Substrate Structure Effects on the pH Dependence of Deacylation of Acyl-Chymotrypsins[1]

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Abstract: A hypothesis that the variation in the apparent pK for deacylation of substituted acyl-chymotrypsins is associated with a preequilibrium hydration of the acyl-enzyme to form a tetrahedral intermediate has been tested. The crux of this test is based upon the fact that in the aminolysis of an acyl-enzyme the tetrahedral intermediate is different from that formed in the hydrolysis reaction so that (a) the equilibrium for formation of a tetrahedral intermediate may differ with an amine nucleophile as compared with water; (b) the rate-determining step in the reaction of an amine may involve tetrahedral intermediate formation rather than breakdown; and (c) the pK of the enzyme histidine residue responsible for activity may vary when a tetrahedral intermediate contains an amine rather than a water molecule. Any of these factors can be expected to result in a difference in the pH dependence for aminolysis as compared with hydrolysis if the hypothesis under review is correct. The apparent pK's for hydrolysis of benzoyl-, *p*-nitrobenzoyl-, *p*-methoxybenzoyl-, 3,5-dinitrobenzoyl-, and furoyl-chymotrypsin are 7.36, 7.22, 7.32, 6.95, and 7.10, respectively. The pK's for reaction of methoxylamine with benzoyl- and furoyl-chymotrypsin are 7.41 and 7.14, respectively. From this it is concluded that the pK variations observed for hydrolysis are not associated with the accumulation of a tetrahedral intermediate which has a substantially different pK than that of the acyl-enzyme.

Ionization constants obtained from pH–rate data may be composite constants which reflect the superimposition of equilibrium processes upon the proton dissociation reaction.[2] In reactions with chymotrypsin the substituent and pH dependence of V_{max} and K_m for N-acetyltyrosine and tryptophan anilides has recently been accounted for in terms of such a process.[3] It has been proposed[3] that in the conversion of the Michaelis complex to the acyl-enzyme a tetrahedral intermediate is formed in a rapid and reversible reaction and that this intermediate accumulates to a significant extent. Recently, Bernhard and coworkers[4] have observed that the pK for hydrolysis of acyl-chymotrypsin intermediates depends, at least in part, upon the electronic properties of the acyl group. A mechanism involving the preequilibrium addition of water to the acyl function was considered as a possible source of these results, and this mechanism was rejected on the basis of results obtained in studies of proton uptake and release accompanying acylation.[5] This conclusion requires further evaluation. To test the hypothesis that the pK variations are associated with the formation of a tetrahedral intermediate we have studied the pH dependence for aminolysis of acyl-chymotrypsins. Changes in the nature of the nucleophile can be expected to change the equilibrium for tetrahedral intermediate formation and, perhaps, the nature of the rate-limiting step so that formation rather than breakdown of the intermediate is rate limiting. Furthermore, the replacement of a water molecule by an amine in a tetrahedral intermediate may result in a change in the acidity of the histidine residue required for activity. Any of these factors will be reflected in the apparent pK for acyl-enzyme aminolysis if the hypothesis is correct that the preequilibrium formation of a tetrahedral intermediate is the source of the pK variations.

The results reported here on the pH dependence for acyl-enzyme hydrolysis are in qualitative but not quantitative agreement with those reported previously.[4] The pK's for aminolysis of furoyl- and benzoyl-chymotrypsin are identical, within experimental error, with those observed for hydrolysis. From this it is concluded that the pK variations observed for hydrolysis are not associated with the accumulation of a tetrahedral intermediate in which the pK of the critical histidine residue is substantially different from that in the acyl-enzyme.

Experimental Section

Materials. *p*-Nitrobenzoylimidazole (mp 122°), furoylimidazole (mp 49°), and *p*-methoxybenzoylimidazole (mp 59°) were prepared as described previously.[6] The melting point of the last mentioned compound is 10–12° lower than that reported previously,[6] and is very different from the 182.5–184° value described by Bernhard, *et al.*[4] Mass spectroscopy[7] revealed a molecular ion at m/e 202, which corresponds to the molecular weight of the acylimidazole, as well as a substantial peak at m/e 152. The latter peak suggests that the substrate is contaminated with anisic acid (mp 184°). A satisfactory elemental analysis of *p*-methoxybenzoylimidazole has been reported previously.[6] Benzoylimidazole and 3,5-dinitrobenzoylimidazole were prepared fresh daily by adding 1 equiv of the acyl chloride to 2 equiv of imidazole dissolved in acetonitrile. After 30 min at room temperature the imidazole hydrochloride was removed by filtration and the acylimidazole solutions were used without further purification. Proflavine sulfate was used as obtained from Mann and methoxylamine hydrochloride was recrystallized twice from ethanol.

Acylation Procedure and Rate Measurements. One milliliter of a 4×10^{-4} M chymotrypsin solution at pH 4.66 in 0.008 M acetate buffer was allowed to react with 0.1 ml of 2.2×10^{-4} M acylimidazole in acetonitrile for 30 min at room temperature. Deacylation was initiated by addition of 0.3 ml of this solution to 2.7 ml of a buffer solution containing 0.78 μmol of proflavin sulfate. The absorbance change at 470 mμ was followed in a Zeiss spectrophotometer using a dye–water blank; the slit width was ordi-

(1) Supported by a grant from the National Institutes of Health (DE 02761). Dr. Vishnu was on leave of absence from the University of Gorakhpur, Gorakhpur, India.

(2) (a) T. C. Bruice and G. L. Schmir, *J. Amer. Chem. Soc.*, **81**, 4552 (1959); (b) W. P. Jencks, *Ann. Rev. Biochem.*, **32**, 639 (1963).

(3) M. Caplow, *J. Amer. Chem. Soc.*, **91**, 3639 (1969).

(4) S. A. Bernhard, E. Hershberger, and J. Keizer, *Biochemistry*, **5**, 4120 (1966).

(5) J. Keizer and S. A. Bernhard, *ibid.*, **5**, 4127 (1966).

(6) M. Caplow and W. P. Jencks, *ibid.*, **1**, 883 (1962).

(7) We are indebted to Dr. W. J. McMurray for assistance with these measurements.

Table I. Rates and pK's for Deacylation of Acyl-Chymotrypsin at 25° [a]

Acyl group	pK[b]	pK_{lit}[c]	$k \times 10^2$, min^{-1} [b]	$k \times 10^2$, min^{-1}, lit.[c]
Benzoyl (hydrolysis)	7.36 ± 0.03	7.49	2.67 ± 0.02	3.90
(methoxylaminolysis)	7.41 ± 0.02		7.69 ± 0.05[d]	
Furoyl (hydrolysis)	7.10 ± 0.01	6.90	11.0 ± 0.04	9.4
(methoxylaminolysis)	7.14 ± 0.03		117.8 ± 1.3[d]	
p-Methoxybenzoyl (hydrolysis)	7.32 ± 0.02	7.34	0.423 ± 0.003	0.72
p-Nitrobenzoyl (hydrolysis)	7.22 ± 0.03	6.86	5.44	5.10
3,5-Dinitrobenzoyl (hydrolysis)	6.95 ± 0.01	6.70	602 ± 3.0	456

[a] Ionic strength maintained at 1.0 with KCl, acetonitrile concentration 0.9%. The buffer was 0.1 M acetate below pH 6, 0.05 M phosphate in the range 6–8 and 0.1 M borate at pH 8 and above. [b] Rate and pK values are followed by the computer-calculated standard error. [c] From results reported in ref 4. [d] The units are M^{-1} min^{-1}.

narily approximately 0.2 mm. This procedure was modified with 3,5-dinitrobenzoylimidazole where the acylimidazole in acetonitrile (0.09 μmol in 0.002 ml) was added directly to 0.12 μmol of enzyme in 3 ml of buffer, the pH of which varied with the individual experiments. Absorbance changes for these reactions were followed in a Cary 15 spectrophotometer with a dye-water blank. Except for the dinitrobenzoyl reactions the pH was recorded at the start of the deacylation process using an identical reaction mixture as that used for the spectral assay, and at the completion of deacylation using the solution used for the rate measurements. The pH was measured using an Orion Model 801 pH meter and a Sargent S-30070-10 electrode which had been calibrated with Beckman standard buffers. The largest pH change during the reaction was 0.03 pH unit and this change was observed in only a handful of reactions. In most reactions the pH change was less than 0.01 unit. First-order rate constants for deacylation were determined from semilogarithmic plots of absorbance–absorbance at infinite time, *vs.* time, and pK's were calculated using a computer program described by Cleland.[8]

Results

The deacylation of acyl-chymotrypsin generates the free enzyme which may bind proflavin. This binding

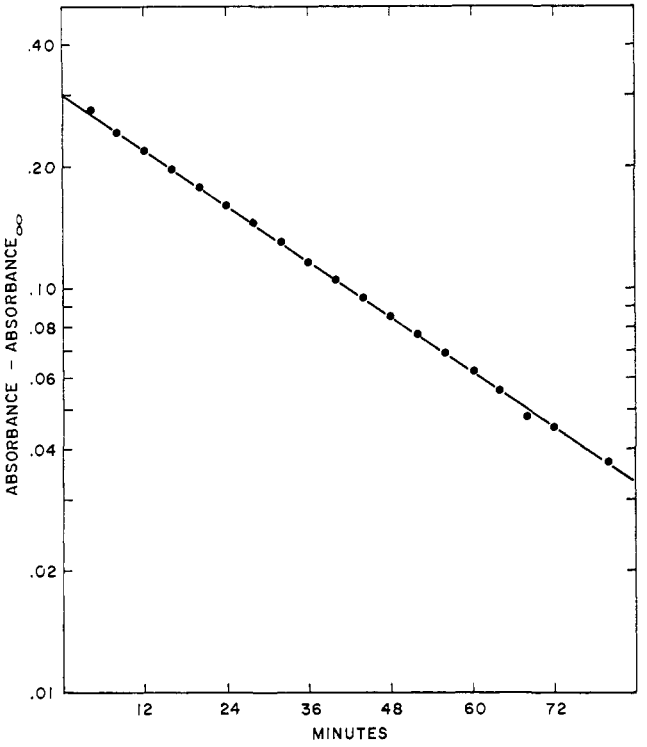


Figure 1. Hydrolysis of benzoyl-chymotrypsin at pH 9.09.

is associated with a significant spectral shift in the proflavin spectrum so that rates of deacylation of acyl-chymotrypsins may be readily followed spectrophotometrically. The results obtained in a typical experiment using this technique, which was introduced by Bernhard and coworkers,[9] are given in Figure 1. As seen in Figure 2 the rate parallels the ionization of a single group with a pK near 7, and the apparent pK's for deacylation along with the rate constant for deacylation of the fully ionized enzyme are summarized in Table I.

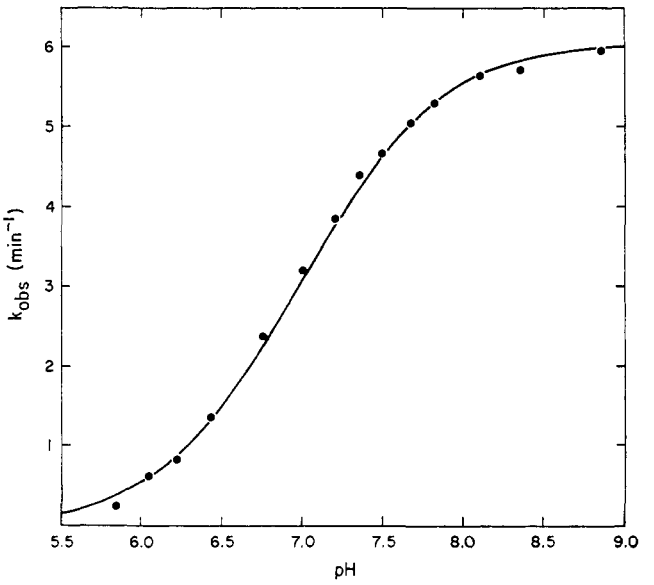


Figure 2. pH dependence of the deacylation of 3,5-dinitrobenzoyl-chymotrypsin. The curve is a theoretical one based upon a pK of 6.95, with the activities of the protonated and ionized forms of enzyme equal to 0 and 6.02 min^{-1}, respectively.

The maximum rates for benzoyl-, *p*-nitrobenzoyl-, and *p*-methoxybenzoyl-chymotrypsin are in very good agreement with those reported earlier,[6] which were obtained using a more laborious and a considerably less exact assay procedure. The pK's of 7.25, 7.25, and 7.40[6] for *p*-nitrobenzoyl-, benzoyl-, and *p*-methoxybenzoyl-chymotrypsins, which were based on relatively few kinetic measurements, have been essentially confirmed.

As seen in Table I there is a substantial disparity between the results reported here and those given by Bernhard, *et al.*[4] For example, the spread in the pK's for deacylation of the acyl-enzymes is only 0.41 pK unit as compared with the 0.79 unit reported previously. The results with furoyl-chymotrypsin are identical with those obtained by Inward and Jencks.[10] In addition to

(8) W. W. Cleland, *Nature*, **198**, 463 (1963).

(9) S. A. Bernhard, B. F. Lee, and Z. Tashjian, *J. Mol. Biol.*, **18**, 405 (1966).

(10) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965).

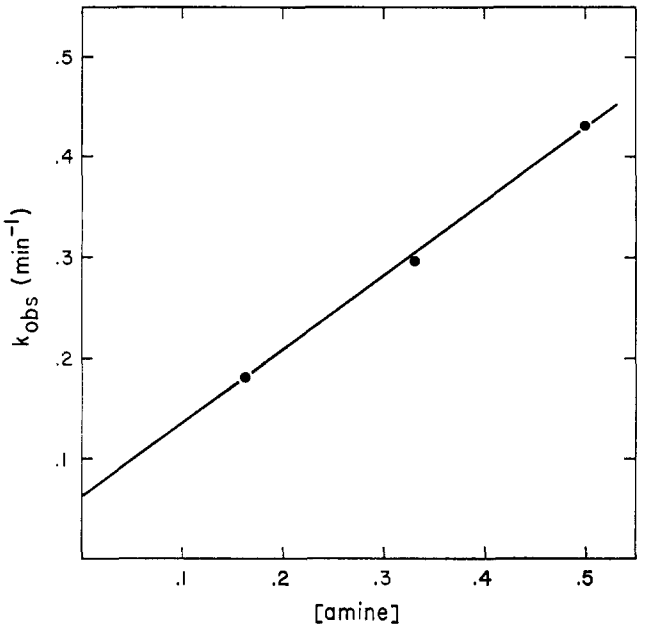


Figure 3. Reaction of methoxylamine with furoyl-chymotrypsin at pH 7.28.

the discrepancies in the pK's, which might possibly be associated with a systematic difference in the method for measuring pH, the rate constants differ by as much as 50% from those reported earlier.[4] We are unable to account for these differences. Since there is little variation in the pK's for activity with the acyl-enzymes previously studied,[6] we conclude that the linear Hammett relationship previously established is not fortuitous, as has been suggested.[4]

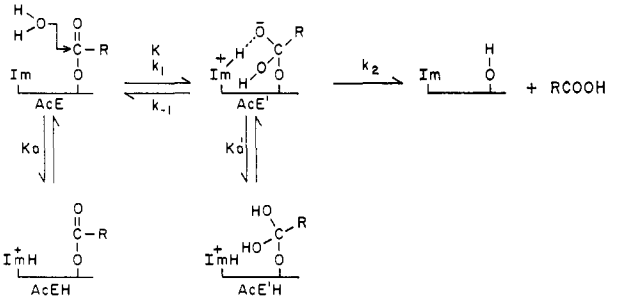
As seen in Figure 3, the rate of reaction of methoxylamine with furoyl-chymotrypsin is proportional to the amine concentration. The precision of the results is reflected by the fact that the intercept values obtained at various pH's , which reflect the hydrolysis of the acyl-enzyme, give a pK of 7.11 (standard error, 0.04) and a rate of 0.110 min^{-1} (standard error 0.0016), which is in excellent agreement with the values obtained from direct studies of the hydrolysis reaction. The corresponding constants calculated from the intercepts obtained from the reaction of methoxylamine with benzoyl-chymotrypsin are pK = 7.31 (standard error 0.06), and 0.0266 min^{-1} (standard error 0.0005) for the rate. In the pH range in which reactions were studied methoxylamine is fully ionized. It should be noted that rate constants obtained from the slope of plots similar to that given in Figure 3 probably do not exclusively reflect the aminolysis reaction since the rate of hydrolysis is increased slightly by the addition of nucleophiles.[10] The increase in hydrolysis is ordinarily only 15–30% of the over-all rate increase brought about by the nucleophilic agent. It is interesting that the ratio of the second-order rate constant for methoxylaminolysis to the pseudo-first-order rate constant for hydrolysis is 10.7 with furoyl-chymotrypsin and only 2.88 with benzoyl-chymotrypsin. This variability is not understood and a more dramatic demonstration of this effect is seen in reactions of hydroxylamine with furoyl-chymotrypsin and acetyltyrosyl-chymotrypsin where the ratio is approximately 200 and 1.0, respectively.[11]

(11) M. Caplow and W. P. Jencks, *J. Biol. Chem.*, **239**, 1640 (1964).

Discussion

The variation in the apparent pK for deacylation of acyl-chymotrypsin derivatives indicates that the dissociation constant is a complex constant which reflects a process in which one or more equilibria are superimposed upon the proton dissociation reaction. The mechanism of this phenomenon, which was first observed by Bernhard and coworkers,[4] remains to be elucidated. In studies of the acylation of chymotrypsin with substituted anilide substrates the apparent pK for acylation has similarly been found to be a complex constant,[3] and the results were interpreted in terms of a mechanism in which a tetrahedral intermediate is formed in a preequilibrium step with breakdown of the intermediate to form the acyl-enzyme acting as the rate-determining step. The deacylation of acyl-chymotrypsins is formulated in terms of a similar scheme in Scheme I. Under conditions where the hydration occurs in a

Scheme I



preequilibrium (*i.e.*, $k_{-1} > k_2$), eq 1 gives the derived apparent K_a, where $K = (AcE')/(AcE)$. This is similar

$$K_a(\text{apparent}) = \frac{K_a K_a'(1 + K)}{K_a' + KK_a} \qquad (1)$$

to an equation given earlier.[3] From eq 1 it is seen that the apparent dissociation constant will be made greater than K_a if $K_a' > KK_a$, *i.e.*, if the histidine residue associated with the tetrahedral intermediate is a relatively strong acid, and if $K > 1$. The latter requirement simply indicates that the protonic equilibria of an intermediate can only affect the apparent pK if it is present at a significant concentration. The assignment $K_a' > KK_a$ appears reasonable because of the favorable hydrogen bonding in the conjugate base AcE'. If $K_a' > KK_a$

$$K_a(\text{apparent}) = K_a(1 + K) \qquad (2)$$

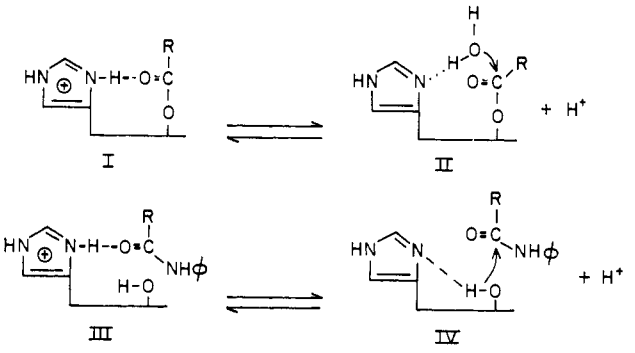
and since variations in electron density will be reflected in K, the apparent K_a will be dependent upon the nature of the acyl function. According to this scheme the substitution of an amine nucleophile for a water molecule can be expected to affect the apparent K_a for deacylation. This will be the case because of effects of the nature of the nucleophile on K, changes in K_a' as a result of the difference in the structure of the tetrahedral intermediate formed in aminolysis as compared with hydrolysis, and also, perhaps, changes in the ratio k_{-1}/k_2, which must exceed unity if tetrahedral intermediate formation is to occur as a preequilibrium process. Information concerning the k_{-1}/k_2 ratio may be inferred from results obtained in studies of the acylation of chymotrypsin with anilides where the available evidence suggests that the breakdown of the tetrahedral intermediate to release the amine and generate the acyl-enzyme is

rate limiting.[3] Since the rate-determining step is identical for a reaction in the forward and reverse direction it may be concluded that the attack step will be rate determining in the reaction of aniline with an acyl-enzyme. If reactions of methoxylamine and aniline are assumed to be equivalent, the attack step will similarly be rate limiting with methoxylamine and since the accumulation of a tetrahedral intermediate is thereby ruled out, the apparent K_a for methoxylaminolysis will be independent of the nature of the acyl group, and in all cases equal to K_a. The results reported in Table I on the pH dependence of the reaction of methoxylamine with furoyl- and benzoyl-chymotrypsin may be taken to indicate that Scheme I (with $k_{-1} > k_2$ and $K > 1$) does not account for the pK variations observed in the acyl-enzyme hydrolysis reaction.

Several other lines of evidence, none of which are conclusive, suggest that Scheme I does not hold. First, Bender and Heck[12] have failed to observe oxygen-18 exchange into the carbonyl group of cinnamoyl-chymotrypsin. This reaction is required if hydrolysis proceeds *via* Scheme I, and if the realtionship $k_{-1} > k_2$ holds. It has been pointed out, however, that oxygen-18 exchange requires that the two oxygen atoms in AcE′ become perfectly equivalent, which may not be the case on an enzyme surface.[12] Also, Charney and Bernhard[13] have noted that were cinnamoyl-chymotrypsin significantly hydrated the spectrum would resemble a styrene derivative rather than a cinnamate ester, as observed. However, because of the instability of acyl-enzymes at neutral pH, spectra of these derivatives have in most cases been recorded at low pH and if $K_a' > K_a$, that is, if the acyl-enzyme is more basic than the tetrahedral intermediate, the hydration process will be repressed under these conditions. No evidence of hydrate formation is apparent from spectra recorded at alkaline pH's with indoleacryloyl-[14a] and furylacryloyl-chymotrypsin,[14b] but the pK's for deacylation of these acyl-enzymes are relatively high and from eq 2 it may be concluded that little accumulation of an intermediate is expected with these acyl-enzymes. What is required for a definitive interpretation using this type of analysis are results obtained at high and low pH with a highly conjugated acyl-enzyme which has a markedly low pK for deacylation. Finally, Keizer and Bernhard[5] have rejected a mechanism similar to the one given in Scheme I, and one described by Bruice and Schmir,[2a] on the basis of results obtained in a study of proton release accompanying acylation of chymotrypsin. In this analysis it is assumed that "... the variability of the apparent K_d for catalytic hydrolysis is a consequence of kinetic details *after* acylation, (so that) one would not anticipate any regular correlation between the empirically determined K_d for hydrolytic activity of the acyl enzyme and the protonic equilibrium accompanying acylation." This analysis is incorrect since if the acyl-enzyme is in equilibrium with an intermediate it cannot be concluded that the intermediate is formed *after* the acyl-enzyme forming step. Thermodynamic data, *i.e.*, proton-release results, are not capable of determining the path of a reaction and there is no evidence as to the order in which the various forms of the acyl-enzyme are generated. Restated, if the relationship $k_{-1} > k_2$ holds, it is impossible, using ordinary methods, to distinguish as to whether an acyl-enzyme or a derived hydrate is the first product of acylation.[15]

A mechanism consistent with the results given here and those obtained previously[4] contains one or more equilibria involving the critical histidine residue and the acyl function in the acyl-enzyme. There is no evidence concerning which protonic form of the imidazole side chain participates in this process since the observed pK may either reflect a decrease brought about by electron withdrawing, or an increase brought about by electron-donating acyl groups. A mechanism, identical with one considered by Bernhard, *et al.*,[4] which involves hydrogen bonding to the acyl function is given in Scheme II.[16] Since the conjugate base I will be stabil-

Scheme II



ized by electron donation the apparent pK will be displaced to a more basic region with increases in electron density about the carbonyl oxygen atom. Similarly, the previously reported results obtained with anilides[3] may be interpreted in terms of structures III and IV, although we consider this unlikely since the K_m for acetyltryptophan amide is *independent* of pH (in the neutral pH range)[18] and in this case the substrate is considerably more basic than the anilide compounds. Structure IV, which is probably implicated in the acylation of the enzyme, has been observed in the X-ray analysis of chymotrypsin, and from the symmetry of acylation and deacylation[19] structure II, which has an analogous compostition, is suggested as an intermediate in deacylation.

The results do not permit conclusive differentiation between the alternative mechanisms previously described for acylation of chymotrypsin with anilides.[3] First, the earlier postulate that a tetrahedral intermediate accumulates in a preequilibrium reaction with anilides does not provide any evidence concerning the rate-determining step in the hydrolysis of an acyl-enzyme. The earlier results simply require that in the reaction of aniline with an acyl-enzyme (specifically, an acetyltryptophanyl-enzyme) the attack step will be rate

(12) M. L. Bender and H. d'A. Heck, *J. Amer. Chem. Soc.*, **89**, 1211 (1967).
(13) E. Charney and S. A. Bernhard, *ibid.*, **89**, 2726 (1967).
(14) (a) S. A. Bernhard and Z. H. Tashjian, *ibid.*, **87**, 1806 (1965); (b) S. A. Bernhard, S. J. Lau, and N. Noller, *Biochemistry*, **4**, 1108 (1965).
(15) Although there is no precedent in nonenzymatic systems, the acyl-enzyme hydrate may be formed directly by SN2 displacement by water on a tetrahedral intermediate formed in the reaction of the enzyme with the acylating agent.
(16) A hydrogen bond[17] between His-57-N$^{\delta 1}$ and Asp-102-O$^{\delta 1}$ has been omitted from the scheme.
(17) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature*, **221**, 337 (1969).
(18) A. Himoe, P. C. Parks, and G. P. Hess, *J. Biol. Chem.*, **242**, 919 (1967).
(19) B. M. Anderson, E. H. Cordes, and W. P. Jencks, *ibid.*, **236**, 455 (1961).

determining. Considering the extreme subtleties involved in determining the nature of the rate-limiting step in nonenzymic acyl transfer reactions,[20] it is not possible from information concerning aminolysis to predict whether attack or breakdown of a tetrahedral intermediate will be rate determining in the hydrolysis reaction.

Also, we believe that the various factors underlying the efficacy of chymotrypsin catalysis are not necessarily important to the same extent with all substrates and the 115,000-fold difference in the rate of deacylation of acetyltyrosyl-[21] and furoyl-chymotrypsin[10] may not involve equivalent reductions in the effectiveness of all components in the catalytic process. In one of two mechanisms previously proposed to account for the pH dependence of reactions of chymotrypsin with anilides, the pK of the Michaelis complex is presumed to vary with different substrates. This mechanism does not account for the substituent dependence of the rates and is considered unlikely because of difficulties in envisioning a simple mechanism for substrate-induced perturbations in the enzyme pK. The results reported here with furoyl-chymotrypsin indicate that the pK may be perturbed by a process not involving a tetrahedral intermediate. Because of the enormous differences in reactivity of nonspecific and specific substrates we do not feel that this is compelling evidence for rejection of a mechanism involving a tetrahedral intermediate. The dissociation process may be more complex than that given in Scheme II and may reflect a combination of factors such as weak interactions in a Michaelis complex and an acyl-enzyme (Scheme II) and the involvement of a tetrahedral intermediate with an appreciably modified pK (Scheme I and ref 3), and the importance of each of these factors may vary with different substrates.

(20) (a) S. A. Shain and J. F. Kirsch, *J. Amer. Chem. Soc.*, **90**, 5848 (1968); (b) W. P. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968).
(21) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

Diffusion-Controlled and Concerted Base Catalysis in the Decomposition of Hemithioacetals[1]

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Abstract: Rate and equilibrium constants are reported for the reactions of benzenethiol, *p*-nitrobenzenethiol, and thioacetic acid with acetaldehyde to form the corresponding hemithioacetals. The hydroxide ion catalyzed breakdown of these hemithioacetals occurs with rate constants near 10^{10} M^{-1} sec^{-1} and is largely or entirely limited by the rate of diffusion-controlled encounter of the substrate and catalyst. The energy of activation is 2–3 kcal/mole and the solvent deuterium isotope effect k_{OH^-}/k_{OD^-} is 1.25. In the synthesis reaction, formation of the carbon–sulfur bond and proton transfer (eq 11) occur rapidly and separation of hydroxide ion from the hemithioacetal (eq 10) is the rate-determining step. Weaker bases catalyze the breakdown reaction at a slower rate with a Brønsted slope β of 0.8 ± 0.03. This indicates that carbon–sulfur bond formation or cleavage and proton transfer are in some sense concerted. Equilibrium constants for hemithioacetal formation are independent of the acidity of the thiol over a range of acidity of 10^7.

The addition to acetaldehyde of the weakly acidic thiols, ethanethiol, methoxyethanethiol, and methyl mercaptoacetate, to form the corresponding hemithioacetals occurs predominantly through a base-catalyzed pathway, although a general acid catalyzed pathway with a large Brønsted coefficient α becomes significant at low pH values. No general base catalysis has been detected for these reactions and there is no significant water reaction, with the possible exception of the reaction with methyl mercaptoacetate. The observed rate constants for the base-catalyzed reaction with methyl mercaptoacetate indicated that the rate constants for breakdown of the hemithioacetal anion and of proton transfer to this anion are very similar, so that a situation is approached in which a diffusion-controlled proton-transfer step becomes rate determining.[2] The experiments described here were carried out to test the prediction[2] that a diffusion-controlled step should become rate determining in the formation and breakdown of hemithioacetals formed from more acidic thiols. As has been reported in a preliminary communication,[3] the results confirm the prediction that a diffusion-controlled step is rate determining, but indicate that the proton transfer in these reactions is in some sense concerted with the formation and cleavage of the carbon–sulfur bond (eq 11), rather than a stepwise process.

Experimental Section

Materials. Thioacetic acid and benzenethiol were redistilled under nitrogen before use. *p*-Nitrobenzenethiol was purified by a modification of the procedure of Willgerodt.[4] Crude thiol (5 g) was dissolved in 25 ml of boiling 5% sodium hydroxide and filtered.

(1) Supported by grants from the National Science Foundation (GB 4648) and the National Institute of Child Health and Human Development of the Public Health Service (HD-01247). R. E. B. was a National Science Foundation Predoctoral Fellow, 1965–1968.

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