Discrete Effects of the Acylamino Proton in a Chymotrypsin Substrate on Different Processes in Catalysis[1]

Michael Caplow* and Curtis Harper

Contribution from the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514.
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Abstract: Substitution of a methyl group for the amide proton of *N*-acetyltyrosine (i) decreases the rate of chymotrypsin acylation by a factor of 2×10^5 with the methyl ester and 1.8×10^3 with the hydroxamic acid; (ii) decreases the rate of acyl enzyme hydrolysis 7.7×10^2-fold (or less) and the rate of hydroxylaminolysis 18-fold (or less); (iii) is without effect on the apparent stability (K_s) of the Michaelis complex with the ester and hydroxamic acid; (iv) destabilizes the acyl enzyme by at least 2.69 kcal/mol (25°). The effect of substrate structure on the enzyme acylation and deacylation reactions is a function of the nature of the leaving group and acyl group acceptor. These results have been accounted for in terms of mechanisms involving a tetrahedral intermediate, with varying rate-limiting steps depending upon the leaving group and nucleophile, and with varying effects of substrate structure on the stability of the transition states leading to and from the tetrahedra lintermediate. An acylchymotrypsin's specificity toward water as the acyl group acceptor with a physiological substrate (*N*-acetyl-L-tyrosylchymotrypsin) is dependent upon the presence of the amide proton. Hydroxylaminolysis of the acyl enzyme predominates over hydrolysis, even at rather low hydroxylamine concentrations, when this proton is replaced by a methyl group.

Replacement of the amide proton of acetyl-L-tyrosine methyl ester with a methyl group is known to profoundly influence reactivity with chymotrypsin.[2] However, the magnitude of this effect is not fully calculable without information concerning the rate-determining step (enzyme acylation or deacylation). We report here results which indicate that enzyme acylation is rate limiting for *N*-acetyl-*N*-methyltyrosine methyl ester. Comparison of the results obtained with methylated derivatives with those obtained with the nonmethylated *N*-acetyltryosine compounds reveals that the rate of enzyme acylation is decreased 2×10^5-fold with the methyl ester and 1.8×10^3-fold with the hydroxamic acid. Hydrolysis of the acyl enzyme is decreased 770-fold or less. Remarkably, the rate of acyl enzyme hydroxylaminolysis is almost unaffected by the methyl substitution. As a result an acetylmethyltyrosylchymotrypsin intermediate undergoes preferential hydroxylaminolysis, even at low hydroxylamine concentrations. Chymotrypsin specificity as a hydrolase with a physiological substrate (acetyltyrosine compounds) is lost when the amide proton is replaced by a methyl group. The dependence of the effect of methyl substitution in acetyltyrosine derivatives on the nature of the reaction (the nucleophile in deacylation and leaving group in acylation) may be accounted for by mechanisms involving a tetrahedral intermediate, with varying rate-limiting steps, depending on the nucleophile and leaving group, and with varying effects of substrate structure on the stability of the transition states leading to and from the tetrahedral intermediate. We have previously accounted for the substituent dependence of K_m, V_{max}, and the pH dependence for acylation of chymotrypsin with anilide substrates in the terms of a mechanism involving a tetrahedral intermediate.[3]

Experimental Section

N-Methyl-L-tyrosine was prepared by the direct methylation of L-tyrosine.[2] The ester hydrochloride, made by the published procedure,[2] was not isolated but used directly for acetylation as per the previous synthesis. The product, *N*-acetyl-*N*-methyl-L-tyrosine methyl ester, mp 131–133° (lit.[2] 132.5–133°), $[\alpha]^{24}D$ −84.1° (*c* 0.96%, pyridine), lit.[2] $[\alpha]^{25}D$ −81.5° (*c* 0.96%, pyridine), was recrystallized repeatedly from ethyl acetate until all traces of material that hydrolyzes rapidly with dilute chymotrypsin disappeared. Methyl resonances were observed by 60-MHz nmr spectroscopy at 1.94 (CH_3–CO), 2.80 (CH_3–N), 3.68 ppm (CH_3–O) (TMS reference, solvent $CDCl_3$). The corresponding hydroxamic acid was synthesized by the method previously used for the unmethylated compound[4] and the sodium salt, which could not be crystallized, was converted to the neutral compound by adding 6 *M* aqueous HCl to an ethanolic solution of the salt. The resultant precipitate was crystallized twice from hot water, mp 189°. *Anal.* Calcd for $C_{12}H_{16}N_2O_4$: C, 57.13; H, 6.39; N, 11.10. Found: C, 56.52; H, 6.61; N, 10.58.

Rates of ester hydrolysis, hydroxylaminolysis, and hydroxamate hydrolysis were followed by the methods described previously.[4] The only important modification made in these procedures was that the enzyme precipitated by $FeCl_3$–HCl was removed by filtration using Celite; this is positively required to obtain optically clear solutions. Reactions were run at 25°, ionic strength 1.0 (with NaCl) with 0.2 *M* Tris buffer.

Results

The rate of hydrolysis of MATM[5] was studied with 5 m*M* substrate (5×10^{-5} *M* enzyme, pH 7.8) and the observed turnover number, equal to 1.06×10^{-2} sec^{-1}, is in good agreement with that calculated (0.97×10^{-2} sec^{-1}) from the published kinetic parameters.[2]

The rate of enzymic hydroxylaminolysis of the ester under similar conditions becomes independent of the hydroxylamine concentration (Figure 1) at a relatively low concentration, and levels out to a rate equal to that for hydrolysis in the absence of hydroxylamine. This result implicates an acyl enzyme pathway for reaction, with acylation as the rate-limiting step.

(1) This investigation was supported by a Public Health Service Research Career Development Award (1-K4-GM-10, 010-01) from the National Institute for General Medical Studies and by a grant (DE03246) from the National Institutes of Health.

(2) R. L. Peterson, K. W. Hubele, and C. Niemann, *Biochemistry*, **2**, 942 (1963).

(3) (a) M. Caplow, *J. Amer. Chem. Soc.*, **91**, 3639 (1969); (b) E. C. Lucas and M. Caplow, *ibid.*, **94**, 960 (1972).

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

(5) Abbreviations used are: ATE, *N*-acetyl-L-tyrosine ethyl ester; ATH, *N*-acetyl-L-tyrosine hydroxamic acid; MATM, *N*-acetyl-*N*-methyl-L-tyrosine methyl ester; MATH, *N*-acetyl-*N*-methyl-L-tyrosine hydroxamic acid.

In earlier studies of the enzymic hydroxylaminolysis of ATE[4,6] it was found that the yield of hydroxamate decreases and then levels out to a constant value with increasing enzyme concentration. This was taken[6] to reflect a mechanism in which the *O*-acylhydroxylamine formed from the acyl enzyme went on to either reacylate the enzyme or react nonenzymically with hydroxylamine to give the *N*-acyl derivative; the former reaction is presumed to predominate at high enzyme concentrations. The effect of this is that at high enzyme concentrations the acyl group has more than one chance to react with water; the first is with the acyl enzyme formed from the ester and the second is with the acyl enzyme formed from the *O*-acylhydroxylamine compound. However, as seen in Table I,

Table I. Yield of Hydroxamate from *N*-Acetyl-*N*-methyl-L-tyrosine Methyl Ester[a]

Hydroxylamine concn, *M*	Enzyme concn, $M \times 10^5$	Yield, %
0.049	5.1	53
0.098	5.1	69
0.49	5.1	90
0.98	5.1	94
0.04	2.1	44.1
0.04	8.3	57.5
0.04	83.0	63.4

[a] Reactions at 25°, ionic strength 1.0 with NaCl, pH 8.16 ± 0.1, 1.8×10^{-5} *M* EDTA, 0.2 *M* Tris buffer.

the yield of hydroxamic acid from MATM *increases* slightly with increasing enzyme concentration. We are unable to account for this result, but the fact that the yield does not decrease indicates that hydroxamate is formed predominantly by an enzymic path. That is, if the *O*-acyl derivative is formed, as it almost certainly is, it reacylates the enzyme so that the product yield correctly reflects the partitioning of the acyl enzyme between water and the nitrogen atom of hydroxylamine. Nonenzymic hydroxylaminolysis of the ester and enzyme autodigestion to give hydroxamate are negligible under the conditions used.

The kinetic constants for MATH hydrolysis by chymotrypsin (pH 8.2, 3×10^{-4} *M* enzyme) are 24 m*M* for K_m and 1.3×10^{-3} sec^{-1} for V_{max}. This reaction is slower than that of the ester so that it does not influence the yield of hydroxamate found in studies of enzymic hydroxylaminolysis of MATM. The rate of hydrolysis of 2.1 m*M* MATH with 10^{-4} *M* enzyme at pH 8.2 is decreased approximately 7.4-fold by 0.1 *M* hydroxylamine. This concentration of hydroxylamine would have no significant effect on the hydrolysis of ATH.[4] The hydroxylamine-induced decrease in the rate of MATH hydrolysis provides further proof that the predominant reaction of the acyl enzyme involves attack of the nitrogen atom since the relative thermodynamic instability of the *O*-acyl derivative[7] would preclude significant conversion of the *N*-acyl to the *O*-acyl derivative. From the arguments previously adduced[4] it may be concluded, from the hydroxylamine-induced rate decrease, that MATH hydrolysis goes *via* an acyl enzyme pathway and that the reaction involves rate-determining acylation of the enzyme.

(6) (a) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kézdy, *J. Amer. Chem. Soc.*, **86**, 3697 (1964); (b) R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **238**, PC 3137 (1963).

(7) W. P. Jencks, *J. Amer. Chem. Soc.*, **80**, 4585 (1958).

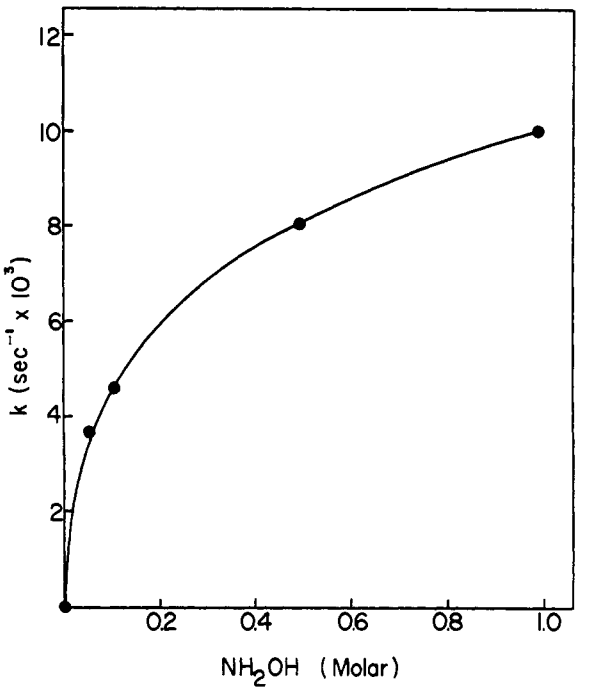

Figure 1. Effect of hydroxylamine on the rate of hydroxamate formation from *N*-acetyl-*N*-methyl-L-tyrosine methyl ester.

The kinetic parameters for reaction of acetyltyrosine derivatives are reported in Table II. The rate constant for hydrolysis of the acyl enzyme formed with MATM was calculated by assuming that acyl enzyme hydrolysis is 10 times as fast as enzyme acylation (acylation is rate limiting). The reported constant is, therefore, a lower limit. The rate constants for acyl enzyme hydroxylaminolysis were calculated from the product yields (ref 4 and Table I) and the relationship, per cent carboxylic acid/per cent hydroxamate = $k_{hydrolysis}/k_{hydroxylaminolysis}$ (NH_2OH). Since only a lower limit is available for $k_{hydrolysis}$ of the methylated acyl enzyme, the value for $k_{hydroxylaminolysis}$ is also a lower limit. However, unless the hydroxylaminolysis of the methylated acyl enzyme is faster than the unmethylated derivative (we consider this unlikely), then the rate constant for acyl enzyme hydrolysis must be less than that for the unmethylated derivative (if the rate constant for hydroxylaminolysis of the methylated acyl enzyme is 1×10^2 M^{-1} sec^{-1}, then the rate constant for hydrolysis is still only 4.6 sec^{-1}).

Discussion

The principal observations described here (Table II) are (i) the partitioning of the acyl group between water and hydroxylamine is in favor of hydrolysis with an acetyltyrosylchymotrypsin intermediate and greatly in favor of hydroxylaminolysis with an acetyl-*N*-methyltyrosyl enzyme (compare $k_{deacylation-hydrolysis}$ and $k_{deacylation-hydroxylaminolysis}$ for ATE and MATM); (ii) substitution of a methyl group for the amide proton on ATE decreases the first- and second-order rate constants for enzyme acylation by 10^5, but has only a small effect on the rate of hydroxylaminolysis of the acyl enzyme; (iii) methyl substitution

Table II. Rates of Reactions of Tyrosine Derivatives with Chymotrypsin[a]

Substrate	K_s, mM	$k_{acylation}$, sec^{-1}	$k_{deacylation-hydrolysis}$, sec^{-1}	$k_{deacylation-hydroxylaminolysis}$, M^{-1} sec^{-1}	V_{max}/K_m,[b] M^{-1} sec^{-1}
N-Acetyl-L-tyrosine ethyl ester	18	5.30×10^{3}[c]	2.0×10^{2}[c]	1×10^{2}	2.75×10^{5}[d]
N-Acetyl-L-tyrosine hydroxamic acid[e]	24	2.35			9.8×10^{1}
N-Acetyl-*N*-methyl-L-tyrosine methyl ester[f]	8.4	2.6×10^{-2}	$\geq 2.6 \times 10^{-1}$	≥ 5.6	3.1
N-Acetyl-*N*-methyl-L-tyrosine hydroxamic acid[g]	24	1.3×10^{-3}			5.4×10^{-2}

[a] Rate constants are for reaction at 25°, at pH's near 8. [b] Second-order rate constant for enzyme acylation in dilute solutions (M. L. Bender and F. J. Kézdy, *Annu. Rev. Biochem.*, **34** (1965). [c] Reference 9. [d] Results of R. J. Foster and C. Niemann, *J. Amer. Chem. Soc.*, 77, 1886, 3370 (1955). [e] Results from reference 4. [f] The value for $k_{acylation}$ was taken from results in ref 2. [g] From this work.

for the amide proton affects the rate of enzyme acylation more than the rate of deacylation (hydrolysis), and the effect on acylation is greater for reaction of the ester than the hydroxamic acid.

Earlier studies have shown that the amide proton and nitrogen atom are not important for the strength of substrate binding: the K_i's for acetyl-D-phenylalanine amide and *O*-acetyl-D-β-phenyl lactamide are similar,[8] as are the K_m's for MATM[2] and ATH[4] and the K_s of ATE.[9,10] It appears that in the Michaelis complex the enzyme's interaction with the acylamino locus of substrates is relatively nonspecific, with modest steric requirements. Also, rate studies indicate that substitution in the acylamino group has limited effects on reactivity; the relative second-order rate constants (normalized for effects on reactivity of the acyl linkage) for acylation of chymotrypsin with L-$C_6H_5CH_2CHXCO_2CH_3$ substrates are[11] CH_3COCH_2, 1; CH_3COO, 0.1; CH_3, 0.4; CH_3CONH, 24. However, a 10^5-fold decrease is seen with MATM as compared with ATE. We take these results to indicate that in going from the Michaelis complex to the rate-limiting transition state for enzyme acylation the enzyme's interaction with the acylamino locus remains rather nonspecific but has increased steric requirements which are seriously violated by the bulkier $CH_3CON-CH_3$ function. The change in steric requirements in going from the Michaelis complex to the transition state indicates that the substrate binding is different in these two species. Whether this entails a change in enzyme and or substrate orientation is not distinguishable here.

The results described here (Table II) may be accounted for by either of the mechanisms outlined in Figures 2 and 3.[12] The essential elements of these schemes are as follows. In both schemes (1) the stability of the Michaelis complex with esters (ATE and MATM) and hydroxamic acids (ATH and MATH) is not influenced by replacement of the acylamino proton by a methyl group. (2) A tetrahedral intermediate is presumed to precede the acyl enzyme and the rate-limiting step is tetrahedral intermediate formation with esters ($k_{-1} < k_2$ and $k_{-1}' < k_2'$, primed constants are for the reactions of the methylated derivatives) and breakdown with hydroxamic acids ($k_{-1} > k_2$ and $k_{-1}' > k_2'$). (3) The acyl enzyme is less stable with the methylated substrates ($k_1k_2/k_{-1}k_{-2} > k_1'k_2'/k_{-1}'k_{-2}'$). (4) Methyl substitution destabilizes the transition state for interconverting the Michaelis complex and tetrahedral intermediate relative to both of these species ($k_1 > k_1'$ and $k_{-1} > k_{-1}'$). In scheme A, (5) the acyl enzyme and tetrahedral intermediate and intervening transition state are equally destabilized by methyl substitution ($k_{-2} = k_{-2}'$ and $k_2 = k_2'$ for both the hydrolysis[12b] and hydroxylaminolysis reactions). In scheme B, (6) the stability of the tetrahedral intermediate is unaffected by methyl substitution ($k_1/k_{-1} = k_1'/k_{-1}'$ for esters and hydroxamates). (7) Methyl substitution destabilizes the transition state for interconverting the tetrahedral intermediate and acyl enzyme to a similar extent as in the acyl enzyme ($k_{-2} = k_{-2}'$ for hydrolysis[12b] and hydroxylaminolysis). (8) The effect of methyl substitution is greater on the k_{-1} step than the k_2 step ($1 < k_2/k_2' < k_{-1}/k_{-1}'$). These relationships are most easily identified by considering the shape of the free energy profiles given in Figures 2 and 3.

The basis of the above assignments follows. That the stability of the Michaelis complex is not influenced by methyl substitution (point 1) is indicated by the equivalence of K_s for ATE and MATM and for ATH and MATH.[10] The assignment of rate-limiting steps (point 2) is extrapolated from results of studies of nonenzymic reactions of esters and amides[13] and from studies of the reaction of chymotrypsin with anilides.[3] Anilide reactions with chymotrypsin have been accounted for by assuming that tetrahedral intermediate breakdown is rate limiting, and a similar

(8) D. W. Ingles and J. R. Knowles, *Biochem. J.*, **108**, 561 (1968).

(9) J. McConn, E. Ku, A. Himoe, K. G. Brandt, and G. P. Hess, *J. Biol. Chem.*, **246**, 2918 (1971).

(10) The possibility that nonproductive binding predominates with MATH and MATM and productive binding predominates with ATH and ATE is very unlikely, since the smaller ATE and ATH substrates would be expected to bind in all of the nonproductive modes available to the methylated substrates *and* in the productive mode. The nonmethylated substrates should, therefore, be bound more tightly; they are not. Of course if nonproductive binding requires a methyl group on the acylamino function (there is no basis for believing this to be the case) then for nonproductive binding to contribute significantly we must assume the unlikely situation where the strength of nonproductive binding of the methylated compounds almost exactly equals that for productive binding of the unmethylated substrates.

(11) M. S. Silver, M. Stoddard, T. Sone, and M. S. Matta, *J. Amer. Chem. Soc.*, **92**, 3151 (1970).

(12) (a) In Figures 2 and 3 the tetrahedral intermediate for ester and hydroxamate reactions is presumed to be less stable than the Michaelis complex; this is not required. Deepening the energy minimum corresponding to the tetrahedral intermediate will not influence the derived conclusions as long as the relative stabilities of intermediates and transition states formed from the methylated and unmethylated substrates remain unchanged from that portrayed here and the formation of the tetrahedral intermediate from the Michaelis complex and acyl enzyme with the methylated substrates is not exergonic. (b) The hydrolysis and alcoholysis of the acyl enzyme are presumed to be equivalent reactions and the decreased rate of acyl enzyme hydrolysis was used to construct the energy profile for the ester acylation reaction.

(13) S. J. Johnson, *Advan. Phys. Org. Chem.*, **5**, 237 (1967).

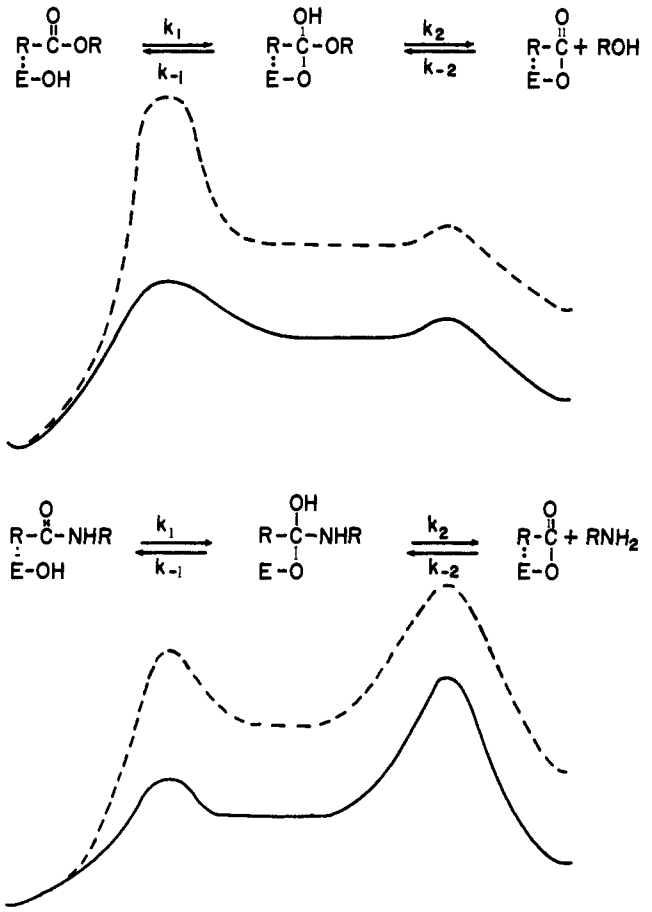

Figure 2. Scheme A shows the free energy profiles for the reaction of esters and hydroxamic acids with chymotrypsin. The solid lines are for reaction of acetyltyrosine derivatives and the dotted lines are for reaction of *N*-acetyl-*N*-methyltyrosine compounds and other nonspecific substrates such as furoate derivatives.

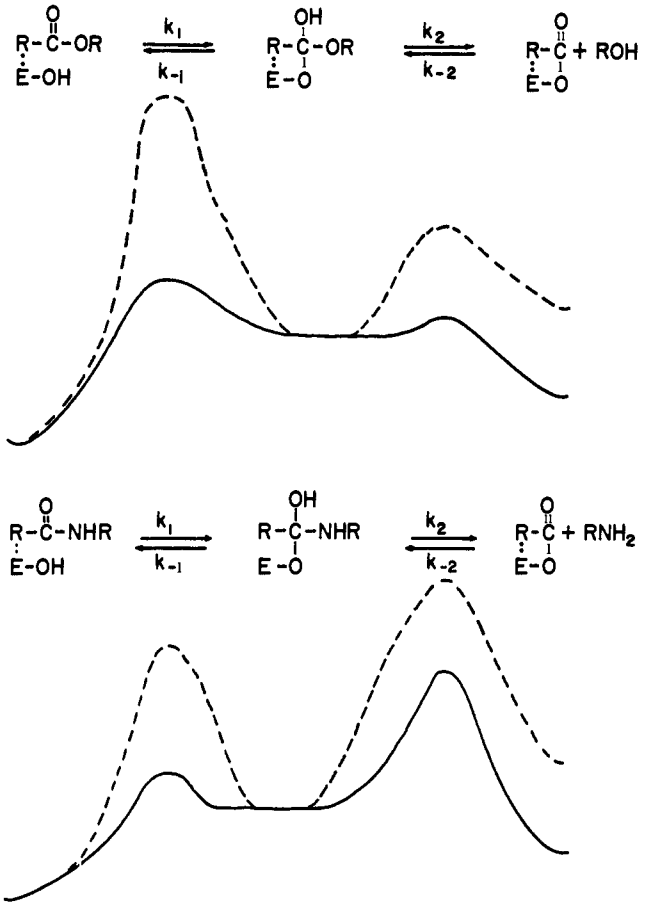

Figure 3. Scheme B shows the free energy profiles for the reaction of esters and hydroxamic acids with chymotrypsin. The solid lines are for reaction of acetyltyrosine derivatives and the dotted lines are for reaction of *N*-acetyl-*N*-methyltyrosine compounds and other nonspecific substrates such as furoate derivatives.

mechanism is presumed for the chemically related hydroxamates. Point 3 comes from analysis of the equilibrium constant for enzyme acylation from the Michaelis complex of the hydroxamates (ATH and MATH)

$$E \cdot RCONHOH \underset{k_r}{\overset{k_f}{\rightleftharpoons}} RCO\text{-enz} + NH_2OH$$

The equilibrium constant for acyl enzyme synthesis (k_f/k_r) at pH 8 is 2.35×10^{-2} *M* with ATH and equal to or less than 2.32×10^{-4} with MATH (only a lower limit was obtained for k_r); methylation destabilizes the acyl enzyme by at least 2.69 kcal/mol (25°). Points 4–8 describe the various possibilities concerning when the methyl-induced instability (point 3) is manifested. In both mechanisms (A and B) it is manifested in the transition states; in mechanism A the effect is also seen in the tetrahedral intermediate and acyl enzyme; in mechanism B in the acyl enzyme only. That the effect of substrate structure on enzyme–substrate interaction in reaction intermediates (Michaelis complex, tetrahedral intermediate, and acyl enzyme) is equal to or less than that in the transition state(s) is expected. The precision and tightness of substrate binding in these relatively stable intermediates are a pale reflection of that in the transition state(s).[14]

(14) R. V. Wolfenden, *Accounts Chem. Res.*, 5, 10 (1972).

In mechanism A the tetrahedral intermediate and acyl enzyme presumably resemble the transition state; they are, therefore, highly sensitive to substrate structure. In mechanism B this is presumably only the case for the acyl enzyme.

Mechanisms A and B account for the results: enzyme acylation with the ester and hydroxamate and acyl enzyme hydrolysis are dramatically decreased with the methylated derivatives; acyl enzyme hydroxylaminolysis is not. This is quantitatively expressed in the following. For both schemes (A and B) the observed rate constant for enzyme acylation is equal to $k_2k_1(ES)/(k_{-1} + k_2)$ and the rate constant for deacylation is $k_{-2}k_{-1}(AcE)(N)/(k_2 + k_{-1})$, where ES, AcE, and N are the Michaelis complex, acyl enzyme, and nucleophile, respectively. For mechanism A, from the assignments made in points 1–5, the ratios of the observed rate constants for enzyme acylation with ATE and MATM and for acylation with ATH and MATH are k_1/k_1' and $(k_1/k_{-1})/(k_1'/k_{-1}')$, respectively. Both of these ratios are greater than 1, and the ratio with the esters (ATE and MATM) exceeds that for the hydroxamic acids (ATH and MATH). Also, the ratio of the rate constants for hydrolysis of the acyl enzymes from ATE and MATM is k_{-1}/k_{-1}', while the ratio of the rate constants for hydroxylaminolysis of the two acyl enzymes is 1. Thus, all of the rates for reaction of the methylated derivatives are expected

to be significantly decreased, *except* that for hydroxylaminolysis of the acyl enzyme. Similar predictions hold for scheme B. From the assignments made in points 1–4 and 6–8 the ratio of the observed rate constants for the enzyme acylation with ATE and MATM and for acylation with ATH and MATH are k_1/k_1' and k_2/k_2', respectively. Again, both of these ratios exceed 1 and the ratio with the esters is larger than that for the hydroxamates. The ratio of the rate constants for hydrolysis of the acyl enzymes formed from ATE and MATM is $(k_{-1}k_2')/(k_{-1}'k_2)$ and that for hydroxylaminolysis is 1. Only the rate of hydroxylaminolysis is not decreased by methyl substitution.

The proposed mechanisms involve a nonequivalent effect of methyl substitution for the acylamino proton on the Michaelis complex and acyl enzyme. Only the acyl enzyme and the transition states involved in its formation are destabilized (in mechanism A the tetrahedral intermediate is also destabilized). This requires a change in substrate binding in the course of acyl enzyme formation from the Michaelis complex. In support of this proposal Henderson[15] has observed a 120° rotation of the C^{α}–C^{β} bond of Ser-195 when this group is acylated. The change in the Ser-195 bond angle, which is required for correct interaction of His-57 with a water molecule, might well be accompanied by a reorientation of the acyl group.

Essentially, we are suggesting a variant of the strain mechanism for catalysis described by Jencks.[16] For MATM we propose here that strain is manifested in a step after the formation of the Michaelis complex rather than in the Michaelis complex, as is the usual case.[16] Knowles[8] has accounted for the low reactivity of MATM as reflecting a methyl-induced prohibition of strain in the Michaelis complex. However, this, the usual strain mechanism, does not appear to account for the fact that the rate of hydroxylaminolysis of the acyl enzyme is little affected by methyl substitution, or that the effect on acylation with the ester is greater than that with the hydroxamic acid.

Our conclusion that the acyl enzyme derived from a nonspecific (methylated) substrate is less stable, relative to the Michaelis complex, than the acyl enzyme derived from a specific substrate was previously arrived at by Epand and Wilson.[17] These workers further concluded that maximum noncovalent interaction of the substrate with the enzyme occurs in the Michaelis complex and that the acyl enzyme and transition state are similarly destabilized with nonspecific substrates. We are in accord with the latter point (only) for the transition state for interconversion of the tetrahedral intermediate and acyl enzyme; Epand and Wilson's formulation omitted consideration of a tetrahedral intermediate. The presumed resemblance of the transition state and acyl enzyme led Epand and Wilson to conclude that the rate of enzyme acylation will be more sensitive to substrate structure than that for deacylation; this is observed. However, as with the strain mechanism discussed above, these assumptions do not appear to account for the difference in sensitivity to substrate structure of the acyl enzyme hydrolysis and hydroxylaminolysis reactions, or for the dependence of the effect of substrate structure on the nature of the leaving group in the acylation reaction.

Earlier results indicate that acyl enzyme hydrolysis, alcoholysis, and hydroxylaminolysis have different structural sensitivities. For example, $k_{\text{hydrolysis acetyltyrosyl}}/k_{\text{hydrolysis furoyl}}$ is 1.1×10^5, $k_{\text{hydrolysis acetylphenylalanyl}}/k_{\text{methanolysis furoyl}}$ is 0.82×10^4, $k_{\text{methanolysis acetylphenylanyl}}/k_{\text{methanolysis furoyl}}$ is 1.3×10^4, while $k_{\text{hydroxylaminolysis acetyltrysoyl}}/k_{\text{hydroxylaminolysis furoyl}}$ is only 4.9×10^2.[4,18]

Comparison of the kinetic parameters of the acetyltyrosine (and acetylphenylalanine) and furoyl derivatives exactly parallels that observed with the acetyltyrosine and acetyl-*N*-methyltyrosine compounds. As with the latter pair, the apparent binding of furoyl derivatives is equivalent to that with acetyltyrosine compounds: K_s for furoylamide is only 14 m*M*. However, unactivated furoyl derivatives such as the amide[19] and hydroxamic acid[4] do not measurably acylate the enzyme. Furoyl enzyme hydrolysis and methanolysis are extremely slow, but hydroxylaminolysis approaches that for the reaction of the most reactive acyl enzyme, the acetytyrosyl derivative. We believe that the acetyltyrosine:furoate results may be accounted for in terms of the same mechanisms described for the acetyltyrosine:acetyl-*N*-methyltyrosine results (Figures 2 and 3, with the dotted lines representing the reactions of the furoyl derivatives). Again, the same predictions hold: enzyme acylation and hydrolysis are slow with furoyl compounds, and acyl enzyme hydroxylaminolysis is less affected.

Our results fit other schemes, including a recently proposed azlactone mechanism,[20] in which the leaving group is lost during enzyme-catalyzed azlactone formation. For this mechanism, symmetry requires that nucleophiles react directly with the azlactone rather than with an acyl enzyme. The low reactivity of MATM and MATH may be accounted for by the difficulty in forming an azlactone without basic catalysis, and the difference in partitioning of the acyl groups of ATE and MATM might reflect a difference in the relative reactivity of water and hydroxylamine with a neutral and cationic azlactone. However, there is no direct evidence supporting an azlactone mechanism at neutral pH's and the small effects observed in studies of acylation of the enzyme with substrates where azlactone formation is precluded (*i.e.*, the CH_3COCH_2 derivative of $C_6H_5CH_2CHXCO_2CH_3$[11]) indicates that this is not an obligatory element in the catalytic scheme.

(15) R. Henderson, *J. Mol. Biol.*, **54**, 341 (1970).

(16) W. P. Jencks, "Current Aspects of Biochemical Energetics," N. O. Kaplan and E. P. Kennedy, Ed., Academic Press, New York, N. Y., and references therein.

(17) (a) R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **239**, 4145 (1964); (b) it was previously concluded[17a] that the hippuryl enzyme is about 2.7 kcal/mol less stable, and the acetyltyrosyl enzyme equivalent to that expected for an acylserine derivative. We have considered the question of the appropriate nonenzymic model reaction for calculating the theoretical thermodynamic stability of an acyl enzyme,[3a] and suggest that the Epand–Wilson calculation underestimates the increased stabilization provided by the acetyltyrosyl moiety. We are unable to conceive of a simple mechanism by which the smaller hippuryl group may be destabilized (relative to the Michaelis complex) without a similar effect on the acetyltyrosyl side chain.

(18) (a) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965); (b) L. W. Cunningham and C. S. Brown, *ibid.*, **221**, 287 (1956); (c) Vishnu and M. Caplow, *J. Amer. Chem. Soc.*, **91**, 6754 (1969); (d) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kezdy, *ibid.*, **86**, 3697 (1964).

(19) J. L. Marini and M. Caplow, *ibid.*, **93**, 5560 (1971).

(20) M. A. Coletti-Previero, C. Axelrud-Cavadore, and A. Previero, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **11**, 213 (1970).