Acylation of Subtilisin A by Aryl Esters: Contribution to Rate Limitation by a Physical Step Preceding General Acid–Base Catalysis†

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ABSTRACT: The specificity ratios $k_c/K_m = k$ for subtilisin A catalyzed hydrolysis of five aryl esters of *N*-(methoxycarbonyl)-L-Phe (McPhe) were determined at pH 7.03 and its pD equivalent. The ratios are independent of the electronic properties of the leaving group substituent. Kinetic solvent isotope effects, ^Dk, increase from about 0.9 to 1.3 as leaving group ability decreases from *p*-nitrophenolate to *p*-methoxyphenolate. The *k* of *N*-(methoxycarbonyl)-L-phenylalanine *p*-nitrophenyl ester (NPE) with native enzyme exhibits a strong temperature dependence; $\Delta H^* = 87 \pm 3$ kJ mol^{-1} and $\Delta S^* = 148 \pm 14$ J K^{-1} mol^{-1} at 25 °C (H_2O). The ^Dk with this substrate is 1.36 at 13.6 °C, declines to 0.89 at 25 °C, and then increases to 1.04 at 39.4 °C. Above neutral pH(D), with McPhe NPE as substrate, the dependence of *k* is for the dissociated form of a single base of $pK_{app} = 7.38 \pm 0.03$ in H_2O and 7.67 ± 0.03 in D_2O. The pK_{app} values are apparently those of the uncomplexed native protein. By contrast, *k* of 3-phenylpropanoic acid (Prop) *p*-nitrophenyl ester exhibits a weaker temperature dependence; $\Delta H^* = 20$ kJ mol^{-1} and $\Delta S^* = -90$ J K^{-1} mol^{-1} (H_2O) at 25 °C. The ^Dk are larger than those for McPhe NPE, decreasing from 1.99 at 20.5 °C to 1.74 at 46.1 °C. These results, combined with those of previous studies, are consistent with limitation of *k* by at least two processes. One process is characterized by negative activation entropy and a normal ^Dk and follows the pH dependence of the uncomplexed enzyme. This process, which dominates *k* of Prop NPE, is probably the chemical acylation of the enzyme. The second process, which dominates *k* of McPhe NPE, is characterized by positive activation entropy, is nearly isotopically silent, and also follows the pH dependence of the free enzyme. This process may be an enzyme conformational change. The contributions of each process to rate limitation were calculated with a semiquantitative model that employs mechanistic assumptions about the origin and magnitude of the intrinsic isotope effects and electronic leaving-group effects on the two steps. Estimates of Hammett ρ and ^Dk values obtained from the model are in reasonable agreement with experimental values for four series of aryl esters.

The endopeptidase subtilisin A (a k a subtilisin Carlsberg) exhibits broad specificity in the hydrolysis of peptide bonds, but preferentially cleaves at positions in which the carbonyl group of the scissile bond comes from hydrophobic residues such as Tyr, Trp, and Phe (Kraut, 1971; Markland & Smith, 1971).[1] Like other serine proteases, this subtilisin variant contains a catalytic triad composed of a serine hydroxyl group (Ser-221), a histidine imidazole group (His-64), and the side-chain carboxyl of aspartate (Asp-47). The catalytic triad likely serves as a general acid–general base proton shuttle or charge relay system in acylation and deacylation of the enzyme (Blow et al., 1969; Matthews et al., 1977). Here we focus on the acylation reaction, which is most readily studied with the specificity ratio $k_c/K_m = k$. This parameter is related to the free energy difference between the uncomplexed enzyme and substrate and the first irreversible TS on the path to acyl enzyme (Schowen, R. L., 1978). The first irreversible TS is reasonably presumed to be the loss of leaving group during collapse of a tetrahedral-like adduct of the enzyme's serine hydroxyl group and the substrate's carbonyl group.

The basic features of the acylation of subtilisin A are doubtless similar to those of other serine proteases. Relatively few mechanistic studies have been reported, however (Polgar, 1973; Polgar & Fejes, 1979; Matta & Staley, 1974; Matta et al., 1976; Jordan et al., 1985), and results of an earlier paper from this laboratory are rather puzzling. For example, there is an inverse relationship between the specificity of the substrate acyl group and the leaving group dependence of acylation of subtilisin A by aryl esters (Matta et al., 1976). For NPEs, *k* decreases in the order AcPhe > BzGly > Prop. Hammett ρ values for the respective aryl ester series are 0.0, 0.4, and 0.9. Only the latter value is near the $\rho = 1.0$ expected for rate-limiting addition of an oxyanionic nucleophile to the carbonyl group of aryl esters, as found in the alkaline hydrolysis of Prop aryl esters (Matta et al., 1976). This suggests no involvement or reduced involvement of leaving group in the TS that limits *k*. Paradoxically, the solvent isotope effect, ^Dk, on the PE of each series is in the range of 2–4 expected if general acid–base catalysis accompanies activation to a rate-limiting TS for chemical acylation.

We now report an extensive investigation of the acylation of subtilisin A by a series of aryl esters of McPhe using several probes, supplemented with similar data for Prop NPE. From this study we conclude that for aryl esters of McPhe, as well as for other aryl ester substrates of subtilisin A, the rate-limiting TSs are generally "virtual" TSs (Schowen, R. L., 1978), or weighted averages of at least two elementary TSs. Some properties of the individual TSs are described. One of these

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[1] Abbreviations: *k*, k_c/K_m; k_L, *k* in H_2O (k_H) or D_2O (k_D); ^Dk, k_H/k_D; k_L(max), pL-independent k_H or k_D; DCC, *N*,*N*′-dicyclohexylcarbodiimide; CI, *N*-*trans*-cinnamoylimidazole; Ac, N^α-acetyl; Bz, N^α-benzoyl; Mc, N^α-(methoxycarbonyl); Prop, 3-phenylpropanoic acid; NPE, *p*-nitrophenyl ester; PE, phenyl ester; TS, transition state; NMR, nuclear magnetic resonance. Amino acids are abbreviated by the usual three-letter designations; chiral acids are of the L configuration.

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TSs is the chemical step of enzyme acylation. Another is probably an enzyme conformational change.

Experimental Procedures

Substrates. Treatment of Phe with methyl chloroformate yielded McPhe as a colorless oil (Elmore & Smith, 1965). Substituted phenyl esters were synthesized by coupling equivalent amounts of the appropriate phenol and McPhe in ethyl acetate with the same equivalent of DCC. Recrystallization of the esters was from $CHCl_3$–hexane: *p*-nitrophenyl, mp 110.5–111.5 °C [lit. mp 99 °C (D isomer) (Elmore & Smith, 1965)]; *p*-cyanophenyl, mp 145.5–147.5 °C; *p*-fluorophenyl, mp 60–70 °C; *p*-acetylphenyl, mp 108–110 °C; *p*-methoxyphenyl, mp 129–130 °C. AcPhe NPE, Prop NPE, and BzGly NPE were available from earlier studies (Matta & Staley, 1974; Matta et al., 1976; Matta & Andracki, 1985). CI was purchased from Sigma.

Enzyme. Stock solutions, 50 mg mL^{-1}, of subtilisin A (Sigma Protease VIII, lots 35F-0206, 75F-0350, and 102F-0265) were prepared daily in 0.5 M KCl. Dilutions were made in the same solvent. Active site content of stock solutions was determined by spectrophotometric titration using CI according to a published method (Schonbaum et al., 1962). The enzyme contained about 60% of active sites on the basis of a M_r of 27 300 (Markland & Smith, 1971). All calculations of $[E]_0$ for kinetics employed the titration values.

Solutions. Buffer ingredients were analytical reagent-grade materials from several sources. Deionized water was brought to a permittivity of 10 MΩ by passage through the activated carbon and deionizing cartridges of a Continental water system. Deuterium oxide (Norrell, 99.8 atom % D) was distilled through a glass apparatus before use.

Solvent isotope effect determinations utilized H_2O solutions of precisely known pH and D_2O solutions of the pD equivalent (Schowen, K. J. B., 1978; Schowen & Schowen, 1982). To prepare buffers in H_2O, accurately weighed amounts of dry Na_2HPO_4 and KH_2PO_4 in a ratio needed to give the desired pH and total phosphate of 0.1 M, in addition to the amount of KCl necessary to give a final ionic strength of 0.5 M, were diluted to volume with H_2O. The final pH was measured to within 0.01 unit on an Orion Model 701A digital pH meter equipped with a Ross combination electrode. Solutions of the pD equivalent were prepared by dilution of precisely the same weights of the buffer pair and KCl in the same volume of D_2O. The D_2O solutions invariably gave a meter reading 0.12 ± 0.02 unit higher than the equivalent H_2O solutions, as expected from an upward shift of 0.52 pK_a unit for the $LPO_4^{2-}/L_2PO_4^-$ (L = H or D) dissociation (Jencks & Salvesen, 1971) and a downward shift in electrode response of 0.40 ± 0.02 unit in D_2O compared with that in H_2O (Schowen & Schowen, 1982; Schowen, K. B. J., 1978).

Solutions for pL dependences consisted of 0.1 M phosphate (pL > 6.0) and 0.1 M acetate (pL < 6.0). Values of pD of D_2O solutions were obtained from pH meter readings according to the relationship pD = pH(meter) + 0.40 (Glasoe & Long, 1960).

Kinetics. Acylations of subtilisin A by McPhe aryl esters and Prop NPE were carried out in the thermostated cell compartment of a Cary-Varian Model 219 spectrophotometer. Addition of 0.1 mL of substrate dissolved in CH_3CN and 0.1 mL of enzyme solution to 3.0 mL of buffer initiated the reaction. Absorbance increases owing to the production of the leaving group were followed at an appropriate wavelength: *p*-nitrophenoxide, 400 nm; *p*-chlorophenol, 275 nm; *p*-fluorophenol, 280 nm; *p*-methoxyphenol, 290 nm; *p*-acetylphenol, 325 nm; *p*-cyanophenol, 290 nm.

Table I: Subtilisin A: k_L and ^Dk for Para-Substituted Esters of McPhe[a]

substituent	σ	$10^{-5}k_H$ (M^{-1} s^{-1})	$10^{-5}k_D$ (M^{-1} s^{-1})	^Dk
NO_2	0.89[b]	2.31 ± 0.04	2.61 ± 0.07	0.89 ± 0.04
CN	0.56	3.99 ± 0.04	4.05 ± 0.03	0.99 ± 0.02
$COCH_3$	0.502	4.06 ± 0.06	4.49 ± 0.05	0.90 ± 0.03
F	0.06	2.62 ± 0.01	1.97 ± 0.07	1.34 ± 0.05
OCH_3	−0.268	2.93 ± 0.02	2.19 ± 0.04	1.34 ± 0.03

[a] At 25 ± 0.1 °C, pH 7.03 and pD equivalent; 3.2% CH_3CN; ionic strength 0.50 M; $[S]_0$ = 0.10 mM; $[E]_0$ = 38.8–45.0 nM. [b] "Enhanced" σ value for *p*-NO_2 (Kirsch et al., 1968); remainder of σ values from Leffler and Grunwald (1971).

In preliminary studies, plots of log $(A_\infty - A_t)$ vs time yielded straight lines through >90% reaction. Final experiments utilized a Bascom-Turner Model 4120 data center to acquire and store evenly spaced voltages related to the absorbance increases through five to seven half-lives. The stored data were treated according to the Guggenheim method (Guggenheim, 1926) to obtain pseudo-first-order rate constants, which were divided by $[E]_0$ to produce the apparent second-order rate constants k. The Guggenheim treatment utilized data from at least three half-lives; the constant difference between readings taken at a series of times and a series of times later was selected to be approximately two half-lives. In all of these studies, two to six H_2O/D_2O pairs of rate constants were obtained at each temperature. Each determination in H_2O was followed by a determination in D_2O to minimize environmental effects on the ^Dk's.

Values of k_H and k_D for acylation of subtilisin A by AcPhe NPE and BzGly NPE were determined in a stopped-flow spectrophotometer by previously described techniques (Matta et al., 1976), according to the pL equivalence method.

Results

Our earlier study of leaving group dependences, pL dependences, and solvent isotope effects in acylation of subtilisin A employed aryl esters of AcPhe, BzGly, and Prop (Matta et al., 1976). Because of the high uncatalyzed hydrolysis rates of the former two classes of esters owing to oxazolinone formation (Matta & Andracki, 1985), mechanistic studies at the desired level of detail were impractical. Activated esters of McPhe are not subject to oxazolinone formation, however. This additional hydrolytic stability enables kinetic studies by conventional spectrophotometry.

Leaving Group Dependence. Specificity ratios k for subtilisin A catalyzed hydrolyses of aryl esters of McPhe were determined in H_2O and in D_2O at pH 7.03 and pD equivalent (Schowen & Schowen, 1982) under pseudo-first-order conditions ($[E]_0 \ll [S]_0 \ll K_m$). These parameters, shown in Table I, exhibit no discernible dependence on leaving group ability. Indeed, McPhe NPE, which has the best leaving group of the five substrates listed in Table I, has the lowest k. A Hammett plot of the k_H data (not shown) gives a negligible slope (<0.01), with the data points scattered about the least-squares best-fit line. The scatter in the data is attributable to a leaving group hydrophobic substituent effect on k; similar effects are seen in the binding of substituted anilides of AcTyr by chymotrypsin (Fastrez & Fersht, 1973).

Solvent Isotope Effects. The ratios of k_H to k_D were taken to produce the ^Dk's of Table I. Values of k_L were also measured for AcPhe NPE and BzGly NPE by stopped-flow spectrophotometry; these were not available from the earlier study. AcPhe NPE gave $k_H = (1.70 \pm 0.03) \times 10^6$ M^{-1} s^{-1} and $k_D = (1.73 \pm 0.03) \times 10^6$ M^{-1} s^{-1}; $^Dk_E = 0.982 \pm 0.034$ at pH 7.16 and pD equivalent. BzGly NPE gave k_H = (3.40

Table II: Temperature Dependence of k_L and ^Dk for the Subtilisin A Catalyzed Hydrolysis of McPhe NPE[a]

temperature (°C)	$10^{-5}k_H$ (M^{-1} s^{-1})	$10^{-5}k_D$ (M^{-1} s^{-1})	^Dk
39.4	6.32 ± 0.21	6.08 ± 0.50	1.04 ± 0.07
35.4	5.41 ± 0.10	5.62 ± 0.34	0.96 ± 0.08
29.8	3.49 ± 0.10	3.72 ± 0.07	0.94 ± 0.05
25.0	2.31 ± 0.04	2.61 ± 0.07	0.89 ± 0.04
20.2	1.24 ± 0.02	1.27 ± 0.03	0.98 ± 0.08
13.6	0.493 ± 0.020	0.365 ± 0.000	1.36 ± 0.08
9.8	0.349 ± 0.020		

[a] Precision in temperature ±0.1 °C; other conditions as described in Table I, except $[S]_0$ = 37.5 μM and $[E]_0$ = 36.2–48.1 nM.

Table III: Temperature Dependence of k_L and ^Dk for the Subtilisin A Catalyzed Hydrolysis of Prop NPE[a]

temperature (°C)	$10^{-4}k_H$ (M^{-1} s^{-1})	$10^{-4}k_D$ (M^{-1} s^{-1})	^Dk
46.1	6.91 ± 0.18	3.97 ± 0.03	1.74 ± 0.04
40.9	6.38 ± 0.04		
36.1	5.64 ± 0.03	3.09 ± 0.07	1.83 ± 0.04
30.6	4.67 ± 0.06		
25.8	3.93 ± 0.06	2.06 ± 0.05	1.91 ± 0.05
20.5	3.09 ± 0.11	1.55 ± 0.06	1.99 ± 0.10
15.7	2.23 ± 0.05		
9.9	1.21 ± 0.04		

[a] Precision in temperature ±0.1 °C; other conditions as described in Table I, except $[S]_0$ = 37.7 μM and $[E]_0$ = 0.349–0.461 μM.

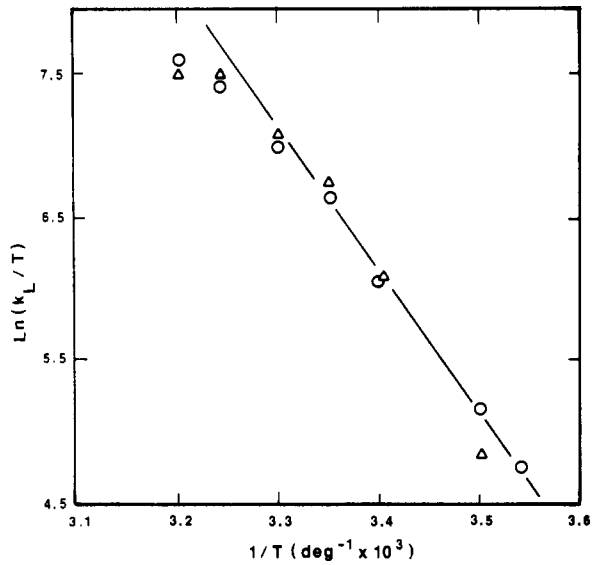


FIGURE 1: Eyring plot of the subtilisin A catalyzed hydrolysis of McPhe NPE from data of Table II as described in the text (circles, H_2O; triangles, D_2O). Least-squares best-fit line for D_2O data, of slightly larger slope, is not shown.

± 0.11) × 10^4 M^{-1} s^{-1} and k_D = (2.80 ± 0.07) × 10^4 M^{-1} s^{-1} at pH 6.67 and pD equivalent; ^Dk = 1.21 ± 0.06.

Temperature Dependences. Temperature dependences of k_L and ^Dk were determined for acylation of subtilisin A by McPhe NPE and the less reactive Prop NPE (Tables II–III). Figures 1 and 2 present Eyring plots of the temperature dependence data. The curvature in the Eyring plot for Prop NPE below 25 °C in H_2O is believed to be the result of aggregation of the substrate and not mechanistically significant.[2] Curvature is also observed in the plot for McPhe NPE at higher temperatures and may be mechanistically significant. We were primarily interested in the energetics of activation at 25 °C. Thus, ΔH^* for Prop NPE in H_2O was estimated from the linear portions of the temperature dependence plots at 25 °C and above, whereas all of the data were used in estimating ΔH^* for this substrate in D_2O. Data points at the two highest temperatures were omitted in calculating ΔH^* for McPhe NPE in both isotopic waters. The activation parameters thus obtained (Table IV) are rough estimates of the energetics at 25 °C, but are sufficiently accurate for our purposes.

[2] Effects of aggregation of this substrate are observed with chymotrypsin A_α at enzyme saturation in metastable substrate solutions of about 3 × 10^{-5} M. The deleterious effect on rate can be so pronounced as to produce lag periods of several minutes. When sufficient substrate has been hydrolyzed to make the remainder monodisperse, a return to normal kinetics, in terms of both order and reaction velocity, is observed (unpublished results of M. S. Matta).

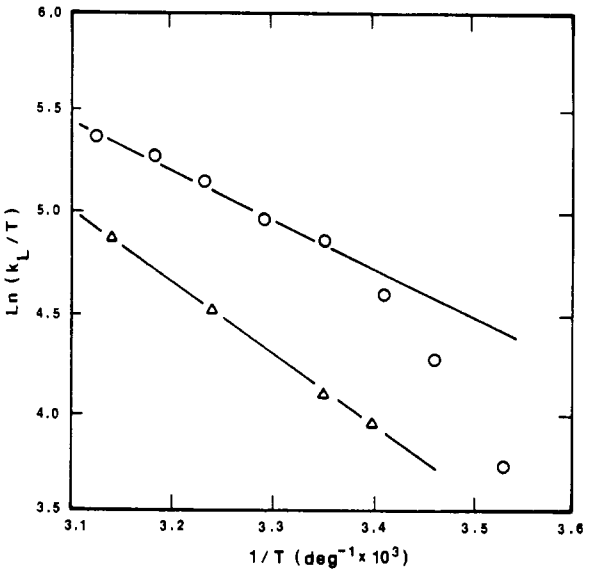


FIGURE 2: Eyring plot of the subtilisin A catalyzed hydrolysis of Prop NPE from data of Table III as described in the text (circles, H_2O; triangles, D_2O).

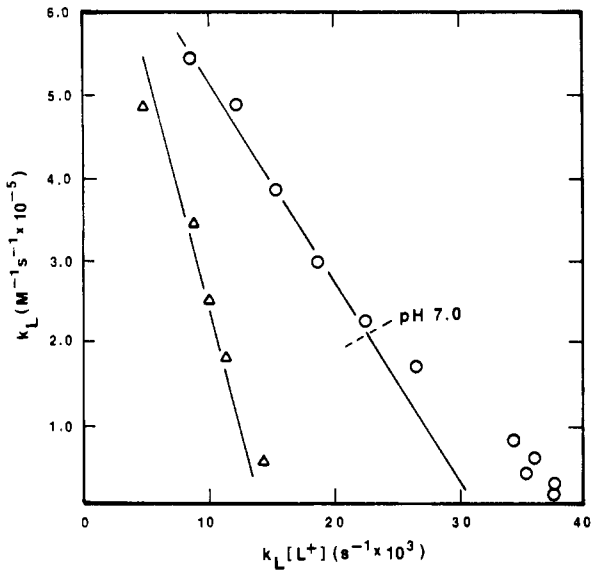


FIGURE 3: pL dependence of the subtilisin A catalyzed hydrolysis of McPhe NPE (circles, H_2O; triangles, D_2O).

pL Dependences. The pL dependence of k for subtilisin-catalyzed acylation by McPhe NPE was determined in the range pH 5.61–7.81 and pD 6.61–8.01. The data are plotted in Figure 3 as k_L vs $k_L[L^+]$. This plotting method, which derives from transformation of eq 1 to eq 2, has some ad-

$$k_L = \frac{k_L(\max)}{1 + [L^+]/K_{app}} \quad (1)$$

$$k_L = k_L(\max) - k_L([L^+]/K_{app}) \quad (2)$$

vantage over plots of k_L vs pL because deviations of the kinetic parameter from linearity in $[L^+]$ are more pronounced (Renard & Fersht, 1973). The plot of the pH data is linear above pH 7, but exhibits positive deviations at lower values of pH; the pD dependence is reasonably linear for all of the data points acquired. Least-squares fitting was used to obtain the slopes and intercepts from the linear portions of each plot. From the slope and intercept in H_2O, $k_H(\max) = (7.59 \pm 0.24) \times 10^5$ M^{-1} s^{-1} and $pK_{app} = 7.38 \pm 0.03$. In D_2O, $k_D(\max) = (7.31 \pm 0.36) \times 10^5$ M^{-1} s^{-1} and $pK_{app} = 7.67 \pm 0.03$. Thus, ${}^Dk(\max) = 1.04 \pm 0.09$. This is slightly larger than the ${}^Dk = 0.89 \pm 0.04$ obtained by the pL equivalence method near pH 7.03 and pD equivalent.

Initial inspection of the pL dependence data focused on two concerns. First, we wished to decide whether the pK_{app} obtained with McPhe NPE is a kinetically perturbed parameter. Kinetic perturbation of the equilibrium pK_a of an uncomplexed enzyme is generally diagnostic of a change in rate-limiting step with pH (Rosenberry, 1975; Quinn & Swanson, 1984; Albery & Massey, 1954; Renard & Fersht, 1973). Thus, such perturbations can be helpful in verifying the existence of virtual TSs. As to this first point, it appears that any kinetic perturbation of subtilisin's equilibrium pK_a upon acylation by McPhe NPE is either small or nonexistent. The experimental value $pK_{app} = 7.38 \pm 0.03$ is well within the mean of 7.39 ± 0.11 obtained previously with three NPEs, three PEs, and AcPhe *p*-acetylanilide (Matta et al., 1976). The range of pK_{app} in the previous determinations is 7.30–7.59, but the differences are attributable to experimental error; no relationship between substrate reactivity and pK_{app} is discernible. The pK_{app} of the kinetic experiments equals the equilibrium $pK_a = 7.2$ obtained by NMR titration of His-64 of native subtilisin A (Jordan et al., 1985), when the higher ionic strength of the kinetic experiments is taken into account.

Second, for the pL equivalence method to give correct ${}^Dk$ near the catalytically influential p*K* of the enzyme, the effect of going from H_2O to D_2O on the equilibrium pK_a's of the buffer and the enzyme must be identical (Schowen, K. B. J., 1978). Since $pK_{app} = pK_a$ in the acylation of subtilisin A by McPhe, $\Delta pK = pK_D - pK_H$ for the buffer and the enzymatic reaction may be compared. With regard to this second point, ΔpK for acylation of the enzyme by McPhe NPE is only 0.29. This is 0.23 unit lower than the $\Delta pK = 0.52$ for the phosphate buffer (Jencks & Salvesen, 1971). From four available examples, three PEs (Matta et al., 1976) and McPhe NPE, the mean $\Delta pK = 0.44 \pm 0.15$ (range 0.28–0.61); i.e., the mean enzyme pK_{app} is about 0.1 unit below that of the buffer. Because we find no relationship between ΔpK and pK_{app} for any of the substrates, we choose to attribute the range of ΔpK values to experimental error in the determinations and not to mechanistic effects. The apparent 0.1 unit difference between enzyme and buffer ΔpK's is small. If the difference exists, its effect is to lower ${}^Dk$'s determined by the pL equivalence method near the influential p*K* by about 15% relative to the true values (Schowen, K. B. J., 1978). An increase of 15% in the mean ${}^Dk = 0.89$, determined by the pL equivalence method, gives ${}^Dk = 1.02$; the calculated value is nearly identical with the ${}^Dk(\max) = 1.04$ determined from the pL-independent kinetics parameters.

The above considerations suggest that the ${}^Dk$ for the acylation of subtilisin A by McPhe NPE is essentially the same at every pL equivalent pair of points above neutrality. No change in rate-limiting step (or mix of rate-limiting steps) with pL is indicated. In later calculations, we regard ${}^Dk$ (pL equivalence) and ${}^Dk(\max)$ as interchangeable.

Discussion

This study was undertaken in an effort to learn why specificity ratios for series of specific activated ester substrates of subtilisin A exhibit negligibly small leaving group dependences, while the PEs of these substrates exhibit fairly large, normal solvent isotope effects. In this discussion, we first develop a qualitative model for this behavior on the basis of the available evidence obtained with various TS probes: reaction rates, leaving group dependence, solvent isotope effects, activation parameters, and temperature dependences of solvent isotope effects.

We next give a kinetic model that explains in a more quantitative fashion the behavior of the systems being investigated. The kinetic model allows us to rationalize the experimental pH dependences, to estimate the contributions to rate limitation of the putative chemical and physical steps, and to examine the relationship between these contributions and the magnitudes of the experimental solvent isotope effects and Hammett ρ values.

The conclusion reached through these considerations is that rate limitation of *k* of McPhe aryl esters, as well as other aryl ester substrates, is governed not only by chemical acylation with its accompanying normal solvent isotope effect but also by an isotopically near-silent physical step which is probably an enzyme conformational change. The shift from predominant rate limitation by chemical acylation to predominant rate limitation by the physical step as the leaving group improves within an ester series explains why PEs of BzGly and AcPhe exhibit ${}^Dk$'s of 2–4, characteristic of general acid–base catalysis, while the leaving group dependences of the series are less than anticipated for rate-limiting chemical acylation.

Rates and Leaving Group Dependences. The lack of leaving group dependence of the McPhe aryl esters suggests that some process other than acylation of Ser-221 limits the rate of reaction. The reactivity of these esters is low, however, if rate limitation is by enzyme–substrate association. The $k_H(\max)$ of McPhe NPE is 7.59×10^5 M^{-1} s^{-1}, but AcPhe NPE gives $k_H(\max) = 3.22 \times 10^7$ M^{-1} s^{-1} with chymotrypsin A_δ at similar conditions (Renard & Fersht, 1973). Enzyme–substrate association partially limits the rate of the latter reaction, with an estimated association rate constant of 6×10^7 M^{-1} s^{-1}. Thus, the rate and leaving group dependence data are consistent with at least partial rate limitation of *k* of McPhe aryl esters, by a step which is neither chemical acylation nor the initial association of enzyme and substrate.

Dependence of Solvent Isotope Effects on Leaving Group. The first three entries of Table I show ${}^Dk$'s of about 0.89–0.99 for substrates with the best leaving groups. These are striking results, since isotopic silence is unexpected for rate-limiting chemical acylation involving general catalysis. Although these isotope effects could be slightly understated, they are reminiscent of the ${}^Dk = 0.91$ obtained in the acylation of porcine pancreatic elastase with the α_1-protease inhibitor (Stein, 1985). The rate of acylation of elastase by the inhibitor is partially limited by a physical process that follows initial contact of the enzyme and substrate but precedes functioning of the general acid–base entity. The latter two entries of Table I, for substrates with poorer leaving groups, give normal ${}^Dk$'s of about 1.3.

The trend in the solvent isotope effects for the McPhe aryl esters is easy to rationalize qualitatively by invoking contributions to rate limitation by chemical acylation, with a normal leaving group dependence of $\rho = 1.0$ and a normal solvent isotope effect of 2–4, and by another process, which exhibits

Table IV: Activation Parameters for Subtilisin A Catalyzed Hydrolysis of McPhe and Prop NPEs[a]

NPE	solvent	ΔG^* (kJ mol^{-1})	ΔH^* (kJ mol^{-1})	ΔS^* (J K^{-1} mol^{-1})
McPhe	H_2O	42.4	87 ± 3	148 ± 14
	D_2O	42.1	100 ± 11	194 ± 18
Prop	H_2O	46.8	20 ± 2	−90 ± 5
	D_2O	48.4	25 ± 2	−78 ± 4

[a] At 25 °C with data of Tables II and III; 1 M standard states; $\Delta G^* = RT \ln (K_b T/hk_L)$, $\Delta H^* = R(-\text{slope})$, and $\Delta S^* = R[\text{intercept} - \ln (K_b/h)]$.

no leaving group dependence and is nearly isotopically silent. Because the properties of the latter process are consistent with a physical change of some kind, we hereafter call this process the "physical step".

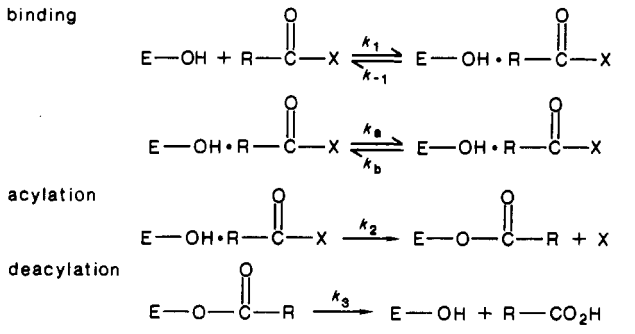
Values of k and ^Dk depend on the relative weights of each step. If the rate constant for the physical step is much slower than chemical acylation by substrates with good leaving groups such as NPEs, k will be independent of leaving group and ^Dk will be very near unity. As the rate of chemical acylation decreases with poorer leaving groups, the acylation step becomes more important in limiting k, and ^Dk becomes normal. There should be a trend toward more normal solvent isotope effects with poorer leaving groups. This is experimentally observed with the McPhe aryl esters. The isotopic silence of ^Dk of McPhe NPE and the leaving group independence of the McPhe aryl esters indicate that k for the former substrate is significantly, and perhaps completely, limited by the physical step.

Activation Parameters and Temperature Dependences of Solvent Isotope Effects. In contrast to McPhe NPE, k of Prop NPE, with a reported $^Dk(\text{max}) = 2.06$ and $\rho = 0.9$ for the aryl ester series (Matta et al., 1976), ought to be predominantly limited by chemical acylation. Comparisons of the activation parameters for these two substrates were therefore of considerable interest.

Activation parameters (Table IV) differ dramatically for the two substrates. Activation to the predominant rate-limiting TS of k for McPhe NPE is characterized by a large ΔH^* and large, positive ΔS^*, but activation of Prop NPE is characterized by low ΔH^* and large, negative ΔS^*. The difference in magnitude and sign of ΔS^* for McPhe and Prop NPEs is inexplicable in terms of structural variations in TSs for chemical acylation (Polgar & Fejes, 1979) of subtilisin by McPhe NPE and Prop NPE. This difference constitutes very good evidence that the processes predominating limitation of k of McPhe NPE and Prop NPE are kinetically distinct.

Except for the activation parameters and solvent isotope effect, the nature of the physical step is ill defined. Perusal of the review on temperature dependences of enzymatic reactions by Laidler and Peterman (1979) provides valuable insights. Every instance of a large, positive ΔS^* on a rate constant for the reaction or temperature-dependent inactivation of an enzyme is connected with a profound reorganization of protein structure, such as a conformational change. Laidler and Peterman attribute the positive ΔS^* values to unfolding of protein structure upon substrate binding. Although the interpretation of the physical change in protein structure reflected in the activation parameters is probably correct, it seems likely that the large ΔH^* and ΔS^* for the physical step limiting the rate of acylation of subtilisin A by McPhe NPE, as well as for other physical processes involving proteins, are dominated by solvation processes. Thus, these processes appear to involve the reorganization of many solvent molecules upon activation and produce little or no net isotope effect. In any

Scheme I



event, a protein conformation change subsequent to enzyme–substrate association but preceding chemical acylation is a reasonable explanation for the activation entropy and solvent isotope effect of the physical step. As in subtilisin BPN (Kraut, 1976), the active site of subtilisin A is probably an extended, shallow cleft. The binding of the substrate could induce significant loosening or tightening of the protein structure, accompanied by disordering or extrusion to bulk water of an ice-like water structure around the hydrophobic side chains of amino acid residues. Possibly the work of desolvation is not compensated by a large number of favorable enzyme–substrate interactions when the substrate is small, so that the conformation change with its attendant desolvation is energetically unfavorable. Rate limitation by the physical step therefore could be limited to kinetic situations in which the substrate is small and chemical acylation is rapid.

Temperature dependences of the ^Dk's are also consistent with the minimum two-step hypothesis. The ^Dk of McPhe NPE becomes normal above and below 25 °C. The increase at 13.6 °C is certainly real, and probably represents the inception of a switch in rat limitation from physical step to chemical acylation. We are less confident of the ^Dk of 1.04 obtained at 39.4 °C. The original results were confirmed in all respects by two investigators, however. This small effect could represent partial limitation of k by yet another step, possibly the initial contact of the enzyme and substrate, at higher temperature. A regular decrease in ^Dk occurs with increasing temperature for Prop NPE. Even with this slow substrate, where chemical acylation dominates k, another kinetic step appears to contribute to rate limitation. This step and the step that dominates rate limitation of k of McPhe NPE may be the same.

Kinetic Model. Being satisfied that at least two processes contribute to k, we considered whether the isotope effect data and leaving group dependences could be used to obtain semiquantitative estimates of the contributions of each process. We also wished to know whether the invariance of pK_{app} could be rationalized analytically, even though multiple steps are rate limiting, since previous proposals that include such steps usually find pK_{app} lower than the pK_a of the free enzyme.

We began by expanding the usual formulation of the kinetic scheme for formation of acyl enzyme to include a transformation following initial contact of enzyme and substrate, but preceding chemical acylation (Scheme I). Rate coefficients for the physical step are designated k_a and k_b, so that the association, acylation, and deacylation rate constants retain their usual notation (Zerner & Bender, 1964). When the association rate constant k_1 is too large to contribute to rate limitation, k is given by eq 3. When $k_2 \gg k_b$, k is completely

$$k = \frac{k_1 k_a k_2}{k_{-1}(k_2 + k_b)} \qquad (3)$$

$$k = k_a/K_s \quad (4)$$

$$k = k_2/KK_s \quad (5)$$

determined by the TS for the formation of the second complex (eq 4), but if $k_b \gg k_2$, k is determined by the TS for chemical acylation (eq 5, $K = k_b/k_a$). When k_2 and k_b are approximately equal, the observed k reflects the weighted average of the TSs for the physical step k_a and the chemical acylation step k_2.

pH Dependence. The pH dependence of k for McPhe NPE is very similar to other aryl esters and AcTyr *p*-acetylanilide. For seven such dependences (Matta et al., 1976), plus that of McPhe NPE, $pK_{app} = 7.39 \pm 0.11$. All of these dependences exhibit positive deviations from eq 1 below neutrality. Scheme II is applicable to the pH behavior above neutrality. According to Scheme II, the pH dependence of k is given by eq 6 (Rosenberry, 1975). i.e., Invariance of pK_{app} will occur if k_a' is

$$k = \frac{k_a k_2\left(1 + \dfrac{k_a'[H^+]}{k_a K_a'}\right)}{\left(1 + \dfrac{[H^+]}{K_a}\right)\left[k_2 + k_b\left(1 + \dfrac{k_a'[H^+]}{k_a K_a'}\right)\right]K_s} \quad (6)$$

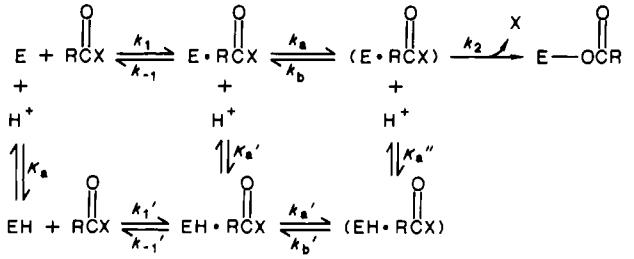
$$k = k_a k_2/[(k_2 + k_b)(1 + ([H^+]/K_a)K_s)] \quad (7)$$

negligible, since eq 6 reduces to eq 7; i.e., pK_{app} closely approximates the pK_a of the free enzyme, regardless of the contributions of the physical step and chemical acylation step to rate limitation of k. Since both the physical step and chemical acylation follow identical pH dependences, the relative contributions of these steps to rate limitation is invariant with pH. Thus the solvent isotope effect should be constant thoroughout the pH range. This expectation is realized within experimental error in the correspondence between the ^Dk(max) and the ^Dk calculated for McPhe NPE at pH 7.03 and its pD equivalent.

The positive deviations in linearity in $[H^+]$ below pH 7 could be interpreted to mean that at low pH the term $k_a'[H^+]/k_aK_a'$ becomes significant, resulting in a plateau in the pH dependence. However, the pH dependence of k for AcTyr *p*-acetylanilide, for which k_2 is reasonably presumed to be completely rate limiting ($k = 78.9\ M^{-1}\ s^{-1}$ at pH 7.0; Matta et al., 1976), exhibits even larger deviations than those for the aryl esters. It seems more likely that the positive deviations are the result of changes in the Linderstrøm–Lang parameter of the protein (Renard & Fersht, 1973) or the incursion of a parallel acid-catalyzed pathway for acylation.

It is informative to compare the pH-dependence behavior of subtilisin A with that of acetylcholinesterase, a serine esterase (Rosenberry, 1975; Quinn & Swanson, 1984). In the latter enzyme a physical step, which sometimes contributes to rate limitation of k, intervenes between enzyme–substrate association and chemical acylation, as we are proposing for subtilisin A. A difference between the two enzymes is that the physical step is pH dependent in the protease but pH independent in the esterase. The pH dependence of k for acetylcholinesterase is also given by eq 6 (Rosenberry, 1975). The uncomplexed enzyme has $pK_a = 6.3$, and this is the value of pK_{app} when acylation is completely rate limiting. When the rate is partially limited by the physical step, however, pK_{app} drops to as low as 5.3. In terms of eq 6, if $k_a' = k_a$ (physical step independent of pH) and $K_a' = K_a$, $K_{app} = (1 + k_2/k_b)K_a$. The lower pK_{app} reflects the ratio k_2/k_b. This ratio is a commitment factor, since it reflects the tendency of the second

Scheme II



noncovalent complex to proceed to products (commitment to catalysis) or revert to enzyme–substrate complex (Northrup, 1977).

Effect of Contributions of Chemical and Physical Steps on Observed Solvent Isotope Effects. With a fair amount of experimental data and a theoretical model in hand, we examined the feasibility of computation of the relative weights of contributions of the physical and chemical steps to rate limitation in the acylation of subtilisin A by various aryl ester substrates. Ideally, these computations would show whether changes in the weighting factors account for the variation in solvent isotope effects with changes in substrate acyl group and leaving group. Table V lists experimental ^Dk values for PE and NPE derivatives of four series of aryl esters. These parameters are compared with calculated values. The remainder of this section describes and gives the results of the computations.

Because we are dealing with various perturbations to k within series of substituted aryl esters, we make the simplifying assumption that the only effect of leaving group substituents on k is electronic. Thus *p*-fluorosubstituents ($\sigma = 0.06$) and *p*-hydro substituents ($\sigma = 0.00$) are considered equivalent, since σ is about the same for both substituents. The assumption may not be strictly correct; there is probably a small hydrophobic substituent effect on k (Fastrez & Fersht, 1973).

Perturbations of the forward steps of an enzymatic reaction with a single rate-limiting step, as in eq 4 and 5, by changes in environmental factors such as temperature, solvent, or isotopic composition of substrate, cause changes in the observed rate constant that reflect the free energy change upon transformation of the enzyme and substrate to the TS for that step. The k's of eq 4 and 5 may therefore be treated as elementary rate constants. We denote the rate constant of the physical step k_a/K_s as k_P; similarly, the rate constant for the isolated chemical acylation step is designated k_C. The observed solvent isotope effect ^Dk is the weighted average of the intrinsic effects on k_P and k_C (eq 8), with the weighting factors given by eq 9–11.

$$^Dk = W_P(^Dk_P) + W_C(^Dk_C) \quad (8)$$

$$W_P = k_2/(k_b + k_2) \quad (9)$$

$$W_C = k_b/(k_b + k_2) \quad (10)$$

$$W_C + W_P = 1 \quad (11)$$

Values of W_P and W_C can be obtained only if ^Dk_P and ^Dk_C are known or can be estimated. Since the intrinsic isotope effects are not known, it is necessary to estimate them. We chose ^Dk_C as 4.4. The magnitude of the effect is based on trial and error, but is about the same as the experimental value of ^Dk of 4.30 found in the acylation of trypsin by BzPhe-Val-Arg *p*-nitroanilide (Elrod et al., 1980). A $^Dk_P = 0.90$ was taken in order to match the experimental results for McPhe NPE, which may be completely rate limited by the physical step at

Table V: Comparison of Experimental and Calculated ^{D}k's and Hammett ρ Values for Subtilisin A Catalyzed Hydrolysis of Activated Esters[a]

substrate	W_C	W_P	^{D}k exptl	^{D}k calcd	k_2/k_b	ρ exptl	ρ calcd	$10^{-5}k_H$(max) (M^{-1} s^{-1})
McPhe NPE	0.010	0.990	0.89,[b] 1.04[c]	0.94	115	0.0	0.0	7.59
McPhe PE[d]	0.080	0.920	1.34[b]	1.29	11.5			8.50[e]
AcPhe NPE	0.087	0.913	0.98[b]	1.21	10.4	0.0	0.3	38.9
AcPhe PE	0.490	0.510	2.61[c]	2.61	1.04			39.7
BzGly NPE	0.140	0.860	1.21[b]	1.39	6.13	0.4	0.4	1.14
BzGly PE	0.620	0.380	3.06[c]	3.07	0.613			0.679
Prop NPE	0.870	0.130	1.91[b]	1.94	0.149	0.9	0.9	1.04
Prop PE	0.985	0.015	2.06[c]	2.08	0.0149			0.125

[a] Experimental isotope effects, ρ values, and k_H(max) from this work or Matta et al. (1976); calculation of ^{D}k by successive approximation of $W_C(4.4) + W_P(0.90)$ or, for the Prop PE and NPE, $^{D}k = W_C(2.10) + W_P(0.90)$; calculated $\rho = \log [(W_P)_{NPE}/(W_P)_{PE}]$. [b] Obtained as ^{D}k(pL equivalent). [c] Obtained as ^{D}k(max). [d] Actually the *p*-fluorophenyl ester (see text). [e] Estimated with k_H of *p*-fluorophenyl ester at pH 7.03, 25 °C, pK_{app} = 7.40, and eq 1; remainder of k_H(max) experimentally determined from pH–rate profiles.

room temperature. These values were used throughout, except where later noted. A sample application of the estimated intrinsic effects is illustrated for McPhe NPE and PE, presented as the first entries of Table V.

Computation was initiated with McPhe PE. W_P and W_C were successively approximated from eq 8, to give the best fit to the experimental ^{D}k = 1.34. The W_C and W_P thus obtained are 0.080 and 0.920; i.e., the TSs of chemical acylation and of the physical step respectively contribute 8% and 92% to limitation of k of the PE. The commitment factor k_2/k_b = 11.5 was calculated from either W_C or W_P by means of eq 9 or 10.

Using k_2/k_b of the PE, we calculated a commitment factor for McPhe NPE. It was assumed that k_2 is increased 10-fold by the change in leaving group. This increase approximates the unitary Hammett ρ value for rate-limiting addition of an oxyanion to an ester carbonyl group, as expected for chemical acylation. In this illustration, the k_2/k_b of 11.5 for the PE was increased to 115. The relative values of k_2 and k_b were incorporated into eq 9 or 10 to produce W_C = 0.010 and W_P = 0.99. The new weighting factors were used to calculate ^{D}k for the NPE with eq 8. As Table V shows, the experimental and computed values of ^{D}k for McPhe NPE are very close. Estimates of W_P, W_C, k_2/k_b, and ^{D}k were made in a similar fashion for the AcPhe and BzGly PEs and NPEs. The agreement of the experimental and theoretical ^{D}k's is also good for the BzGly and AcPhe esters.

The final entries of Table V are Prop NPE and PE. Agreement between the experimental and computed ^{D}k for Prop NPE cannot be obtained when $^{D}k_C$ = 4.4 is assumed for the PE. In this instance, a good fit of experimental and calculated isotope effects was obtained if $^{D}k_C$ = 2.1 was assumed.

Effect of Contributions of Chemical and Physical Steps on Observed Hammett ρ Values. Partial rate limitation of k by two steps, one of which is leaving group dependent and the other leaving group independent, ought to produce curved Hammett plots. Scatter in k within a series, say due to hydrophobic substituent effects, is likely to mask this curvature in experimental plots. Thus, we anticipated that theoretical ρ values obtained from the two-step model of rate-limitation, and estimated from only the PE and NPE of a given series, should approximate the experimental values.

It is easily shown that if K_s and K are unaffected by the leaving group substituent and $(k_2)_{NPE} = 10(k_2)_{PE}$, ρ is given by eq 12, provided that only TSs for k_P and k_C contribute to

$$\rho = \log \frac{k_{NPE}}{k_{PE}} = \log \frac{(W_P)_{NPE}}{(W_P)_{PE}} \qquad (12)$$

limitation of k. As shown in Table V, the experimental ρ values, taken from the complete series, and the theoretical values, calculated from only the W_P of the NPE and PE of each series, are in agreement for the McPhe, BzGly, and Prop esters. The predicted value of ρ for AcPhe esters is 0.3, but no dependence of k on leaving group is actually observed (Matta et al., 1976). AcPhe PE and NPE are more rapidly hydrolyzed than the same esters of the other series. Although the calculated ρ value is in anticipated direction, contributions of enzyme–substrate association to rate limitation or breakdown of one or more of the other assumptions of the model may adversely affect the AcPhe ester results. In general, however, departures from the extensive explicit and implicit assumptions involved in the computations of solvent isotope effects and Hammett ρ values evidently are of minor importance or cancel in the final results.

Conclusions. The specificity ratio k for the subtilisin A catalyzed hydrolysis of McPhe aryl esters is limited by at least two processes. One of these processes is chemical acylation of the enzyme, and the other is physical and probably involves a change in enzyme conformation. Chemical acylation exhibits a leaving group electronic dependence, substantial normal kinetic solvent isotope effect, small ΔH^*, and large negative ΔS^* and follows the pH dependence of the uncomplexed enzyme. The physical step exhibits no leaving group dependence, is nearly isotopically silent, has a large ΔH^* and large positive ΔS^*, and also follows the pH dependence of the uncomplexed enzyme. Contributions of each process to rate limitation of k, computed for PEs and NPEs of McPhe, AcPhe, BzGly, and Prop, indicate that chemical acylation contributes roughly 1% (McPhe NPE) to 98.5% (Prop PE) of the rate limitation of k. The physical step contributes roughly 1.5% (Prop PE) to 99% (McPhe NPE) of the rate limitation of k. Within these series of esters, a decreased contribution of physical step, as chemical acylation becomes slower with poorer leaving groups, causes a corresponding increase in the magnitude of ^{D}k. The physical step dominates rate limitation of k of the most rapidly hydrolyzed substrates, so that the leaving group dependence is smaller than anticipated. The apparent paradox described in the introduction is therefore resolved. Values of k for PEs of BzGly and AcPhe, at the low-reactivity end of their respective series, are primarily rate limited by chemical acylation. These substrates therefore exhibit ^{D}k's of 2–4 characteristic of general acid–base catalysis. The more reactive members of the series are primarily rate limited by the electronically and isotopically insensitive physical step. Values of ^{D}k decline for these members, and the leaving group dependences of the series are less than anticipated for rate-limiting chemical acylation.

Supplementary Material Available

Derivations of rate law, weighting factors, and relationship

between weighting factors and Hammett ρ values (8 pages). Ordering information is given on any current masthead page.

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Serine Hydroxymethyltransferase: Mechanism of the Racemization and Transamination of D- and L-Alanine†

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ABSTRACT: The reaction specificity and stereochemical control of *Escherichia coli* serine hydroxymethyltransferase were investigated with D- and L-alanine as substrates. An active-site H228N mutant enzyme binds both D- and L-alanine with K_d values of 5 mM as compared to 30 and 10 mM, respectively, for the wild-type enzyme. Both wild-type and H228N enzymes form quinonoid complexes absorbing at 505 nm by catalyzing the loss of the α-proton from both D- and L-alanine. Racemization and transamination reactions were observed to occur with both alanine isomers as substrates. The relative rates of these reactions are quinonoid formation > α-proton solvent exchange > racemization > transamination. The observation that the rate of quinonoid formation with either alanine isomer is an order of magnitude faster than solvent exchange suggests that the α-protons from both D- and L-alanine are transferred to base(s) on the enzyme. The rate of racemization is 2 orders of magnitude slower than the formation of the quinonoid complexes. This latter difference in rate suggests that the quinonoid complexes formed from D- and L-alanine are not identical. The difference in structure of the two quinonoid complexes is proposed to be the active-site location of the α-protons lost from the two alanine isomers, rather than two orientations of the pyridoxal phosphate ring. The results are consistent with a two-base mechanism for racemization.

Serine hydroxymethyltransferase (SHMT)[1] (EC 2.1.2.1) catalyzes the reversible interconversion of serine and glycine with tetrahydrofolate serving as the one-carbon carrier (Schirch, 1982). In addition to this physiological reaction, SHMT catalyzes several other reactions characteristic of pyridoxal-P enzymes:

$$\text{allothreonine} \rightleftharpoons \text{glycine} + \text{acetaldehyde} \quad (1)$$

$$\text{aminomalonate} \rightarrow \text{glycine} + CO_2 \quad (2)$$

$$\text{glycine} + \text{lipoate} \rightarrow NH_2CH_2\text{-lipoate} + CO_2 \quad (3)$$

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