DETERMINATION OF THE BINDING CONSTANT OF A SPECIFIC ESTER AND A SPECIFIC AMIDE SUBSTRATE TO α-CHYMOTRYPSIN[1]

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Kinetic investigations of the chymotrypsin-catalyzed hydrolysis of specific substrates have relied almost entirely on the steady state approach (Bender and Kézdy, 1965), which yields combinations of rate and equilibrium constants. An elucidation of the catalytic process, however, requires a knowledge of the individual rate constants and of the enzyme-substrate binding constants. In this paper we are reporting the determination of overall binding constants, K_S', of specific substrates to α-chymotrypsin (CT) at selected pH values. Both acetyl-L-tryptophan ethyl ester (ATrEE) and acetyl-L-tryptophan amide (ATrA) were used. Binding constants of specific ester or amide substrates to CT have not been reported previously.

Fig. 1 shows an oscilloscope trace obtained in a typical experiment in which proflavin (F) and enzyme (E) were mixed with ATrEE in a stopped flow apparatus and the concentration of the enzyme-proflavin complex (EF) determined spectrophotometrically at 465 mμ (Bernhard and Lee, 1964) as a function of time. Interpretation of results depends on: (A) the fact that proflavin competes with substrate for the CT-substrate binding site (Wallace, Kurtz,

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and Niemann, 1963; Weiner and Koshland, 1965); (B) observations of Bernhard and Gutfreund (1965) that the characteristic absorption spectrum of the EF complex can be used for the detection of transients in trypsin- and CT-catalyzed reactions; (C) relaxation experiments of Havsteen and Eigen (1965) which indicate that at pH 6.7 the dissociation rate constant for the EF complex is 250 $sec.^{-1}$; (D) the observation that CT-specific substrate complexes, as detected by spectral changes of the enzyme at 290 mμ (Wootton and Hess, 1962; Labouesse, Havsteen, and Hess, 1962; Moon, Sturtevant, and Hess, 1965) are formed in less than 2×10^{-3} sec. under the conditions used in the experiments; and (E) the equation, given below, which was originally shown (Hartley and Kilby, 1954; Gutfreund and Sturtevant, 1956) to apply to the CT-catalyzed hydrolysis of p-nitrophenyl acetate:

$$(1)\quad E + S \overset{K_S'}{\rightleftharpoons} ES \xrightarrow{k_{23}} EP \xrightarrow{k_{34}} E + P$$

where P is the hydrolysis product and $k_{23} > k_{34}$.

Four distinct steps were seen in most of the stopped flow experiments: (I) A rapid decrease in EF which occurs just within the time resolution of the instrument, 3×10^{-3} sec. This process is seen when enzyme-proflavin solution is mixed either with the amide or ester substrate, or with buffer alone. This step is considered to be the dissociation of the EF complex, brought about by dilution or formation of ES. (II) A deerease in EF, observable in Fig. 1 as a decrease with a $t_{1/2}$ of .01 sec. The $t_{1/2}$ of this step, which is seen with ATrEE but not with ATrA, depends on the initial substrate concentration, S_o, and is considered to reflect the rate of formation of EP (see Equation 1). (III) A time interval (about 5 sec in Fig. 1) during which EF remains constant. This time interval depends on S_o and is considered to represent the steady state concentration of EP. (IV) Finally, an increase in the concentration of EF, considered to be due to the decomposition of EP.

When such stopped flow experiments are performed at various S_o concentrations, it is possible to calculate K_S' and k_{23} of Equation 1 under the follow-

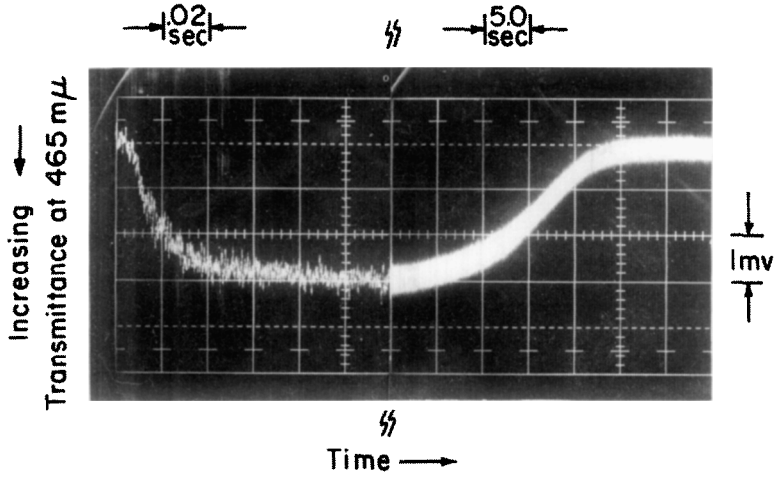

Figure 1

Photographs of oscilloscope traces of transmittance at 465 mμ in two stopped flow experiments with ATrEE at pH 6.0 (see Table I). Expt. I time scale is 0.02 sec/cm; expt. II time scale is 5 sec/cm. Recording of the initial fast increase in transmittance at 465 mu (Step I--see text) would have required a third time scale, and this is not shown.

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ing conditions: (i) $S_o > E_o < F_o$; (ii) $S_o \gtrsim K_S'$; and (iii) EF and ES in equilibrium with E, S_o, and F. Then: (2) $E_o = E+ES+EF+EP$. (3) $K_S' = (E)(S_o)(ES)^{-1}$. (4) $K_F = (E)(F)(EF)^{-1}$. (5) $d(EP)/dt = k_{23}ES - k_{34}EP$. (6) $\ln[(EP)_{ss} - (EP)_t] = \ln[(A_{465})_t - (A_{465})_{ss}] = -k_{obs}t + C$. The subscripts ss and t refer to concentrations at steady state and at any time before steady state conditions have been reached; A_{465} refers to absorbance at 465 mμ. (7) $k_{obs} = k_{23}S_o[(K_S'/K_F)F + K_S' + S_o]^{-1} + k_{34}$. Condition (ii) above allows the k_{34} term to be neglected, so that: (8) $k_{obs} = k_{23} - k_{obs}K_S'[(F/K_F)+1](S_o)^{-1}$.

A typical plot of the pH 5.0 data according to Equation 6 is shown in the inset of Fig. 2. It can be seen that the reaction follows first order kinetics for over 90% of the reaction. Similar results were obtained at all substrate concentrations used, at both pH 5.0 and pH 6.0. The k_{obs} values obtained at pH 5.0 are plotted against substrate concentration according to Equation 8 in Fig. 2. The K_S' and k_{23} values obtained from these experiments are compared with the steady state kinetic parameters in Table I.

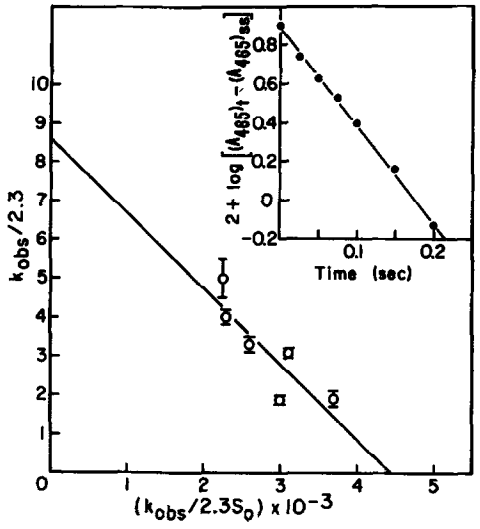

Figure 2

Inset: Typical plot of data from a photograph of an oscilloscope trace of the pre-steady state portion of the ATrEE reaction at pH 5.0 (see Table I), according to Equation 6. The line shown is that calculated by the method of least squares, and gives a value of $k_{obs}/2.3 = 4.89$ sec^{-1}.

Main Plot: A plot, according to Equation 8, of the data from three experiments at pH 5.0 under conditions described in footnote a of Table I. The line is calculated by the method of least squares.

In the CT-catalyaed hydrolysis of ATrA, there was observed only the very rapid decrease in EF (Step I), which is not shown in Fig. 1. Thereafter, EF was observed to be constant for several minutes. It was therefore possible to obtain difference spectra between solutions containing enzyme, proflavin, and substrate, and solutions containing enzyme and proflavin only, in a time sufficiently short so that S_o does not change by more than 1.7% under the most unfavorable experimental conditions used. When $S_o \gg E_o$, then: (9) $E_o = E+ES+EF$ and (10) $K_S' = (E_o-EF-ES)(S_o)(ES)^{-1}$; then from Equation 4 one obtains: (11) $K_S' = [(K_F)(EF)(S_o)](F)^{-1}\{E_o-EF[(K_F/F)+1]\}^{-1}$. Therefore, spectrophotometric determination of EF at various S_o concentrations allows computation of K_S' from Equation 11. The value obtained in these experiments is compared with the steady state kinetic parameter K_m(app) in Table I.

TABLE I

Substrate	pH	Stopped Flow Experiments			Steady State Kinetic Parameters [d]	
		K'_S (M)	k_{23} (sec^{-1})	K_m(app) [c] calculated (M)	K_m(app) observed (M)	k_{cat} observed (sec^{-1})
N-acetyl-L-tryptophan ethyl ester [a]	5.0	0.9×10^{-3}	20	3.6×10^{-5}	8.3×10^{-5}	.84
"	6.0	0.5×10^{-3}	140	1.7×10^{-5}	-	-
N-acetyl-L-tryptophan amide [b]	5.7	6.3×10^{-3}	-	-	3.3×10^{-3}	.025
"	8.0	5.7×10^{-3}	-	-	4.8×10^{-3}	.044

[a] Evaluated as described in the text from experiments on the Gibson-Durrum stopped flow apparatus at 28°. At pH 5.0 in 0.1 M acetate and 0.29 M KCl: 1.0×10^{-5} M α-CT (Worthington three-times crystallized), 5.0×10^{-5} M proflavin (Mann Laboratories), and 0.5×10^{-3} to 2.25×10^{-3} M ATrEE (Mann). At pH 6.0 in 0.1 M potassium phosphate and 0.1 M KCl: 1.0×10^{-5} M α-CT, 4.7×10^{-5} M proflavin, and 0.5×10^{-3} to 2.7×10^{-3} M ATrEE. Values of K_F used were 3.7×10^{-5} M at pH 5.0 and 4.1×10^{-5} M at pH 6.0.

[b] Evaluated as described in the text from experiments on Cary 14 and Cary 15 spectrophotometers equipped with 0-0.1 and 0-1.0 slide wires. Experiments were performed at 23°. At pH 5.7 in 0.1 M potassium phosphate and 0.1 M KCl: 4.0×10^{-5} M α-CT, 4.0×10^{-5} M proflavin, and 1.0×10^{-3} to 10.5×10^{-3} M ATrA. At pH 8.0 in 0.1 M potassium phosphate and 0.1 M KCl: 4.1×10^{-5} M α-CT, 4.0×10^{-5} M and 5.0×10^{-5} M proflavin, and 2.0×10^{-3} to 14.0×10^{-3} M ATrA. Three experiments were done at pH 5.7 and two at pH 8.0. Values of K_F used were 4.0×10^{-5} M at pH 5.7 and 2.4×10^{-5} M at pH 8.0.

[c] Calculated from the k_{23} and K'_S values determined in these experiments, and k_{cat} values from the literature, with the assumption that for esters $k_{34} = k_{cat}$, according to the equation $K_m(app) = [k_{34}/(k_{23}+k_{34})]K'_S$. The value of k_{cat} used at pH 6.0 is an extrapolated value.

[d] Bender, Kézdy, and Gunter (1964).

It has been shown by Bender, Kézdy, and Gunter (1964) that the steady state kinetic data is consistent with the applicability of Equation 1 to the CT-catalyzed hydrolysis of specific substrates. Consequences of the mechanism shown in Equation 1 for the CT-catalyzed hydrolysis of specific substrates have been discussed in detail by these authors, and include: (1) A K'_S value

which is similar for a particular specific substrate amide and its corresponding ester. (2) The rapid formation of an intermediate (as EP) in the ester hydrolysis, followed by the rate-limiting decomposition of this intermediate; this requires different values for K_S' and $K_m(app)$. (3) The rate-limiting formation of an intermediate (as EP) in the amide hydrolysis, if EP exists at all; this requires the same values for K_S' and $K_m(app)$. As can be seen in Table I, direct proof for (1), (2), and (3) has been obtained, for the first time, in the experiments presented.

REFERENCES

Bender, M. L., and Kézdy, F. J., Ann. Rev. Biochem., 34, 49 (1965).

Bender, M. L., Kézdy, F. J., and Gunter, C. R., J. Am. Chem. Soc., 86, 3714 (1964), and preceding papers.

Bernhard, S. A., and Gutfreund, H., Proc. Natl. Acad. Sci. U.S., 53, 1238 (1965).

Bernhard, S. A., and Lee, B. F., Abstracts, VIth Internatl. Congr. Biochem., New York, 1964.

Gutfreund, H., and Sturtevant, J. M., Biochem. J., 63, 656 (1956).

Hartley, B. S., and Kilby, B. A., Biochem. J., 56, 288 (1954).

Havsteen, B. H., and Eigen, M., personal communication, 1965.

Labouesse, B., Havsteen, B. H., and Hess, G. P., Proc. Natl. Acad. Sci. U.S., 48, 2137 (1962).

Moon, A Y., Sturtevant, J. M., and Hess, G. P., J. Biol. Chem., 240, 4204 (1965).

Wallace, R. A., Kurtz, A. N., and Niemann, C., Biochemistry, 2, 824 (1963).

Weiner, H., and Koshland, D. E., Jr., J. Biol. Chem., 240, PC 2764 (1965).

Wootton, J. F., and Hess, G. P., J. Am. Chem. Soc., 84, 440 (1962).