 and at pH 4.3. Above pH 4.3 the reaction is too fast to be measured by stopped flow techniques. It can be seen in Fig. 1 that at each pH, measurements by both methods yielded the same intercept of the abscissa, which is a measure of k_{23}/K'_S. Values for k_{23}/K'_S of $1.2 \pm 0.1 \times 10^5$ M^{-1} sec^{-1} and $10 \pm 1 \times 10^5$ M^{-1} sec^{-1} were obtained at pH 3.5 and pH 4.3 respectively.

Fig. 2A shows progress curves obtained by analogue computer for the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester (ATE) at pH 6 under the conditions $E_o = S_o = F_o < K'_S$. The computation was made with use of k_{23}, k_{34}, and K'_S values measured when $S_o \gg E_o < F_o$, since under these conditions the calculation of the constants is straightforward. The curve computed for EP_2 formation (O in Fig. 2A) is compared with a curve obtained experimentally under comparable conditions (● in Fig. 2A); good agreement is observed. Fig. 2B shows the computed progress curves for the same experiment in the absence of proflavin.

Barman and Gutfreund (1966) investigated the chymotrypsin-catalyzed hydrolysis of N-furylacryloyl-L-tyrosine ethyl ester (FATyrE) under the conditions $E_o \cong S_o < K'_S$, measuring absorbance change due to substrate binding at 340 mμ, and liberation of ethanol. Their progress curves for ΔD_{340} and for alcohol release look similar to the curves for EP_2 and P_1, respectively, in

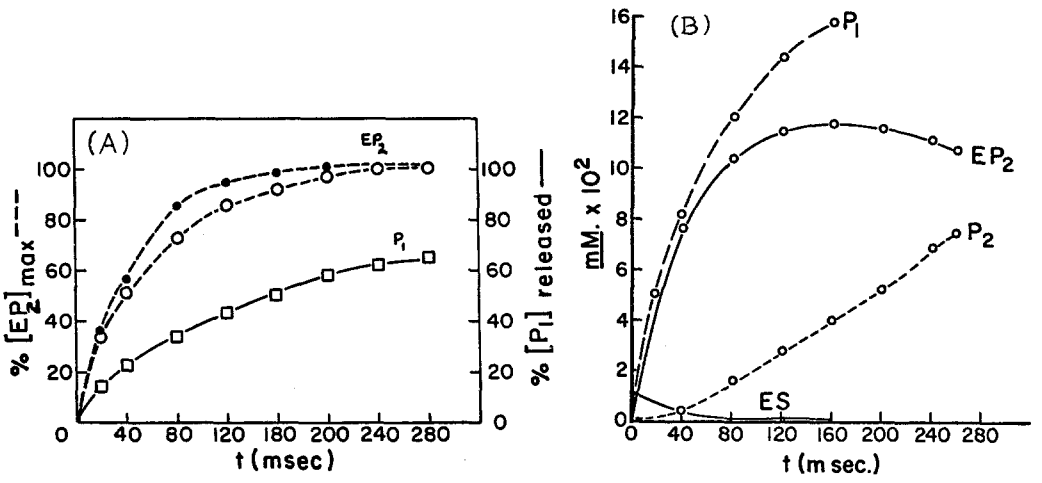

Fig. 2

The α-chymotrypsin-catalyzed hydrolysis of ATE at pH 6.0. $E_o=S_o=0.2$ mM. In presence (Fig. 2A) and absence (Fig. 2B) of 0.2 mM proflavin (F_o). All open symbols refer to curves computed by means of an analogue computer (Model EAI-TR-48, Electronic Associates, Inc.). The computed curves are based on the experimentally obtained k_{23}, k_{34}, K'_S, and K_{EF} values of 140 sec^{-1}, 3 sec^{-1}, 1.3 mM, and 44 μM respectively. These values were obtained under the conditions $S_o \gg E_o$; pH 6.0, with 0.1 M phosphate buffer and KCl to give a final ionic strength of 0.39; and temperature = 28±0.3°. The rate constants pertaining to the formation and dissociation of the enzyme-substrate complex are known (Havsteen, 1967) to be large compared to k_{23} and k_{34}, and it was assumed that the same is true for the corresponding rate constants pertaining to the ES complex. The solid circles in Fig. 2A are taken from an oscilloscope trace of transmittance at 480 mμ in a Gibson-Durrum stopped flow apparatus, obtained in an experiment in which the concentrations after mixing were $E_o=S_o=F_o=0.2$ mM. Other conditions were temperature = 25±0.3°; pH 6.0, with 0.1 M phosphate buffer and KCl to give a final ionic strength of 0.39. ATE (Lot J 1322), chromatographically pure, was obtained from Mann. Other materials were as described under Fig. 1.

Fig. 2B. Barman and Gutfreund observed that the $t_{1/2}$ value for the rise of the ΔD_{340} curve is 2 msec, while the $t_{1/2}$ value for alcohol release is 2 1/2 times larger, or about 5 msec. On the assumption that there is a single turnover of the enzyme, these authors concluded that alcohol (P_1 in Fig. 2B) is not released concomitantly with the formation of a species (considered to be EP_2 in Fig. 2B) which absorbs at 340 mμ.

This method of analysis for the ATE data in Fig. 2B leads to similar results: a $t_{1/2}$ value for P_1 release (75 msec) which is about 3 times larger

than the $t_{1/2}$ value for the rise of the EP_2 curve. (In the experiments with both ATE and FATyrE, P_1 release appears to be a monomolecular process for over 90% of reaction.)

The same method of analysis, including the assumption of a single turnover of the enzyme, led Barman and Gutfreund (1966) to the conclusion that proflavin release is not a measure of p-nitrophenol release in the trypsin-catalyzed hydrolysis of N-carbobenzoxy-L-tyrosine p-nitrophenyl ester. Similar interpretation of the curves for ATE in Fig. 2A would indicate that in this reaction also, proflavin release is associated with a process faster than P_1 release. However, the Fig. 2A curves were calculated on the basis of concomitant P_1 release and EP_2 formation; the non-identity of the progress curves for P_1 and EP_2 is due to turnover of the enzyme and the formation of P_2 as well as P_1. Values of $t_{1/2}$ calculated from the curves in Fig. 2 have no obvious relation to the actual rate constants which were used for computing the curves.

The conclusion to be drawn from these studies is that the investigation of the chymotrypsin-catalyzed hydrolysis of ATN indicates that the proflavin displacement method measures the formation of p-nitrophenol, and that therefore P_1 liberation occurs simultaneously with the formation of EP_2. The apparently contradictory experiments of Barman and Gutfreund (1966) actually support this conclusion; the results they obtained are explained by the indication that under their experimental conditions the reaction does not proceed with a single turnover of the enzyme. Under given experimental conditions, the possibility of obtaining a single turnover of the enzyme depends on both K'_S and k_{23}/k_{34}. In this respect, ATE is a better substrate than the corresponding tyrosine ester: for ATE, K'_S = 1.3 mM and k_{23}/k_{34} = 46 at pH 6, and for the corresponding tyrosine substrate K'_S = 22 mM and k_{23}/k_{34} = 24 at pH 5 (Himoe, Brandt, and Hess, 1967).

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