Chymotrypsin Catalysis. Evidence for a New Intermediate. II[1]

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Abstract: The kinetics of the reaction of chymotrypsin with *N*-acetyl-L-tyrosine *p*-methoxy- and *p*-chloroanilide have been studied and the principal observations are as follows. (1) V_{max} for the methoxyanilide is larger (11.4 fold) than that of the chloroanilide at pH 7.95. (2) The pK influencing V_{max} with the methoxyanilide is higher than that for reaction with the chloroanilide so that the relatively greater reactivity of the former compound diminishes at lower pH's. (3) K_m is smaller (16.2 fold) for the chloroanilide than the methoxyanilide at pH 7.95 and this difference shows signs of disappearing at pH 6.2. These results have been accounted for by a previously proposed scheme[3d] in which there is a coupling of a substituent-dependent catalytic step—tetrahedral intermediate formation—with substrate binding and proton dissociation from the active-site histidine residue. To test this proposal we have studied the binding of the above anilides to a catalytically inactive form of the enzyme which has a methyl group on N-3 of the active-site histidine residue. The modified enzyme has unchanged affinity for proflavin, from which it is concluded that the binding site is intact. In support of the hypothesis that the substituent and pH dependence of anilide binding reflect a coupling of catalysis with proton and substrate binding, the binding of anilides to the catalytically inactive derivative shows no sensitivity to either electronic substituents or pH (in the pH 7.95–6.2 range). The role of pretransition-state proton transfer in chymotrypsin catalysis is evaluated.

Previous studies of the CT[2]-catalyzed hydrolysis of anilides[3] have shown that in several ways the kinetics of this reaction are unusual. The rate-determining step of the reaction is acylation of the enzyme, so that V_{max} is the rate constant for enzyme acylation and the observed K_m is presumably the true dissociation constant of the Michaelis–Menten complex. Although V_{max} would be expected to depend on the nature of substituents in the leaving group, it is not immediately apparent why the pH dependence of V_{max} and the magnitude and pH dependence of K_m should be markedly substituent dependent, as they are observed to be.[3] One of us[3d] has proposed a mechanism for the hydrolysis which does account for these observations. The work presented here was undertaken to test this proposal further.

Experimental Section

Materials. *N*-Acetyl-L-tyrosine was converted to the anilide by the method of Anderson, *et al.*[4] A solution of 1.12 g (5 mmol) of the acid and 0.6 ml of *N*-methylmorpholine in 75 ml of tetrahydrofuran (freshly distilled off calcium hydride) was cooled to 0°. The solution was stirred vigorously as 0.65 ml of isobutyl chloroformate was added, followed 1 min later by 5.5 mmol of the aniline dissolved in tetrahydrofuran. The reaction vessel was flushed with nitrogen and sealed, and the solution was stirred for 2 hr at room temperature. After the solvent was removed under vacuum, trituration with 0.1 *N* hydrochloric acid solidified the oily mass. Both anilides were contaminated with colored impurities and these were removed as follows. For *p*-methoxyanilide the solid was ground up in 1 *N* hydrochloric acid, using a mortar and pestle, until it was white. The harvested precipitate was washed with water, and recrystallized twice from acetone: yield, 0.95 g; mp 218–219° (lit. 220–221°,[3c] 205–206°[3b]). For *p*-chloroanilide the solid was dissolved in ethyl acetate and the solution washed with portions of 0.1 *N* sodium hydroxide solution until the aqueous layer no longer became colored. After washing the ethyl acetate phase and drying over magnesium sulfate the solvent was removed under vacuum until crystals began to form. The solution was then heated to boiling and petroleum ether was added to produce opalescence; crystallization occurred on cooling. The yield was increased by harvesting crystals which deposited after a few minutes from the alkaline extracts. These were washed with water, and treated in the same way as the original solid: yield, 0.42 g; mp 228–230° (lit. 224–225°,[3c] 227–228°[3b]).

Three-times crystallized CT was obtained from Worthington, lots CDI OBK and CDI OLB. Anhydro-CT was prepared as described by Weiner, *et al.*[5] Methyl-CT was prepared as described by Nakagawa and Bender,[6] except that after modification of the enzyme was complete the solution was applied to a G-25 Sephadex column and the protein eluted with water. The protein was isolated by lyophilization. The activity of the modified enzymes was determined by titration with *p*-nitrophenyl acetate and was found to be less than 0.5% of that of the native enzyme.

Proflavin was purified as described previously.[7] DMF was purified by shaking with solid potassium hydroxide, and then solid calcium oxide, followed by distillation under reduced pressure.

Kinetic Studies. CT solutions were made up daily in 10^{-3} M hydrochloric acid and stored at 2°; concentrations were determined spectrophotometrically using $\epsilon_{280} = 5 \times 10^4$.[8] Reactions were carried out in stoppered 1-cm cuvettes in the thermostated cell compartment of a Zeiss PMQ II spectrophotometer. Production of the aniline was monitored at 305 nm. The molar absorbance change for the reactions at each pH was determined by allowing several of the reactions to go to completion. Values of V_{max} and K_m were obtained by a computer fit to the Michaelis–Menten equation as described by Hanson, *et al.*[9]

Binding Studies. Protein solutions were either filtered through a millipore filter (BDWPO 1300) or centrifuged for 1 hr at 10^5 G in a Beckmann Model L ultracentrifuge to remove all insoluble matter. Concentrations of the solutions were obtained assuming $\epsilon_{280} = 5 \times 10^4$ for both the native and modified enzymes. The concentration of proflavin solutions was determined using $\epsilon_{465} = 1.62 \times 10^4$.[7] The value of ϵ_{465} for proflavin in 5% DMF–water (v/v) was determined over the pH range 7.95–6.25 and was found to be pH independent and equal to $2.00 \pm 0.01 \times 10^4$ ODU M^{-1} cm^{-1}. Proflavin solutions were protected from light when not in use. Difference spectra were determined using matched 5-cm path-length cells. All measurements were made in the thermostated cell compartment of a Zeiss PMQ II spectrophotometer.

(1) Supported by a grant from the National Institutes of Health (DE03246).

(2) Abbreviations used are: CT, chymotrypsin; DMF, dimethylformamide.

(3) (a) T. Inagami, S. S. York, and A. Patchornik, *J. Amer. Chem. Soc.*, **87**, 126 (1965); (b) L. Parker and J. H. Wang, *J. Biol. Chem.*, **243**, 3729 (1968); (c) T. Inagami, A. Patchornik, and S. S. York, *J. Biochem. (Japan)*, **65**, 809 (1969); (d) M. Caplow, *J. Amer. Chem. Soc.*, **91**, 3639 (1969).

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(5) H. Weiner, W. N. White, D. G. Hoare, and D. E. Koshland, *ibid.*, **88**, 3851 (1966).

(6) Y. Nakagawa and M. L. Bender, *Biochemistry*, 9, 259 (1970).

(7) J. L. Marini and M. Caplow, *J. Amer. Chem. Soc.*, **93**, 5560 (1971).

(8) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1958).

(9) K. R. Hanson, R. Ling, and E. Havir, *Biochem. Biophys. Res. Commun.*, 29, 194 (1967).

The dissociation constant (K_D) for the proflavin–enzyme complex was determined by measuring the absorbance difference (A_{ED}) at 465 nm between solutions containing a fixed (low) concentration of proflavin ([D]) and varying amounts of enzyme ([E]) and a reference solution that was identical except that the enzyme was omitted. The conditions used were [E] > [D], and therefore eq 1 holds.[10a]

$$\frac{1}{[E]} = \frac{[D]\Delta\epsilon_{465}}{K_D}\frac{1}{(A_{ED} - A_E)} - \frac{1}{K_D} \quad (1)$$

In this equation A_E is the absorbance of the enzyme at 465 nm, which was determined for each enzyme solution used, and $\Delta\epsilon_{465}$ is the difference between the molar extinction coefficients of the proflavin–enzyme complex and the free dye. The equation has the same form as the Michaelis–Menten equation and the values of K_D and $\Delta\epsilon_{465}$ were calculated using the computer program described above.

The binding of the anilides to methyl-CT was determined by competitive binding with proflavin. Positive absorbance differences were measured by using reference solutions containing fixed concentrations of proflavin and enzyme and varying concentrations of anilide, and sample solutions which were identical except that the anilide was omitted. Since [anilide] > [enzyme] > [proflavin] the dissociation constant of the anilide–enzyme complex is given by eq 2,[10a] where [S_T] is the total concentration of anilide, [E_T] the total

$$K_S = \frac{K_D[ED][S_T]}{[E_T][D_F] - K_D[ED] - [ED][D_F]} \quad (2)$$

concentration of enzyme, [D_F] the concentration of unbound proflavin, and [ED] the concentration of enzyme–proflavin complex. Control experiments showed that absorption by the anilides was negligible at 465 nm, and that there was no detectable interaction (as determined by comparison of dye + anilide *vs.* dye absorbance) between the anilides and proflavin.

Results

The dissociation constants for the complexes between proflavin and CT, methyl-CT, and anhydro-CT are given in Table I. Values of K_D and $\Delta\epsilon_{465}$ are the same

Table I. Complexation of Proflavin with Chymotrypsin, Methylchymotrypsin, and Anhydrochymotrypsin[a]

Enzyme	$K_D \times 10^5$, M[b]	$\Delta\epsilon_{465} \times 10^{-4}$, ODU M^{-1} cm^{-1} [b]
CT	3.66 (0.30)	1.85 (0.10)
Methyl-CT	3.41 (0.17)	1.76 (0.03)
	4.19 (0.20)	1.87 (0.03)
Anhydro-CT	28.7 (8.21)	0.536 (0.113)
	29.6 (11.7)	0.296 (0.059)

[a] Reactions at 25° in 0.04 M phosphate buffer, pH 7.45, ionic strength 0.1. The proflavin concentration was *ca.* 6×10^{-6} M and the enzyme range was 2–25 $\times 10^{-5}$ M. [b] Calculated values are followed by the computer-calculated standard error.

for CT and methyl-CT, and these differ markedly from those for anhydro-CT. The K_D for anhydro-CT agrees well with that determined by Weiner, *et al.*,[5] by equilibrium dialysis at 2° and pH7. However, the difference in $\Delta\epsilon_{465}$ observed in two different experiments is disturbing. The only known difference in the history of the samples for these two experiments is that the one with the lower value had been stored at 2° for 10 days while the other was used freshly prepared. Storage at 2° for 4 weeks as the solid did not affect the parameters obtained with methyl-CT. We have confirmed the previous report[5] that the solubility of anhydro-CT is markedly reduced as compared to the native enzyme (to *ca.* 8 mg/ml at pH 7 and 20°). The solubility of the altered enzyme increases markedly above and below pH 7. The altered proflavin binding and decreased solubility suggests that the enzyme structure is drastically changed in the synthesis of anhydro-CT and the mechanistic conclusion previously derived[5] from studies of this derivative are, therefore, subject to considerable uncertainty.

Because of the low solubility of the anilides, and in order to compare our results with those of Inagami, *et al.*,[3c] the kinetic and binding studies were carried out in 5% DMF–water (v/v). Table II gives the parameters

Table II. Binding of Proflavin to Methylchymotrypsin at 25° [a]

pH	$K_D \times 10^5$, M[b]	$\Delta\epsilon_{465} \times 10^4$, ODU M^{-1} cm^{-1} [b]
7.95	1.35 (0.09)	1.55 (0.05)
7.97	1.30 (0.08)	1.60 (0.05)
7.94	1.29 (0.06)	1.62 (0.03)
6.70	2.56 (0.12)	1.32 (0.04)
6.66	2.49 (0.11)	1.40 (0.04)
6.25	4.31 (0.48)	1.46 (0.11)
6.26	4.42 (0.41)	1.46 (0.10)

[a] Reactions in 5% DMF–water (v/v) in 0.08 M phosphate buffer (pH 6.2 and 6.6) and 0.17 M Tris (pH 7.9), ionic strength 0.095. The proflavin concentration was *ca.* 7×10^{-6} M and the enzyme concentration was 3–25 $\times 10^{-5}$ M. [b] The calculated values are followed by the computer-calculated standard error.

for the proflavin–methyl-CT complex under the experimental conditions used in the studies with the anilides. The pH independence of the molar extinction coefficient and the increase in K_D with decreasing pH parallels the effects of pH on the proflavin–CT complex.[7]

Dissociation constants for the anilide–methyl-CT complexes are given in Table III. The large standard

Table III. Binding of Acetyl-L-tyrosine Anilides to Methylchymotrypsin[a]

Anilide	pH	K_S,[b] mM	No. of determ
p-Methoxy-	7.95	12 (2)	11
	6.66	16 (3)	10
	6.25	11 (2)	9
p-Chloro-	7.95	11 (2)	10
	6.66	13 (3)	8
	6.25	13 (3)	15

[a] Reactions at 25° in 5% DMF (v/v), ionic strength 0.095, 0.08 M phosphate buffer (pH 6.2 and 6.6) or 0.17 M Tris (pH 7.9). The proflavin and enzyme concentrations were *ca.* 6.5×10^{-6} and *ca.* 1.5×10^{-4} M, respectively. The anilide concentrations were 0.6–4 $\times 10^{-3}$ M with the methoxyanilide and 0.6–2.5 $\times 10^{-3}$ M with the chloroanilide. [b] The standard error is given in parentheses.

errors arise because the low solubilities of the anilides prevent achieving more than 30% saturation of the enzyme with the *p*-methoxyanilide and 20% with the *p*-chloroanilide. Hence, the measured absorbance differences are small (0.01–0.04 ODU), and the values of K_S are determined in a sensitive region of the titration curve. The major source of experimental error is in making up solutions, since the measured absorbance difference is only *ca.* 1–5% of the absorbance of the sample solution, so that small errors in concentrations are magnified. We consider the maximum possible

(10) (a) K. G. Brandt, A. Himoe, and G. P. Hess, *J. Biol. Chem.*, **242**, 3973 (1967); (b) according to Scheme I the apparent pK affecting V_{max} and K_m will be identical. The large experimental error with the tryptophan compounds[3d] prevents a conclusive analysis of this point.

undetected contribution by anilide and anilide–proflavin absorbance to be 0.002. Therefore, the effect on K_S of a systematic error of ±0.002 in the observed absorbance difference was computed. In most cases this produced a change of ±5–20% in K_S. The largest effects, which occurred in only a very few instances with the *p*-chloroanilide at pH 6.66 and 6.25, were a reduction of 50% and an increase of 100% in K_S. Since the value of K_S depends on the value used for K_D, values of K_S were computed using K_D − SE, K_D, and K_D + SE (SE = standard error in Table II). Typical results for the *p*-methoxyanilide were: pH 7.95, 9–12–16 (m*M*); pH 6.66, 12–16–24; pH 6.25, 5–11–40. Results for the *p*-chloranilide were similar. A generous estimate of the error in K_S therefore seems to be ±50% at pH 7.95 and 6.66. At pH 6.25 the true value probably lies between one-half and twice that observed. Since the same K_D is used for calculating K_S with different anilide substrates, in studies at a similar degree of saturation the uncertainty in K_D is without significant effect on the ratio of anilide binding constants.

Table IV summarizes the results of the kinetic studies.

Table IV. Kinetic Parameters for the Chymotrypsin-Catalyzed Hydrolysis of Acetyl-L-tyrosine Anilides[a]

Anilide	pH	K_m, m*M*[b]	$V_{max} \times 10^2$, sec^{-1} [b]
p-Methoxy-	7.90	13.0 (2.3)	23.0 (3.4)
	6.65	16.3 (4.2)	17.5 (3.9)
	6.25	16.1 (1.7)	10.8 (0.8)
p-Chloro-	7.90	0.81 (0.05)	2.02 (0.07)
	6.65	1.40 (0.06)	1.92 (0.05)
	6.21	2.60 (0.19)	1.91 (0.09)

[a] Reactions at 25° in 1.17 *M* Tris (pH 7.9) or 0.08 *M* phosphate (pH 6.6 and 6.2) buffer, ionic strength 0.095, 5% DMF (v/v). The enzyme concentration was 3–3.3 × 10^{-5} *M* and the anilide concentrations were 1–3.6 m*M* and 0.3–1.2 m*M*, respectively, for the methoxy- and chloroanilide. From 11 to 16 kinetic measurements were made to determine each set of kinetic parameters. [b] The computer-calculated standard error is given in parentheses.

The large standard errors in the kinetic parameters for the *p*-methoxyanilide arise because it is not possible to use concentrations of anilide greater than 25% of K_m. Where comparison with the results of Inagami, *et al.*,[3c] is possible the agreement is good. The pH dependence of the kinetic parameters of the *p*- and *m*-chloroanilides[3c] parallels that reported here for reaction of the para chloro compound. The general pattern of the results is similar to that obtained with acetyltryptophan anilides.[3d]

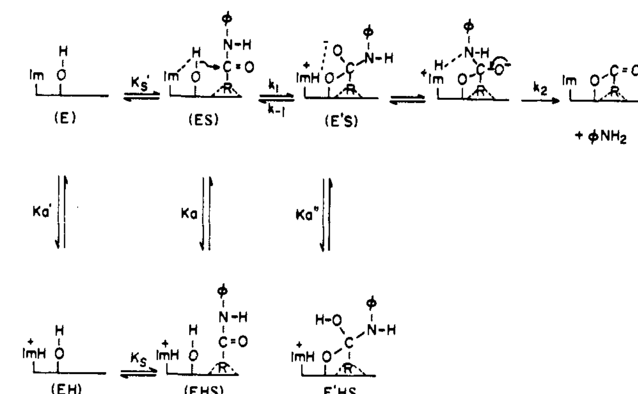
Discussion

Results obtained in kinetic studies of acetyltyrosine anilides (Table IV) are similar to those obtained previously with the corresponding acetyltryptophan derivatives.[3d] Study of the latter compounds required the use of supersaturated substrate solutions and the agreement of the results indicates that the earlier studies were not seriously influenced by this factor.

The principal findings described here and previously[3d] are: (a) V_{max} for the methoxyanilide is larger than that of the chloroanilide at pH 7.95; (b) the p*K* influencing V_{max} with the methoxyanilide (6.15–6.30) is higher than that for reaction with the chloroanilide (less than 6.21) so that the relatively greater reactivity of the former compound diminishes at lower pH; (c) K_m is smaller for the chloroanilide than the methoxyanilide at pH 7.95 and this difference disappears with the acetyltryptophan anilides at pH 5.5 and is starting to disappear with the acetyltyrosine anilides at pH 6.2.[10b]

These results may be accounted for by the previously proposed scheme[3d] (Scheme I) in which a tetrahedral

Scheme I



intermediate is formed from the Michaelis complex in an equilibrium reaction (*i.e.*, $k_{-1} > k_2$) with rate-limiting breakdown of the tetrahedral intermediate to the acyl enzyme. The impact of this equilibrium is to superimpose a substituent dependent process (k_1/k_{-1}) upon two presumably substituent independent processes, K_a and K_s. As a result, substrate binding and proton dissociation may be substituent sensitive, depending upon the magnitude of k_1/k_{-1}.

The essential element in this interpretation is that a component of the catalytic mechanism, reaction of the serine nucleophile with the substrate carbonyl group, is superimposed upon the substrate binding process. Jencks, *et al.*,[11] have interpreted the substituent dependence for anilide binding to chymotrypsin as reflecting a transformation of the substrate part way along the reaction coordinate in the binding process; tetrahedral intermediate formation is considered to be an extreme case of this phenomenon. A similar interpretation has been advanced to account for the relatively poorer binding of NADD as compared with NADH with alcohol dehydrogenase.[12]

The result most strongly supporting the hypothesis that substrate binding is coupled with the catalytic process is that when the enzyme activity is repressed by protonation of His-57, the substituent dependence of binding decreases or disappears.[3d] To test this interpretation we have studied the substituent and pH dependence of anilide binding to methyl-CT. This derivative contains a methyl group on N-3 of His-57 so that the electron pair (on N-3) which is presumed to coordinate with the proton of Ser-195 is unavailable (without disrupting the aromatic resonance of the imidazole ring) in either the conjugate acid or base form of the enzyme. The activity of the so modified enzyme is negligible.

That the active site is intact may be inferred from the observation of equivalent binding with CT and methyl-CT of proflavin,[13] *N*-acetyl-L-tyrosine ethyl ester,[13]

(11) W. P. Jencks, B. Schaffhausen, K. Tornheim, and H. White, *J. Amer. Chem. Soc.*, 93, 3917 (1971).
(12) K. Bush, H. R. Mahler, and V. J. Shiner, *Science*, 172, 478 (1971).
(13) R. Henderson, *Biochem. J.*, 124, 13 (1971).

indole,[14] and *N*-acetyl-L-tyrosine *p*-methoxyanilide. In agreement with the above hypothesis we have found that ablation of catalysis is accompanied by loss in the sensitivity of binding to substituents and pH. The equivalent binding of the methoxyanilide to the intact and methylated enzymes may be taken to indicate that in this case the equilibrium for tetrahedral intermediate formation is unfavorable and has little influence on binding. At high pH the apparent binding for anilides is equal to $K_s'/(1 + K)$ where $K = k_1/k_{-1}$; electron donation by the methoxy group would be expected to make K small.

The results described here for methyl-CT render unlikely an alternate explanation for the pH dependence of the apparent K_m; namely, the possibility that the acid dissociation constant for the Michaelis complex is substrate dependent, so that in some cases $K_a \neq K_a'$ (Scheme I). In these cases K_m (apparent) will be pH dependent. If this interpretation were correct, binding to methyl-CT might be expected to be pH dependent when this is the case for the native enzyme. This has not been found; binding to methyl-CT is pH independent for both anilides.

From the above, it may be concluded that for reactions in which substrate binding is coupled with the catalytic process static studies of enzyme–inhibitor complexes, such as may be achieved with X-ray crystallographic techniques, do not reveal important aspects of the binding process. This is also the case for study of substrate binding to a catalytically inactive enzyme. This problem may be circumvented, at least in part, by the use of transition-state analogs rather than substrates or inhibitors.[15]

Concerning Pretransition-State Proton Transfer in Chymotrypsin Catalysis. Wang and Parker[3b,16] have previously accounted for the substituent dependence of V_{max} for acylation of chymotrypsin by anilide substrate in terms of a "pretransition-state proton transfer" mechanism. According to this proposal, acylation involves attack by the serine anion which is generated by proton donation to the imidazole of His-57. Prior to attack of the serine anion, the so-formed His-57 conjugate acid serves to protonate the nitrogen atom of the bound anilide. While the substituent dependence for V_{max} (but *not* K_m) is consistent with this mechanism, several serious objections may be raised. First, the proposal requires a different mechanism for acylation of the enzyme with amide and ester substrates since the lower basicity of the latter compounds precludes a sufficient rate for substrate protonation by the His-57 conjugate acid.[3b] There is, however, no evidence for a different mechanism for acylation with ester and amide substrates or for the reverse process, the alcoholysis and aminolysis of acyl enzyme.[17] Also, as Williams has pointed out,[18] the proposed mechanism requires unreasonable estimates of the basicity of the anilide nitrogen atom. Finally, the rate constant for generation of the serine anion by proton transfer to His-57 is required to be equal to 10^{13}–10^{14} sec^{-1} to account for the observed rates.[16] A rate constant of that magnitude was derived from studies of proton transfer in ice[19] where the proton was presumably more mobile because of the existence of preformed hydrogen bonds. However, recent studies[20] of proton transfer in ice indicate that the value derived by Eigen and DeMayer is too large by one or two orders of magnitude, so that precedent is lacking for a proton-transfer rate large enough to satisfy the requirements of the mechanism of Wang and Parker.

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