Anomalous pH Dependence of k_{cat}/K_M in Enzyme Reactions. Rate Constants for the Association of Chymotrypsin with Substrates†

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ABSTRACT: Under normal circumstances the pH dependence of k_{cat}/K_M follows the titration curve of the active site of the free enzyme. However, if at the optimum pH for the reaction the rate determining process in the term k_{cat}/K_M is the association of the enzyme and substrate the above rule may break down. As the pH is changed the chemical steps will slow down and eventually may become rate determining. This will cause a change in rate determining step with pH and an anomalous pH dependence of k_{cat}/K_M. The pK_a of the active site of δ-chymotrypsin at 25°, 5% Me_2SO, and $\mu = 0.95$ was found from kinetic experiments to be 6.83. However, the pH dependence of k_{cat}/K_M for the hydrolysis of acetyl-L-tryptophan *p*-nitrophenyl ester under these conditions follows a titration curve of $pK_a = 6.50$ and a maximum value of 3.1×10^7 sec^{-1} M^{-1}. This is consistent with the association of enzyme and substrate being partially rate determining in the acylation reaction at high pH and the association rate constant being 6×10^7 sec^{-1} M^{-1}.

The rate constants for enzyme–substrate complex formation are generally fast. They are often beyond the time scale of stopped-flow techniques due to the association reaction being completed during mixing and in the case of highly reactive substrates also inaccessible to temperature-jump methods due to the overall chemical reaction being completed during the incubation of reagents. However, under certain circumstances otherwise unobtainable rate constants may be inferred from steady-state kinetics after making mechanistic assumptions (Eigen and Hammes, 1963). We wish to present a novel application of this method.

The values of k_{cat}/K_M for some highly activated specific substrates of chymotrypsin are so high as to suggest rate determining association of the substrate with the enzyme[1] (Philipp and Bender, 1973). If this is so then there should be a change in rate determining step with pH giving rise to an anomalous pH dependence of k_{cat}/K_M. The pH dependence of k_{cat}/K_M is usually considered to give the pK_a values of the groups at the active site of the enzyme (or on the substrate) (Alberty and Massey, 1954). But, if in the case of an enzyme such as chymotrypsin whose activity depends on the ionization of a base, the rate determining step changes from a chemical step at low pH to the diffusion-controlled encounter of the enzyme with the substrate at high pH then k_{cat}/K_M will follow an ionization curve governed by a pK_a below that of the catalytically important base, as is illustrated in Figure 1.

Anomalous pK_a values in the pH dependence of the Michaelis–Menten parameters may arise in general from changes of rate determining step with pH (Fersht and Requena, 1971; Schmidt and Westheimer, 1971). The above analysis is a special case of this.

Materials and Methods

δ-Chymotrypsin was obtained from Sigma (lot 20C-0150) and contained 88% of the theoretical number of active sites (Fastrez and Fersht, 1973b). Acetyl-L-tryptophan *p*-nitrophenyl ester was obtained from Cyclo. Other materials have been described elsewhere (Fastrez and Fersht, 1973a,b).

Absorbance measurements were made on a Gilford 2400 spectrophotometer. Rapid reactions were monitored on the same apparatus using a stopped-flow mixing device of dead-time 4 msec and path length 1 cm. The output was recorded on Polaroid film using a Tektronix 564B storage oscilloscope.

The hydrolysis of a 10^{-4} M solution of acetyl-L-tyrosine *p*-acetylanilide was followed by the first-order increase in absorbance at 340 nm due to *p*-acetylaniline release using 1-cm cuvets and conventional spectrophotometry. The hydrolysis of acetyl-L-tryptophan and acetyl-L-phenylalanine *p*-nitrophenyl esters was monitored by the increase in absorbance of *p*-nitrophenolate at 400 nm or *p*-nitrophenyl at 340 nm using stopped-flow spectrophotometry as follows. One syringe of the mixer contained a freshly prepared solution of enzyme in buffer at ionic strength 0.95 and 5% Me_2SO. The other syringe contained a freshly prepared solution of the *p*-nitrophenyl ester obtained by diluting 0.5 ml of a stock ester solution in Me_2SO with 9.5 ml of 1 M KCl. Both solutions were incubated at 25° prior to mixing into the thermostated observation chamber. The total progress curve of the reaction was recorded. Each experiment was repeated in triplicate. Some experiments were performed at low pH using conventional techniques.

The experimental conditions along with the results are summarized in Tables I and II and Figure 3.

Results

The determinations of the Michaelis–Menten parameters for the hydrolysis of specific *p*-nitrophenyl ester substrates by chymotrypsin are difficult using conventional techniques for two reasons: at high pH the spontaneous hydrolysis rate is high and the accurate determination of k_{cat}/K_M requires that the reaction be monitored at substrate concentrations below

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[1] The rate determining step for the *overall* enzymic reaction is of course a function of substrate concentration as saturation kinetics are observed. In this paper we are concerned with the rate determining step in the term k_{cat}/K_M which in the particular case of chymotrypsin involves the acylation of the enzyme rather than the overall rate of hydrolysis.

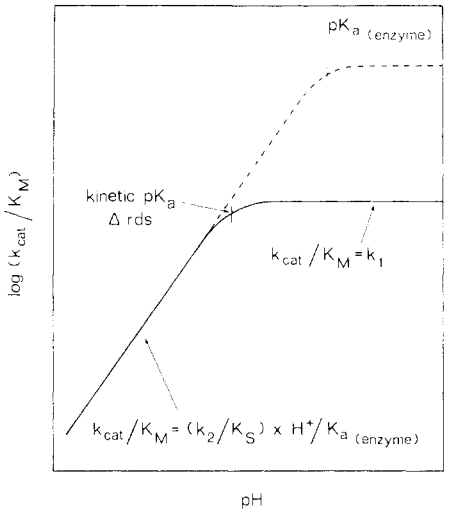


FIGURE 1: Illustration of how a change in rate determining step from a chemical event at low pH to the encounter of enzyme and substrate at high pH may lead to an anomalous pK_a in the k_{cat}/K_M–pH profile.

the K_M values which are typically 1–2 μM. The two problems were overcome by using stopped-flow spectrophotometry. The short time scale is fast with respect to spontaneous hydrolysis and also very small absorbance changes may be monitored without problems of long-term stability of the apparatus. A typical trace is illustrated in Figure 2 where it may be seen that absorbance changes as low as 0.005 could be accurately measured. Typically, concentrations of $K_M/5$ to $3K_M$ were used to determine k_{cat}/K_M. High substrate levels of 25–50K_M were also used to check k_{cat}. k_{cat}, K_M, and k_{cat}/K_M were obtained from the total progress curves of the reaction from the tangents to the curve at various substrate concentrations. The concentration of substrate was determined at each point by the difference in absorbance between it and the end point trace. In this procedure the determination of k_{cat}/K_M does not involve the difference in extinction coefficient between the nitrophenol and ester. This is useful as *p*-nitrophenol has a pK_a of 7.05, which is in the middle of the pH range studied. The determination of k_{cat} and K_M required the measurement of the extinction coefficient of *p*-nitrophenol at each pH.

Acetyl-L-tyrosine *p*-acetylanilide was chosen as a slowly reacting substrate to check the pH dependence of k_{cat}/K_M. The high value of K_M enables k_{cat}/K_M to be determined from a single experiment at a substrate concentration well below K_M. An initial concentration of about $K_M/50$ was hydrolyzed by the enzyme to give an excellent first-order increase in A_{340} as the products were released with rate constant (k_{cat}/K_M)·[chymotrypsin]. We have shown that the pH dependence of k_{cat}/K_M determined in this way agrees well with that determined from Lineweaver–Burk plots, but is experimentally easier and more reproducible and accurate (A. R. Fersht and M. Renard, manuscript in preparation).

δ-Chymotrypsin was used throughout the study due to the

TABLE I: Summary of Experimental Conditions and Results for the Hydrolysis of Acetyl-L-phenylalanine *p*-Nitrophenyl Ester by δ-Chymotrypsin.[a]

pH	[Enzyme] ($\times 10^6$ M)	[Substrate] ($\times 10^6$ M)	k_{cat} sec^{-1}	K_M ($\times 10^6$ M)	k_{cat}/K_M ($\times 10^{-7}$ sec^{-1} M^{-1})
5.67	0.16	0.47–11.25	9.31	1.79	0.52
5.91	0.16	0.39–2.34	14.9	1.80	0.83
6.03	0.16	0.39–1.37	17.5	1.52	1.15
6.10	0.16	0.7–9.72	22.6	1.79	1.26
6.20	0.16	0.63–4.3	26.5	2.5	1.06
6.42	0.16	0.34–9.0	39	2.35	1.66
6.62	0.16	0.47–4.4	43	1.89	2.29
6.77	0.16	0.7–6.6	64.4	2.67	2.41
6.86	0.16	0.27–1.9	79	2.97	2.67
7.00	0.16	0.56–7.9	83	2.58	3.22
7.30	0.16	0.34–5.0	93.6	2.26	4.14
7.41	0.16	0.44–1.96	105	2.63	4.01
7.52	0.16	0.22–1.6			4.63
7.66	0.16				4.96
7.66	0.16	0.28–4.8			4.99
2.09[b]	14	1.6–104	0.0042	6.68	6.3×10^{-5}
2.59[b]	2.9	0.6–110	0.011	2.95	3.7×10^{-4}
3.07[b]	2.9	1.4–133	0.03	3.6	8.2×10^{-4}
4.59[b]	0.09	0.3–77	0.98	1.2	8.3×10^{-2}
5.73	11.2	2–48	10.2		
6.42	0.58	1–71	37.1		
6.77	0.58	1–60	65		
7.30	0.58	1–60	106		
7.67	0.58	1–60	121		

[a] 25°, μ = 0.95, 5% Me_2SO. [b] Monitored by conventional slow methods.

TABLE II: Summary of Experimental Conditions and Results for the Hydrolysis of Acetyl-L-tryptophan *p*-Nitrophenyl Ester[a] by δ-Chymotrypsin.

pH	[Enzyme] ($\times 10^6$ M)	[Substrate] ($\times 10^6$ M)	k_{cat} (sec^{-1})	K_M ($\times 10^6$ M)	k_{cat}/K_M ($\times 10^{-7}$ sec^{-1} M^{-1})
5.68	0.16	0.31–4.8	4.4	1.03	0.426
5.91	0.16	0.27–3.4	6.99	1.02	0.70
6.04	0.16	0.16–3.4	7.25	0.70	1.04
6.11	0.16	0.31–4.7	9.29	1.03	0.90
6.20	0.16	0.31–4.2	10.6	1.13	0.943
6.42	0.16	0.27–5.4	15.4	1.26	1.23
6.62	0.16	0.39–3.9	20.1	1.17	1.72
7.00	0.16	0.18–3.4	30.0	1.22	2.46
7.22	0.16	0.37–2.8	42.6	1.89	2.25
7.29	0.16	0.38–4.5	45.5	1.87	2.44
7.32	0.16	0.34–2.36	42	1.6	2.69
7.40	0.16	0.36–3.2	50	1.72	2.91
7.48	0.16	0.34–3.4	52	1.85	2.81
7.56	0.16	0.38–2.84	48.5	1.72	2.82
7.56	0.16	0.36–3.9	53	1.78	2.97
7.66	0.16	0.34–4.2	57	2.1	2.77
5.68	0.6	3–55	4.4		
6.11	0.6	3–55	10.2		
6.42	0.6	3–55	17.5		
6.78	0.6	3–55	31.7		
7.00	0.6	3–55	38.8		
7.32	0.6	3–55	50.7		
7.66	0.6	3–55	61.7		

[a] 25.0°, μ = 0.95, 5% Me_2SO.

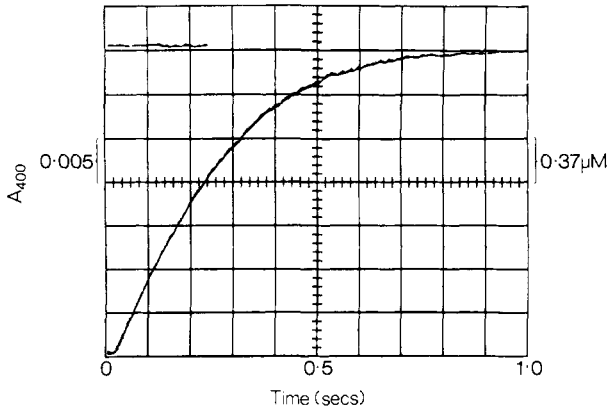


FIGURE 2: Typical trace of the release of *p*-nitrophenolate against time used for the determination of k_{cat}/K_M. Acetylphenylalanine *p*-nitrophenyl ester (10 μM) was mixed with 0.32 μM enzyme in pH 7.66 buffer in the stopped-flow spectrophotometer.

artifacts which may occur from the equilibration of the inactive and active conformations of the enzyme (Fersht, 1972a). Under our conditions there was less than 1% of the inactive conformation at pH 7.6 and only 5% at pH 6 so that it could be neglected.

The results at 25° and ionic strength 0.95 are summarized in Tables I and II.

The important experimental observations are: k_{cat}/K_M for the hydrolysis of acetyltyrosine *p*-acetylanilide fits an ionization curve of $pK_a = 6.83$ and $(k_{cat}/K_M)_{max} = 389$ sec^{-1} $_M{}^{-1}$ (Figure 3); k_{cat}/K_M for the hydrolysis of acetylphenylalanine *p*-nitrophenyl ester fits an ionization curve of $pK_a = 6.84$ and $(k_{cat}/K_M)_{max} = 5.6 \times 10^7$ sec^{-1} $_M{}^{-1}$, but there is a positive deviation below pH 6.2 (Figure 4); k_{cat}/K_M for acetyltryptophan *p*-nitrophenyl ester fits an ionization curve of $pK_a = 6.50$ and $(k_{cat}/K_M)_{max} = 3.1 \times 10^7$ sec^{-1} $_M{}^{-1}$ (Figure 5). k_{cat} is well behaved for both nitrophenyl esters; the values for the AcPhe and AcTrp compounds fit an ionization curve of $pK_a = 6.83$ and 6.90 and $(k_{cat})_{max} = 139$ and 65 sec^{-1}, respectively (Figure 6).

The pK_a value controlling k_{cat}/K_M for the hydrolysis of acetyltryptophan *p*-nitrophenyl ester by δ-chymotrypsin is some 0.33 unit below that found for well-behaved substrates. This deviation is well beyond the limits of experimental error, which we estimate to be not more than ±0.15 pH unit at the very most.

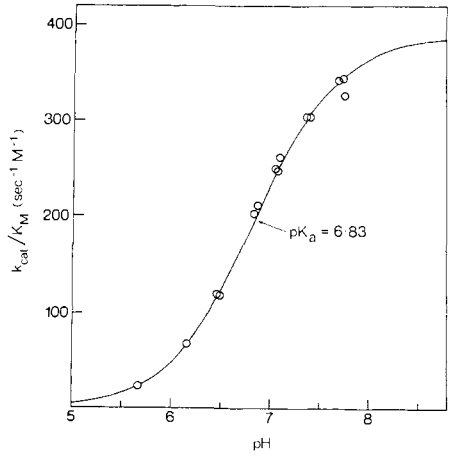


FIGURE 3: k_{cat}/K_M against pH for the hydrolysis of acetyltyrosine *p*-acetylanilide by δ-chymotrypsin at 25°, 5% Me_2SO and μ = 0.95.

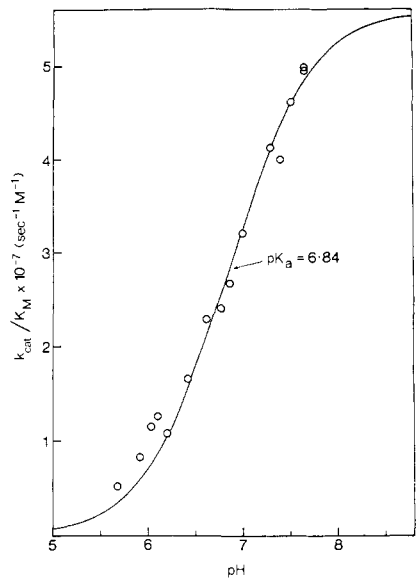
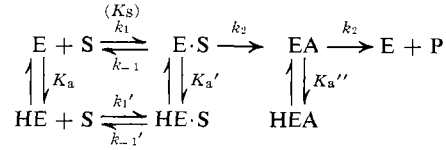


FIGURE 4: k_{cat}/K_M against pH for the hydrolysis of acetylphenylalanine *p*-nitrophenyl ester by δ-chymotrypsin at 25°, 5% Me_2SO, and μ = 0.95.

Discussion

The mechanism of the hydrolysis of esters and amides by chymotrypsin is well established. The initial formation of a noncovalently bound enzyme–substrate complex is followed by the acylation of the enzyme. This chemical step is catalyzed by the basic form of a histidine residue of $pK_a \simeq 6.8$ as is also the subsequent deacylation of the acyl-enzyme (Bender and Kézdy, 1964; McConn *et al.*, 1971; Fastrez and Fersht 1973b).

SCHEME I



Under normal circumstances the binding steps are fast and k_{-1} is much greater than k_2. The catalytic constant k_{cat}/K_M is then equal to $k_2(k_1/k_{-1})$, *i.e.* k_2/K_S, and its pH dependence follows a titration curve of ionization constant K_a, the value

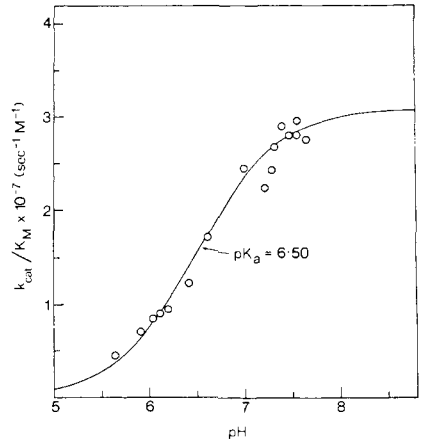


FIGURE 5: k_{cat}/K_M against pH for the hydrolysis of acetyltryptophan *p*-nitrophenyl ester by δ-chymotrypsin at 25°, 5% Me_2SO, and μ = 0.95.

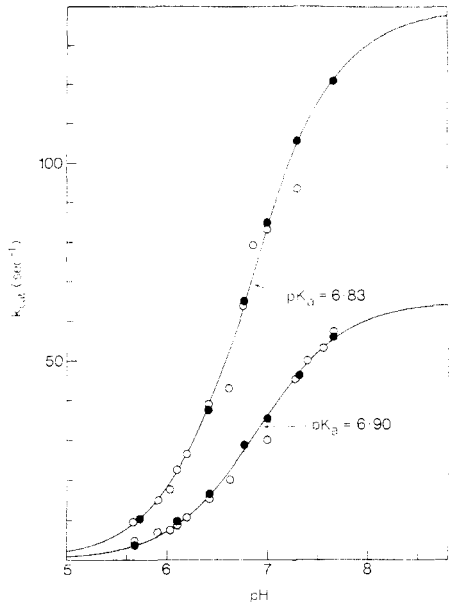


FIGURE 6: Plots of k_{cat} against pH for the hydrolysis of acetylphenylalanine *p*-nitrophenyl ester (upper curve) and acetyltryptophan *p*-nitrophenyl ester (lower curve) at 25°, 5% Me_2SO, and μ = 0.95: (●) values from high initial substrate concentrations; (○) from low substrate concentrations (less accurate).

for the histidine in the free enzyme (Alberty and Massey, 1954). This has been confirmed by Bender *et al.* (1964). But if $k_2 \simeq k_{-1}$ then at high pH $k_{cat}/K_M = k_1k_2/(k_{-1} + k_2)$. And if $k_2 \gg k_{-1}$, $k_{cat}/K_M = k_1$. The pH dependence of k_{cat}/K_M is then more complicated (eq 1). The pH dependence may be divided into two cases.

$$k_{cat}/K_M = \frac{k_2\{k_1 + k_1'([H^+]/K_a)\}}{\{1 + ([H^+]/K_a)\}\{k_2 + k_{-1} + [H^+](k_1k_{-1}/k_1'K_a)\}} \quad (1)$$

Case A, $k_1 = k_1'$. Equation 1 simplifies into eq 2. The $k_{cat}/$

$$k_{cat}/K_M = \frac{k_2k_1}{k_2 + k_{-1} + [H^+](k_{-1}/K_a)} \quad (2)$$

K_M–pH profile is a simple sigmoid with apparent ionization constant K_{app} given by

$$K_{app} = K_a(k_2 + k_{-1})/k_{-1} \quad (3)$$

The limiting value of k_{cat}/K_M is given by

$$(k_{cat}/K_M)_{max} = k_1k_2/(k_{-1} + k_2) \quad (4)$$

Case B, $k_1 \neq k_1'$. The k_{cat}/K_M–pH profile is a double sigmoid curve defined by two ionization constants: $K_1 = K_a(1 + (k_1/k_1')[(k_2 + k_{-1})/k_{-1}])$ and $K_2 = K_a(1 + (k_1'/k_1)[k_{-1}/(k_2 + k_{-1})])^{-1}$. If $k_2 \gg k_{-1}$ there will be two plateau regions in the curve—see Figure 7. At high pH $k_{cat}/K_M = k_{-1}k_2/(k_1 + k_2)$ as before but there will be a lower pH plateau where $k_{cat}/K_M = k_1'k_2/(k_2 + k_{-1} + k_1'k_{-1}/k_1)$. The low pH p$K_a$ is due to the change in rate determining step from acylation to association. The high pH pK_a, which is approximately equal to the real pK_a of the enzyme, is due to the increase in the association rate constant as the active site histidine becomes deprotonated. But if $k_2 \simeq k_{-1}$ and $k_1 > k_1'$ the two plateaus merge

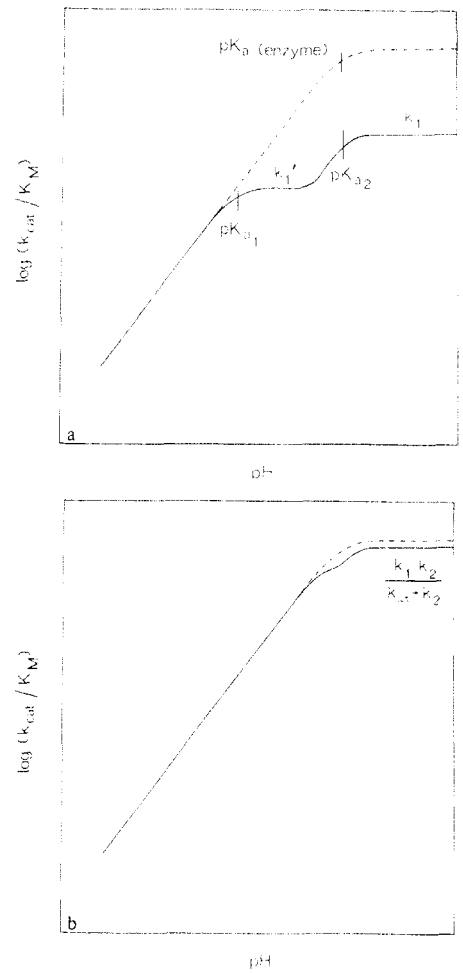


FIGURE 7: Illustration of the pH dependence of k_{cat}/K_M in case B. (a) The rate determining step at high pH is the diffusion-controlled encounter of the substrate and enzyme, but the rate constant is higher for the unprotonated form of the enzyme. (b) The association step is only partially rate determining and the double sigmoid tends to merge into a single.

and the curve will approximate to a simple sigmoid curve of ionization constant $\sim K_a$. Indeed it may be easily demonstrated that if $K_1 = 4K_2$ and $k_{cat}K_M$ at high pH is twice that at low pH the analytical form of the curve is an *exact* sigmoid of ionization constant $2K_2$. In case B where $k_2 \simeq k_{-1}$ it is experimentally very difficult to analyze the pH dependence of k_{cat}/K_M. Case A is simple to analyze. The apparent pK_a of the enzyme is lower than determined for slowly reacting substrates. Case B approximates to case A if $k_1 \simeq k_1'$.

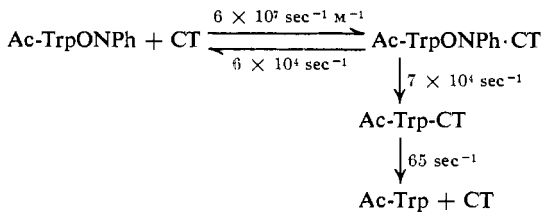
pK_a of δ-Chymotrypsin. Before searching for deviations in k_{cat}/K_M–pH profiles the true pK_a of the enzyme must be known. This is assumed to be the value observed for the hydrolysis of slowly reacting substrates as found by Bender *et al.* (1964). The value obtained from acetyltyrosine *p*-acetylanilide, which is a representative substrate (A. R. Fersht and M. Renard, manuscript in preparation), is 6.83. This is confirmed by examination of k_{cat}–pH profiles for the ester substrates. Hammond and Gutfreund (1955) found that k_{cat} for the deacylation of acetylphenylalanine-chymotrypsin follows the pK_a of k_{cat}/K_M. Bender *et al.* (1964) found that k_{cat} for the deacylation of acetyltryptophan-chymotrypsin gives a pK_a value 0.9 unit above that for k_{cat}/K_M. We find that the pK_a for the deacylation rate constant of acetylphenylalanine-chymotrypsin is 6.83 and that for the tryptophan case is 6.90 under our conditions.

Hydrolysis of Acetyl-L-tryptophan p-Nitrophenyl Ester. k_{cat}/K_M for the hydrolysis of this ester fits a sigmoid curve of $pK_a = 6.50$, 0.33 unit below the pK_a of the enzyme. A change of rate determining step with pH is indicated. The simplest explanation is the analysis given above. However, a more complex mechanism with additional intermediates and involving parallel reaction paths could generate the same situation. We shall make the plausible, but somewhat arbitrary, interpretation of the data that the association rate constant is partially rate determining in the acylation of the enzyme at high pH. The analysis of steady-state data in this way usually involves mechanistic assumptions (Eigen and Hammes, 1963).

The analysis of case A appears appropriate as the curve is a simple sigmoid. k_1 and k_1' must be approximately equal. k_2 is determined from eq 3 to be equal to $1.1k_{-1}$. Substituting this and the observed limiting value of k_{cat}/K_M of 3.1×10^7 sec^{-1} M^{-1} into eq 4 gives $k_1 = 5.9 \times 10^7$ sec^{-1} M^{-1}, *i.e.* the rate constant for the association of acetyltryptophan *p*-nitrophenyl ester with δ-chymotrypsin is about 6×10^7 sec^{-1} M^{-1}. This may be compared with values of 6.2×10^6 sec^{-1} M^{-1} for furylacryloyl-L-tryptophanamide at 15° (Hess *et al.*, 1970), 10^8 sec^{-1} M^{-1} for proflavine at 12° (Havsteen, 1967), both obtained from temperature-jump experiments, and 1.5×10^7 sec^{-1} M^{-1} for trifluoroacetyl-D-tryptophan at 32° (Smallcombe *et al.*, 1972), determined by nuclear magnetic resonance.

The value of 6×10^7 sec^{-1} M^{-1} is about an order of magnitude below that expected for a diffusion-controlled encounter between the molecules (Eigen and Hammes, 1963) and presumably reflects the orientational and other small requirements of the process.

k_2 cannot be directly determined by this procedure but may be estimated by assuming a value of K_S (*i.e.*, k_{-1}/k_1). The dissociation constants of acetyl-L-tryptophanamide and ethyl ester from the enzyme are 5 and 2×10^{-3} M, respectively. The nitrophenyl ester will bind more tightly due to the additional hydrophobic group (Fastrez and Fersht, 1973a) so that K_S is expected to be $\sim 1 \times 10^{-3}$ M. Assuming this value, k_{-1} is 6×10^4 sec^{-1} so that k_2 is 7×10^4 sec^{-1}. It should be noted that these values are for productive binding; additional nonproductive modes will give lower observed experimental values for K_S and k_2 (*cf.* Fastrez and Fersht, 1973a).



Hydrolysis of Acetyl-L-phenylanine p-Nitrophenyl Ester. The plot of k_{cat}/K_M against pH fits well a sigmoid curve of $pK_a = 6.84$ for the region above pH 6.2. Below pH 6.2 there is a significant positive deviation; the points are about 40% high on average. Normally this would be considered as experimental error and ignored. In view of the previous discussion and the high value of 5.6×10^7 sec^{-1} M^{-1} for k_{cat}/K_M at pH 7.6 we feel that this is probably an example of case B where $k_{-1} \sim k_2$ and $k_1 > k_1'$. This gives the merged double sigmoid which is extremely difficult to distinguish from a simple sigmoid curve. In these circumstances it is not possible to analyze the curve for the association rate constant or even to prove conclusively from the pH dependence that there is a partial change in rate determining step. However, as these experiments were performed under identical conditions to those for the acetyltryptophan derivative and yield a clearly different pK_a value for k_{cat}/K_M this is strong evidence for a partial change in rate determining step as a function of pH for the acetyltryptophan *p*-nitrophenyl ester.

Interpretation of k_{cat}- and k_{cat}/K_M-pH Profiles. The simple theory that the pH dependence of k_{cat} gives the pK_a of the enzyme–substrate complex and that of k_{cat}/K_M the pK_a of the free enzyme (Alberty and Massey, 1954) breaks down when different steps of the reaction depend on different protonic states of the enzyme (Fersht and Requena, 1971; Schmidt and Westheimer, 1971). The apparent pK_a values obtained in these cases are combinations of the real ionization constants and ratios of *rate* constants. The apparent pK_a values do not represent the titration of the enzyme so that, in principle, they may be detected by titration experiments such as proton release or uptake on substrate binding (Fersht, 1972b) or by straightforward pK_a determination by nuclear magnetic resonance for example. Also, as the pH dependence of K_M should give the pK_a values of both the enzyme and the enzyme–substrate complex (Dixon, 1953), the real pK_a values, in theory, may be determined in this way and compared with the rate constant data (Fersht and Requena, 1971).

Artifactual pK_a values may also occur when there is nonproductive binding which is pH dependent (Fastrez and Fersht, 1973a). This will then cause anomalous pK_a values in the pH dependence of k_{cat} (but not of k_{cat}/K_M). In this case the observed pK_a value of the enzyme–substrate complex is composed of the real ionization constant and the *equilibrium* constants for nonproductive and productive binding. As this pK_E is composed of equilibrium constants only, it represents a real titration of the enzyme and the artifact cannot be detected by titration experiments or by Dixon (1953) plots on K_M.

Some caution must be applied in interpreting the pH dependence of Michaelis–Menten parameters and assignments of pK_a values. Fortunately the exceptions to the simple theory of Alberty and Massey (1954) are rare so that the large amount of useful information obtained from pH–rate data has been interpreted correctly. The exceptions to the rule provide additional kinetic information.

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Mechanism of the Reaction Catalyzed by the Catalytic Subunit of Aspartate Transcarbamylase. Kinetic Studies with Carbamyl Phosphate as Substrate†

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ABSTRACT: The catalytic subunit prepared by treatment of native aspartate transcarbamylase with *p*-mercuribenzoate has been used to investigate the mechanism of the reaction. The results of initial velocity, product inhibition, dead-end inhibition, isotope transfer, and binding studies are consistent with the reaction having a random mechanism for which the rate of interconversion of the central complexes appears to be slow compared with all other steps of the reaction sequence. Further, they indicate that the mechanism involves the formation of three dead-end complexes: enzyme–aspartate–P_i, enzyme–aspartate–carbamyl aspartate, and enzyme–carbamyl phosphate–carbamyl aspartate. In addition, initial velocity and product inhibition studies have been made using the catalytic subunit obtained by subjecting the native aspartate transcarbamylase to limited proteolytic digestion by trypsin. The resulting data are qualitatively similar to those obtained with the mercurial catalytic subunit and in accord with the reaction approximating to a rapid equilibrium, random mechanism with three dead-end complexes. This postulate contrasts with previous conclusions that the reaction has an ordered mechanism. Dissociation constants have been determined for the reaction of substrates with various enzyme forms. However, some of these are not precise because of the difficulties associated with obtaining accurate, steady-state kinetic data under conditions where carbamyl phosphate binds strongly to the enzyme.

Both the native form of aspartate transcarbamylase, isolated from *Escherichia coli*, and the catalytic subunit derived from it by treatment with *p*-mercuribenzoate catalyze the reaction: carbamyl phosphate + aspartate → carbamyl aspartate + P_i. From investigations of the binding of succinate, an inhibitory analog of aspartate, to the catalytic subunit, it was reported that the binding was dependent on the presence of carbamyl phosphate (Changeux *et al.*, 1968). Thus, it was inferred that the reaction involving aspartate would occur *via* an ordered mechanism with carbamyl phosphate as the first substrate to add to the enzyme. This conclusion was supported by the results of steady-state kinetic studies from which it was concluded that carbamyl aspartate was released from the enzyme before P_i (Porter *et al.*, 1969).

By contrast with the above findings, the results of Collins and Stark (1969) showed that succinate did combine with the catalytic subunit although its reaction was considerably enhanced in the presence of carbamyl phosphate. In addition, these authors showed that the catalytic subunit is capable of binding aspartate. Moreover, aspartate influences the rate of digestion of the native enzyme by proteolytic enzymes (McClintock and Markus, 1968, 1969). These results raise the possibility that substrates bind in a random manner to both the native enzyme and catalytic subunit.

At the time of publication of the paper by Porter *et al.* (1969), steady-state kinetic investigations were being carried out with the catalytic subunit which can be obtained by limited digestion of the native aspartate transcarbamylase with trypsin (Heyde *et al.*, 1973) and whose physicochemical properties are similar to those of the subunit obtained using *p*-mercuribenzoate (Gerhart and Holoubek, 1967). Since it was considered that the mechanism of the reaction catalyzed by the latter catalytic subunit had not been definitely established, the kinetic investigations were extended to include work with this enzyme form. From the results of these studies, it has been concluded that the reactions catalyzed by both catalytic subunits conform to a random mechanism which appears to be of the rapid equilibrium type and which involves the formation of three dead-end complexes. The conclusion is supported by the data obtained from isotope transfer and binding studies as well as by the results of kinetic investigations with acetyl phosphate as a substrate (Heyde and Morrison, 1973).

Experimental Section

Materials. L-Aspartic acid (A grade), [2,3-^{14}C]succinic acid (10.2 mCi/mmol), carbamyl aspartate (DL-ureidosuccinic acid, A grade), dilithium carbamyl phosphate (B grade), and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid[1] (TES, A grade) were obtained from Calbiochem. The L isomer of carbamyl aspartate was prepared by the method of Korn (1957) and converted to the sodium salt by passage of a solution through a column of Zeo-Karb 225 (Na^+ form). Carbamyl phosphate was purified by repeated precipitations from solu-

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[1] Abbreviation used is: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.