Transition State Charge Distribution in Reactions of an Acetyltyrosylchymotrypsin Intermediate

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SUMMARY

The previously reported results implicating general base catalysis in the deacylation of furoylchymotrypsin have been confirmed with a specific acylenzyme, acetyltyrosylchymotrypsin. For nonaromatic amines extending over a range of basicity of 10^{-6} the rate varies by a factor of only four; the rate constants for the reaction of these amines are equal to approximately 200 M^{-1} s^{-1}. Methoxyamine and ammonia are 16- and 10-fold, respectively, less reactive than the other amines. With four piperidine-substituted anilines of pK 2.2 to 6.3 the rate is less than that for nonaromatic amines of equivalent basicity and the Brønsted slope is equal to 0.52. Some unknown factor apparently prevents effective proton abstraction from attacking anilines.

There is a deuterium isotope effect on the rates of reaction of ethylamine, formylhydrazine, and p-chloroaniline with the acetyltyrosylenzyme and this effect is equal to that for hydrolysis of the acylenzyme. The effects of basicity and deuterium oxide are consistent with a mechanism involving concerted (and effective) proton abstraction from the attacking amine. An alternate stepwise mechanism involving a rate-limiting diffusion of protonated His-57 away from an anionic tetrahedral intermediate, which predicts a zero Brønsted β for basic amines and a β near unity for amines of pK less than 7, is not supported by the results.

In enzyme-catalyzed reactions in which a proton is transferred from a heteroatom it is widely accepted that general base catalysis is involved. However, the evidence for this is inferential. Consistent with the presumed importance of enzyme-mediated general base catalysis is the observation of a deuterium isotope effect in a number of reactions involving proton abstraction1. The most direct evidence is Inward and Jencks' result on the effect of nucleophile basicity on the reaction of furoylchymotrypsin with amines and alcohols (3). The results indicate that there is little charge development on the nucleophile in the transition state for acyl transfer. It has reasonably been assumed that this results from effective proton abstraction from the nucleophile by an enzyme general base (His-57). We report here a similar study with an intermediate formed from a "natural" substrate, the acetyltyrosylchymotrypsin intermediate. The rate of hydrolysis of this derivative is 1.1 \times 10^{4} times that of the furoylenzyme. We find that the reaction of nonaromatic amines is virtually independent of basicity, while aniline nucleophiles show a modest dependence on amine pK. For the former compounds, in reactions with a transition state which is expected to resemble that of the physiological catalytic process, the previously derived conclusion (3) that general acid-base catalysis contributes to catalysis apparently holds.

EXPERIMENTAL PROCEDURES

Materials—N-Acetyl-L-tyrosine amide was prepared by gently warming 50 ml of a concentrated aqueous ammonia solution of the methyl ester (1 g) for 1 hour. The solution was taken to dryness under vacuum and the product, crystallized several times from ethanol-water, had m.p. 224.8 to 225, literature 222 to 224 (4).

N-Acetyl-L-tyrosine ethylamide was made by reacting 3 g of the methyl ester with 5 ml of ethylamine at 0° for 24 hours. The resultant solid, obtained after evaporation of excess amine, is recrystallized from ethyl acetate-ligroin; m.p. 195 to 195.5.

\[ \text{C}_{9}\text{H}_{12}\text{NO}_{2} \]

Calculated: C 62.40, H 7.71, N 11.20

Found: C 62.08, H 7.20, N 11.75

N-Acetyl-L-tyrosine trifluoroethylamide was made by mixing 0.42 g (1.88 meq) of N-acetyl-L-tyrosine, 0.688 ml (9.4 meq) of trifluoroethyamine, and 5.0 ml of dimethylformamide. After the solution was cooled to 0°, 0.386 g (1.88 meq) of diethylcarboimidide was added; the solution was allowed to warm to room temperature and stirred overnight. Following the removal of the insoluble urea and solvent (under vacuum) the product was crystallized from an ethyl acetate-ligroin mixture to give a material of m.p. 170 to 172.

\[ \text{C}_{9}\text{H}_{12}\text{NO}_{2}\text{F}_{3} \]

Calculated: C 51.2, H 5.28, N 9.19, F 18.7

Found: C 51.5, H 5.19, N 9.16, F 18.75

N-Acetyl-L-tyrosine semicarbazide was synthesized as described for the N-formylphenylalanine derivative (5). The product was crystallized from water to give what appears to be the monohydrate, with a m.p. of 208 to 210.
C₈H₁₅N₃O₄
Calculated (for the monohydrate): C 48.4, H 0.94, N 18.8
Found: C 48.33, H 0.95, N 18.5

The N-acetyl-L-tyrosine hydrazide (6) and p-chloro- and p-methoxyanilides were prepared as described previously (7).

Preparation of N-acetyl-L-tyrosine p-acetylanilide was accomplished by adding 1 g of diecylohexylcarbodiimide (4.9 meq) to an ice-chilled mixture of 1 g (4.5 meq) of acetyl-L-tyrosine and 0.605 g (4.5 meq) of p-acetylalanine dissolved in a minimal volume of dimethylformamide. The reaction was allowed to reach room temperature and stirred for 5 hours. After the addition of 0.1 ml of glacial acetic acid the mixture was refrigerated overnight. The oil obtained after filtering off the area and evaporation of the solvent under vacuum was crystallized from acetone to give a solid with m.p. 255 to 255.1.

Calculation of Amide Yield

The azide method described by Inagami et al. (8) was used to make the p-dimethylaminomaleide of N-acetyl-L-tyrosine. The product was crystallized from ethyl acetate to m.p. 212.3 to 213.6.

N-[14C]Acetyl-L-tyrosine methyl ester was synthesized by the procedure previously described for N-methyltyrosine methyl ester (9), using radioactive acetylchloride. The product was recrystallized to constant specific activity (7.0 × 10⁴ cpm per μmole).

All buffers and amines used in the deuterium oxide experiments were depleted of exchangeable protons by first dissolving these substances in deuterium oxide and then evaporating off the solvent under vacuum. α-Chymotrypsin was three times crystallized material obtained from Worthington.

Rates of acetyltyrosylchymotrypsin aminolysis were determined by product analysis using isotope dilution for quantitation. After incubating enzyme, [14C]acetyltyrosine methyl ester and amine in a buffered solution (see Table I for reaction conditions), for a time previously shown, and calculated (from the results in Ref. 10), to be adequate to hydrolyze quantitatively the ester the reaction was stopped by heating the reaction tube in a boiling water bath. It is necessary to stop the reaction since the amide product may be further hydrolyzed to the carboxylic acid. Using the rate constants for amide hydrolysis, it was calculated that negligible further reaction is expected under these conditions, and this was verified in a number of cases by showing that the amide yield was independent of time over a period substantially greater than that which elapsed for the reaction in which the product yield was first determined. The nonenzymic reaction was either calculated or shown to be insignificant under the reaction conditions. A weighed quantity (50 to 100 mg) of the appropriate nonradioactive amide was then dissolved in the reaction mixture and either the amide was isolated from the cooled reaction or the mixture was taken to dryness under vacuum and the amide was extracted from the resultant solid with an organic solvent. The isolated amide was recrystallized to constant specific activity and identified by melting point. The yield of amide was determined by weight or spectrophotometrically, and the radioactivity was measured with a liquid scintillation counter using Aquasol counting fluid. A control experiment in which amine was added after completion of the enzymic hydrolysis of the ester was run to determine the extent of co-crystallization of carboxylate with the amide. The background so-determined was ordinarily negligible (less than 1% of the radioactivity found associated with the amide); however, this is not the case for the morpholine amide and the high background prevented measurement of the reactivity of morpholine. In the case of p-acetyl-

In the case of methoxyamine, the amide could not be synthesized and the product amide was isolated by extraction from the reaction mixture after the ester disappearance was complete. This was done by first adding nonradioactive acetyltirosinate and then evaporating the reaction mixture to dryness under vacuum. The resultant solid was extracted with hot ethyl acetate in successive portions to extract the amide quantitatively. Background counting levels were observed in subsequent extracts.

The reaction of formylhydrazine was determined by quantitating the proton release associated with the reaction of the enzyme with acetyltirosine methyl ester in the presence of varying concentrations of formylhydrazine. At the pH where the study was carried out the formation of amide does not result in the release of a proton. The pH of the reaction was maintained constant with a pH-stat and the ratio of the volume of titrant added in the presence of amine to the volume delivered in the absence of amine gives the percentage of acid formed. This method is feasible if the reaction may be run at a pH at least 2 pH units above the pK of the amine and if there is appreciable (at least 10%) amide formation.

RESULTS

In reactions of competing nucleophiles with a common substrate the product ratio is determined by the rate constants and nucleophile concentrations as defined in the relationship:

\[ \frac{\text{Yield of product 1}}{\text{Yield of product 2}} = \frac{k_1 \times [\text{concentration nucleophile 1}]}{k_2 \times [\text{concentration nucleophile 2}]} \]

In this study of the reaction of acetyltyrosylchymotrypsin with water and amines the alternate products are carboxylic acid and amide, and the rate constant for acylamidase hydrolysis is equal to 193 s⁻¹ (10). From the product ratio the rate constant for acylenzyme aminolysis may be determined. This method is most accurate when the yield of one product is low so that variations in the yield have a maximal effect on the ratio of the products; in most of the studies reported here the yield of amide ranged from 1 to 10%.

The so-determined rate constants for acetyltyrosylchymotrypsin aminolysis are summarized in Table I and a Brønsted plot relating amine basicity and reactivity is given in Fig. 1.² The reactivity of nonaromatic amines is essentially independent of basicity and if a slope (β) were to be established from related compounds such as trifluoroethylamine and ethylamine, or hydrazine, semicarbazide, and formylhydrazine, it would be near zero. A similar conclusion may be derived even without such classification of the amines; over a range of basicity of 10⁻⁴ the rate constants vary by a factor of about four. The β for the reaction of anilines is equal to 0.52.

The rate constant given for the reaction of hydroxylamine is that for the reaction of the nitrogen atom, as indicated by the

² We are grateful for the assistance of Dr. Curtis Harper for having obtained the rate constant for reaction of hydrazine with the acyl enzyme.
The $O$-acyl derivative is presumed to reacylate the enzyme (12, 13) and only the stable products, the carboxylic acid and $N$-acyl derivative, are formed enzymically in reactions with high enzyme concentrations. The low reactivity of ammonia was previously seen in the study with furoylchymotrypsin (3). We do not understand the basis of this deviation.

A statistical correction is made for both the basicity (two sites for protonation) and reactivity (two sites for reaction) with hydrazine. For $p$-dimethylaminoaniline the basicity has been adjusted by subtracting 0.3 unit from the pK. This is a minimal correction which only reflects the fact that there are two potential sites for protonation, but does not take into account the fact that the measured pK is almost certainly that for the more basic tertiary nitrogen atom, while the site for nucleophilic attack is the slightly less basic primary amino group. It was previously found that tertiary amines do not react with furoylchymotrypsin (3). The necessity to study the various amines under different pH conditions is not believed to influence the results. It was found in studies with $p$-chloroaniline that the partitioning of the acetyltyrosylzyme is pH-invariant over the range pH 5.5 to 7.5. In studies with formylphenylalanylchymotrypsin the partitioning with water and formylhydrazine is pH-invariant over the range 4.5 to 7.5 (14). This result is not consistent with the previously proposed mechanism for chymotrypsin-catalyzed amide hydrolysis (5).

The rate of reaction of ethylamine, formylhydrazine, and $p$-chloroaniline with acetyltyrosylchymotrypsin is decreased in deuterium oxide (Table I). This is inferred from the observation that the product ratio (amide to carboxylic acid) is unchanged in $D_2O$. The rates of hydrolysis of a number of acylenzymes are decreased approximately 3-fold by the deuterium substitution (15) and the lack of an effect on the product ratio indicates that the rate of acylanzyme aminolysis is affected identically. The rate constants for re-
reaction conditions and assay were such in these studies that a rather small difference in the isotope effects on hydrolysis and aminolysis could be detected: from the standard deviations observed in the deuterium oxide results (Table I) it is estimated that there is less than a 20% difference in the isotope effects for the two reactions.

**Discussion**

We report here a study of the reaction of amines with a specific acyl enzyme, the acetyltirosylchymotrypsin intermediate. This acylchymotrypsin is among the most reactive of those studied and the conclusions derived from such an investigation can be expected to reflect the elements of the catalytic process which are manifested in the physiological role of the enzyme. It is emphasized that although chymotrypsin probably does not function in vivo as a catalyst for amide bond synthesis, as is the case here, the principle of microscopic reversibility requires an identical transition state for amide bond cleavage. Therefore, the results reported here are directly applicable in considerations of the physiological reaction, enzyme acylation by amines, the first covalent change of the substrate in amide hydrolysis.

The rates of reaction of nonaromatic amines with acetyltirosylchymotrypsin are virtually independent of amine basicity. This is consistent with there being little or no positive charge development on the amine in the transition state for aminolysis. If there were significant charge development, as there is in the reference amine protonation reaction, electron-donating groups would stabilize this charge and the rate constants would correlate with amine basicity.

The amine reactivities are reported in terms of second order rate constants. Although we have not observed any saturation of an amine receptor site we consider it likely that there is a site for binding of the amines before nucleophile reaction. That there is a site suggested by the prior observation that with amines and alcohols of comparable basicity, reactivity is correlated with hydrophobicity (3). Also, the 20-fold greater reactivity (kcat/Km) of Ac-Phe-Ala-NH2 as compared with ac-Phe-Gly-NH2 (16) suggests that there is a leaving group site in the acylation reaction which, by the principle of microscopic reversibility, must be available as the nucleophile site in the reaction of amines with the acylenzyme. We consider it extremely unlikely that a cancellation of structural effects on binding and reactivity has generated the results reported here. For one thing in the case of amines with fairly similar structure such as ethylamine and trifluoroethylamine, or hydrazine, formylhydrazine, and semicarbazide, where the amine binding would be expected to be relatively constant, there is little change in reactivity despite the wide range in basicity.

Our interpretation of the low Brønsted β for aminolysis is the same as that provided previously (3): charge development does not occur because His-57 effectively removes the proton from the attacking amine as the attack step proceeds. This is a form of intramolecular concerted base catalysis. Recent developments (17) from studies of acid-base catalysis make this result unexpected.

Sufficient evidence has accumulated to permit the derivation of a rule concerning the requirements for concerted general acid-base catalysis (18). Such catalysis "...can occur only (a) at sites that undergo a large change in pK in the course of the reaction and (b) when ... the pK of the catalyst is intermediate between the initial and final pK values of the substrate site." Requirement b is not met in the reaction of amine nucleophiles with pK values near or greater than that of His-57. In Equation 1 the pK of imidazole is not between that of the amine (pK near 20) and the dipolar intermediate (pK near that of the conjugate acid of the amine (18)).

\[
R_2NH + RCOX \xrightleftharpoons{K_a} R_2N-O-C-R + X^-
\]

This does not preclude the involvement of His-57 as a catalyst; catalysis may occur by a stepwise mechanism as shown in Equation 2. In this scheme the catalyst "rescues" the dipolar intermediate from breakdown (via the k-1 step) to starting materials. With basic amines the rates are diffusion-controlled (they may be slow because of an unfavorable equilibrium prior to the diffusion step) with diffusion of the conjugate acid of the catalyzing base away from the amionic tetrahedral adduct as the rate-limiting step. The rates are similar for such amines (β is near zero) since there is a cancellation of effects of basicity on the kcat/Km and k/k-1 equilibria. The corresponding rate-limiting reaction in the enzymic process would be the movement away from the amionic intermediate of the His-57 conjugate acid by a conformational change in the enzyme. For amines which are somewhat less basic than His-57 a similar mechanism will hold, but since the kcat/k-1 and k/k-1 equilibria are similar, this appears unlikely (7, 19, 20). We are able to envision an addition compound (17). Although the evidence is not conclusive, this appears unlikely (7, 19, 20). We are able to envision a number of other factors which may account for the persistence of a concerted mechanism in the enzymatic process. However, there is no evidence on this subject and the question remains open.

The results with aniline nucleophiles are inexplicably different from those obtained with the nonaromatic amines. For some unknown reason there is significant charge development on the

*The implications of a stepwise and concerted mechanism for the enzymic process were brought to our attention by Dr. W. P. Jencks.*
amino acid in the transition state. Jencks et al. had predicted such an effect from an analysis of the rates of enzyme acylation with the corresponding anilides and the equilibrium constants for anilide formation (21).

As described above the isotope effect is independent of amine basicity. This is not in accord with the results reported by Wang and Parker (22) who found that in the acylation of the enzyme with substituted acetyltyrosine anilides the isotope effect systematically varies with electronic effects, with $k_{\text{H}}:k_{\text{D}}$ equal to 2.76 ± 0.2 with the m-chloroanilide, and 1.47 ± 0.4 with the p-methoxyanilide. This result was taken to support the "pretransition state proton transfer" theory. No isotope effect is expected in the equilibrium for enzyme acylation with the anilides, since protons are neither taken up nor released in the reaction. Therefore, a similar substituent dependence is predicted for the isotope effect in the acylenzyme aminolysis reaction; however, none is observed. Although we have not looked at this effect with all of the amines the identity of the isotope effect in the reaction of the three amines studied, which vary substantially in structure and basicity, makes it likely that the other amines behave similarly. We cannot account for the discrepancy with the requirements of the earlier reported (22) results. However, in the previous studies (22) the steady state kinetic parameters were obtained with relatively insoluble substrates which only fractionally saturated the enzyme, so that the results may be subject to a fairly large error. Also, the organic solvent concentration used previously was substantially greater than that used here and this may somehow affect the reaction.

REFERENCES
1. Jencks, W. P. (1969) Catalysis in Chemistry and Enzymology pp. 310-312, McGraw-Hill Book Co., New York
2. Pollock, E., Hoog, J. L., and Schowen, R. L. (1973) J. Amer. Chem. Soc. 95, 908-909
3. Inward, P. W., and Jencks, W. P. (1965) J. Biol. Chem. 240, 1986-1990
4. Thomas, D. W., MacAllister, K. V., and Niemann, C. (1951) J. Amer. Chem. Soc. 73, 1548-1552
5. Fersht, A. R., and Requena, Y. (1971) J. Amer. Chem. Soc. 95, 7079-7087
6. Hoggness, D. S., and Niemann, C. (1953) J. Amer. Chem. Soc. 75, 884-890
7. Lucas, E. C., and Caplow, M. (1972) J. Amer. Chem. Soc. 94, 900-903
8. Iiagami, T., Paschke, A., and York, S. S. (1969) J. Biochem. (Tokyo) 65, 809-819
9. Peterson, R. L., Hubble, K. W., and Niemann, C. (1963) Biochemistry 2, 942-946
10. Cunningham, L. W., and Brown, C. S. (1956) J. Biol. Chem. 221, 287-299
11. Caplow, M., and Jencks, W. P. (1964) J. Biol. Chem. 239, 1649-1652
12. Efand, R. M., and Wilson, I. B. (1963) J. Biol. Chem. 238, PC 3138-3140
13. Bender, M. L., Clement, G. E., Gunter, C. R., and Kézdy, F. J. (1964) J. Amer. Chem. Soc. 86, 3697-3702
14. Zeeberg, B., Caswell, M., and Caplow, M. (1973) J. Amer. Chem. Soc. 95, 2734-2735
15. Bender, M. L., Clement, G. E., Kézdy, F. J., and Heck, H. O. A. (1964) J. Amer. Chem. Soc. 86, 3680-3689
16. Baumann, W. K., Bezzoehio, S. A., and Dutler, H. (1970) Fed. Eur. Biochem. Lett. 8, 267-269
17. Jencks, W. P. (1972) Chem. Rev. 72, 705-718
18. Jencks, W. P. (1972) J. Amer. Chem. Soc. 94, 4731-4732
19. Caplow, M. (1969) J. Amer. Chem. Soc. 91, 3639-3645
20. O'Leary, M. H., and Klutetz, M. D. (1972) J. Amer. Chem. Soc. 94, 5585-5589
21. Jencks, W. P., Schaffhausen, B., Tornheim, K., and White, H. (1971) J. Amer. Chem. Soc. 93, 3917-3922
22. Parker, L., and Wang, J. H. (1968) J. Biol. Chem. 243, 3729-3734
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