Experimental Section

Materials. Enzymes, substrates, and alkylating agents were purchased or synthesized as described in the preceding paper in this issue.[3] Bromoacetanilide was a generous gift of Dr. W. B. Lawson, New York State Department of Health, Albany.

Synthesis of N-Ac-Ala-Gly-Gly-OMe. To 775 mg (3.8 mmol) of L-alanylglycylglycine (Ala-Gly-Gly, Nutritional Biochemicals Corp., Cleveland, Ohio) slurried in 125 mL of dry methanol at −60 °C was added 10.0 mL (139 mmol) of thionyl chloride in 50 mL of $CHCl_3$. The solution was allowed to warm to room temperature with constant stirring, after which the solvents were removed in vacuo, followed by redissolution of the residue in 50 mL each of pyridine and water. At 0 °C, 4.0 mL (116 mmol) of acetic anhydride was added with vigorous stirring, followed by a second 4.0 mL 15 min later. The reaction proceeded 30 min longer, followed by evaporation and chromatography on silica gel (14.5 × 3.8 cm) in 300-mL steps of 5, 10, and 15% methanol–chloroform. The acetylated tripeptide methyl ester, *N*-Ac-Ala-Gly-Gly-OMe, eluted in the last two steps: yield 530 mg (54%); mp 133–135 °C; MS parent peak at 259 ($A + 1/A = 0.124 \pm 0.004$; calcd., 0.124), fragmentation peaks at *m/e* 171, 144, 114, and 87 for $CH_3CONHCHCH_3CONHCH_2CO^+$, $^+NCH_2CONHCH_2CO_2CH_3$, $CH_3CONHCHCH_3CO^+$, and $^+NCH_2CO_2CH_3$, respectively; ^1H NMR (^2H_2O, DSS internal reference) 4.28 (quartet, C_α-H of Ala), 4.04 and 3.96 (2s, α-CH_2 of each Gly), 3.75 (s, methyl ester CH_3), 2.03 (s, CH_3 of acetyl group), 1.39 ppm (doublet β-CH_3 of Ala).

Procedures. Enzyme alkylation, assay, and purification have been described in the preceding paper.[3] *p*-Nitrophenyl acetate kinetics were measured spectrophotometrically on a Unicam SP 1800 instrument at pH 7.16 (0.09 M phosphate), 25 °C, 6.9% CH_3CN (to dissolve the substrate), at 347.8 nm where an isosbestic point for nitrophenol was used: $\Delta\epsilon = 5.06 \times 10^3\ M^{-1}\ cm^{-1}$. The pH–rate profile was measured in phosphate buffers as well.

Ac-Tyr-OEt and *N*-Ac-Ala-Gly-Gly-OMe kinetics were measured with a Radiometer pH-stat system at pH 8.0, 0.1 M, KCl, 25 °C. All kinetic data were processed on a Data General Nova 1220 minicomputer by either linear least squares or a nonlinear regression analysis written by M. H. Klapper based on CURFIT by Bevington.[10]

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Protonic Reorganization in Catalysis by Serine Proteases: Acylation by Small Substrates[1]

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Abstract: The pH (pD)–rate profiles for acylation of α-lytic protease in protium and deuterium oxides by *p*-nitrophenyl acetate show p*K* values of 5.92 and 6.60, well below the enzyme ionization pK values of 6.70 and 7.35. This is attributed to a pH-induced change in the rate-determining step. The data are consistent with an initial acylation of active-site histidine (protolytically assisted, $k_H/k_D = 2.4$), followed by an intramolecular N → O acyl shift to active-site serine by parallel specific-acid-catalyzed ($k_H/k_D = 0.5$) and general-acid-catalyzed ($k_H/k_D = 2$) routes. The magnitude of $pK(D_2O) - pK(H_2O)$ and a proton inventory of the general-acid-catalyzed N → O acyl shift both suggest that deprotonation of α-lytic protease generates an unusual protonic site with a "loosely bound" proton. The β-deuterium isotope effect, $k_{3H}/k_{3D} = 0.98$, for the same step confirms nucleophilic interaction at carbonyl in the transition state. An abbreviated proton inventory for acylation of α-chymotrypsin by *p*-nitrophenyl acetate is consistent with a "loosely bound" proton there also. A proton inventory for acylation of elastase by *N*-(carbobenzyloxy)-L-alanine *p*-nitrophenyl ester is linear, suggesting one-proton catalysis and indicating that if "loosely bound" reactant-state protons are present, they are catalytically silent. The general picture, from this work and that of others, is that the catalytic response of serine proteases to small, "unnatural" substrates is highly variable, both in site of nucleophilic attack and involvement of protolytic catalysis. Probably mutual transition-state interactions over an extended region of both enzyme and natural-substrate structure are required to bring into active function the full catalytic capability with which the serine proteases have been endowed by biological evolution.

Enzymes of the serine hydrolase class[2] catalyze the hydrolysis of acyl substrates, such as peptides, other amides, and esters, by the double-displacement (acylation–deacylation) mechanism of eq 1. Their catalytic power is generally supposed to derive, at least in part, from general acid–base catalysis[3] because (a) their active sites contain an assembly of carboxylate and histidine units[4] which are chemically suited for such a purpose[5] and which are biologically conserved from bacteria to mammals[6] and because (b) their catalytic rates are commonly reduced by two- to fourfold in deuterium oxide.[7]

$$E\text{–}OH + RCOX \rightarrow EOCOR + HX \quad (1a)$$

$$EOCOR + H_2O \rightarrow E\text{–}OH + RCO_2^- + H^+ \quad (1b)$$

The carboxylate–histidine assembly (the "charge-relay system";[4,5] hereafter ABCE, for "acid–base catalytic entity") has been examined in stable states of the enzymes by NMR techniques.[8-10] One of the protons associated with this assembly displays an unusual chemical shift,[8] but NMR titrations of the bacterial enzyme α-lytic protease in which the histidine has been isotopically labeled at carbon[9] or nitrogen[10] are in disagreement

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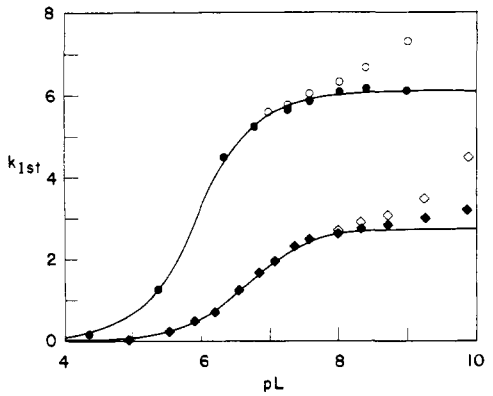

Figure 1. First-order rate constants (plotted as 10^4k, s^{-1}, for an enzyme concentration of 0.630 mg mL^{-1}) for acylation of α-lytic protease by 4.6–4.7 × 10^{-5} M *p*-nitrophenyl acetate, as a function of pL (pH in H_2O, circles on upper curve; pD in D_2O, diamonds on lower curve) at 25.0 ± 0.1 °C, μ = 0.043. The filled circles and diamonds are for the enzymatic acylation reaction only. The open circles and diamonds are for the total appearance of *p*-nitrophenol from both background and enzymatic reactions.

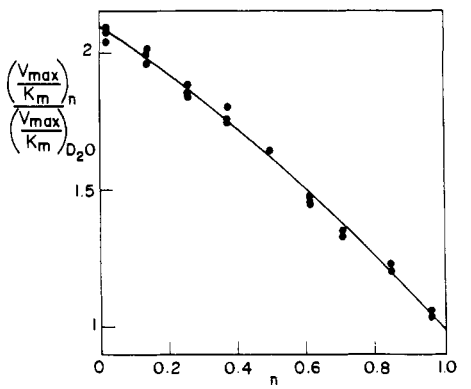

Figure 2. A proton-inventory plot, of partial solvent isotope effect, k_E^n/k_E^1, for acylation pf α-lytic protease by 4.9 × 10^{-5} M *p*-nitrophenyl acetate at pH 8.07 and equivalent (Tris buffers) as a function of *n*, the atom fraction of deuterium in binary mixtures of protium and deuterium oxides as solvent at 25.00 ± 0.05 °C, μ = 0.025. k_E^0 = 19.8 ± 0.2 M^{-1} s^{-1}.

as to the relative basicity of the carboxylate and histidine functions. These studies are valuable for the important task of structural characterization of the enzymes in their stable solution forms, but an account of their catalytic power will necessarily have to focus on the transition states rather than the stable states.[11] For the particular purpose of investigating the role of transition-state protonic reorganizations at exchangeable sites, such as would be involved in acid–base catalysis, solvent kinetic isotope effects can be useful.[7,12,13] In the present report, we want to consider protonic reorganization in transition states for α-lytic protease from *Sorangium* and for porcine elastase and some supporting data for α-chymotrypsin. A previous study of these structurally and functionally related[14,15] enzymes was made by Hunkapiller, Forgac, and Richards,[16] who measured the values of k_{cat} ($\equiv k_{ES}$) for the specific-substrate analogue Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide in mixtures of light and heavy water (atom fraction of deuterium *n*). They found that these proton inventories were described by $k_{ES}^n/k_{ES}^0 = (1 - n + n[0.60])^2$ for α-lytic protease and by $k_{ES}^n/k_{ES}^0 = (1 - n + n[0.68])^2$ for elastase. Spectroscopic evidence was given that the rate-determining step under observation was leaving-group expulsion from the tetrahedral intermediate formed from the substrate and the active-site serine hydroxyl. The proton inventories are consistent with a model in which two protons of the enzyme structure are participating in catalytic bridges,[13] each proton contributing an isotope effect of about 1.7 (thus an overall effect of about 2.8) with α-lytic protease and about 1.5 (overall about 2.2) with elastase. These two protons are most logically considered those of the ABCE[16] assisting in fission of the carbon–nitrogen bond of the tetrahedral adduct.

The finding of two-proton catalysis with these serine proteases contrasted with a report from our group of one-proton catalysis in the deacetylation of acetyl-α-chymotrypsin.[17] Although there are models for multiple-proton catalysis which can be fit to the one-proton data if a large number of other protons are also postulated to make small contributions,[18] the simplest interpretation would seem to be that small, unnatural substrates react by a one-proton mechanism like that employed by general catalysts in model systems.[19] When the substrate structure is elaborated sufficiently to permit the remote-subsite interactions known[20] to be important in serine proteases, then a compression across the hydrogen-bond structure of the ABCE brings two-proton catalysis into play.[13,21]

We now report investigations of protonic reorganization features in catalytic transition states of α-lytic protease, porcine elastase, and α-chymotrypsin which are exposed through the use of unnatural or less nearly natural substrates.

Results

pH and pD Rate Profiles for α-Lytic Protease and *p*-Nitrophenyl Acetate. Spectrophotometric determination of the first-order production of *p*-nitrophenol and *p*-nitrophenoxide at low substrate concentrations yielded the rate constants plotted in Figure 1, with the D_2O values scaled up by a factor of 1.63 to correct for the different enzyme concentrations employed (0.630 mg mL^{-1} in H_2O, 0.386 mg mL^{-1} in D_2O). At higher basicities, a background reaction of *p*-nitrophenyl acetate with buffer and lyate species begins to contribute. The rate of this background reaction was measured in the absence of enzyme, but with otherwise identical conditions, and subtracted from the total rate in the presence of enzyme. When the data for the enzymatic reaction alone are considered, the rate constant k_E ($\equiv k_{cat}/K_m$) is given by eq 2, where

$$k_E = k_E^{lim}[K_a/(K_a + a_H)] \quad (2)$$

k_E^{lim} is the limiting value in basic solution, K_a is an apparent ionization constant, and a_H is the hydrogen-ion activity expressed in molar units, for both H_2O and D_2O. The slight deviations at pD 9–10 in D_2O will be discussed later. Conversion to linear form and least-squares determination of k_E^{lim} and K_a yields

$$k_E^{lim} = 19.27 \pm 0.27\ M^{-1}\ s^{-1}\ (H_2O)$$

$$k_E^{lim} = 8.61 \pm 0.15\ M^{-1}\ s^{-1}\ (D_2O)$$

$$10^6K_a = 1.20 \pm 0.08\ M\ (H_2O)$$

$$10^6K_a = 0.25 \pm 0.02\ M\ (D_2O)$$

The solvent isotope effects are

$$k_E^{lim}(H_2O)/k_E^{lim}(D_2O) = 2.24 \pm 0.05$$

$$K_a(H_2O)/K_a(D_2O) = 4.83 \pm 0.47$$

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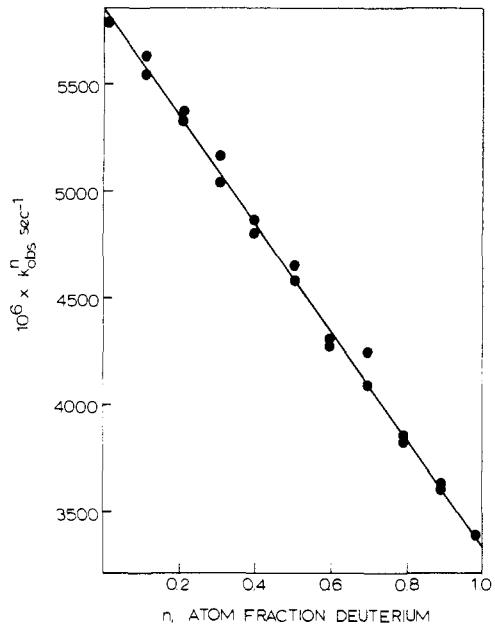

Figure 3. A proton-inventory plot for acylation of elastase by 3.01×10^{-5} M N^{α}-(carbobenzyloxy)-L-alanine *p*-nitrophenyl ester at pH 8.00 and equivalent (0.0281 M Tris-HCl, 0.0219 M Tris) at 25.00 ± 0.03 °C, M = 0.028. First-order rate constants at an enzyme concentration of 1.6 ng mL^{-1} are plotted vs. *n*, the atom fraction of deuterium in binary mixtures of protium and deuterium oxides as solvent.

The values of pK_a, which should refer to a free enzyme ionization,[22] are 5.92 ± 0.03 (H_2O) and 6.60 ± 0.03 (D_2O). These values differ strongly from the values from pH, pD profiles with *N*-acetyl-L-valine methyl ester (6.70 [H_2O], 7.35 [D_2O]) obtained by Kaplan and Whitaker[23] and from the similar H_2O values from NMR titrations.[9,10] We consider this distinction mechanistically important; its significance is discussed later.

Proton Inventory: α-Lytic Protease with *p*-Nitrophenyl Acetate. Rate constants are shown in Figure 2 for the reaction in mixtures of H_2O and D_2O at "equivalent"[12] pH (constant buffer ratio). All pH values are on the flat region of the pH/pD–rate profile so that whether the value of p$K(H_2O)$ – p$K(D_2O)$ for the buffer matches that for the enzyme is not important. Here again background contributions have been evaluated and removed. The nonlinear dependence of k_E on atom fraction of deuterium is discussed below.

Proton Inventory: Elastase with N^{α}-(Carbobenzyloxy)-L-alanine *p*-Nitrophenyl Ester. Figure 3 presents similar data for porcine elastase with an ester having the "natural" *N*-acyl-L-alanine structure for an elastase substrate. Here k_E depends linearly upon *n*.

β-Deuterium Secondary Isotope Effect: α-Lytic Protease with *p*-Nitrophenyl Acetate. In order to test whether nucleophilic interaction is occurring at the substrate carbonyl group in the transition state for acylation of α-lytic protease, in the plateau region of the pH–rate profile, the isotope effect for CH_3CO_2Ar vs. CD_3CO_2Ar was determined in both protium and deuterium oxides. First-order rate constants at 25.00 ± 0.05 °C, μ = 0.025, pH 8.07 and equivalent (Tris buffer), enzyme concentration 16 μM, were: 10^6k (s^{-1}) = 336 ± 4 (CH_3, H_2O), 342 ± 2 (CD_3, H_2O); 180 ± 4 (CH_3, D_2O), 183 ± 2 (CD_3, D_2O). The effect is inverse and small: $k_E(CH_3)/k_E(CD_3) \sim 0.98$ with a standard deviation of 1–2%. No difference could be detected between the two isotopic solvents.

Table I. Kinetic Parameters for Solvolysis of *p*-Nitrophenyl Acetate in Three Binary Mixtures of Protium Oxide and Deuterium Oxide (Atom Fraction of Deuterium, *n*) Catalyzed by Bovine α-Chymotrypsin at 25.00 ± 0.05 °C and μ = 0.0056, pH 7.99$_s$ and Equivalent[a]

n^b	10^5k_{ES}, s^{-1}	10^3K_m, M	k_E, M^{-1} s^{-1}
0.000	513, 533	425, 443	1208, 1208
0.473	369, 369	348, 370	1063, 1000
	384	380	1010
0.946	217, 217	329, 307	67, 71

[a] All solutions contained 0.0056 M Tris-HCl and 0.0044 M Tris. [b] Calculated from volumetric composition of mixed buffers. [c] Enzyme concentration 9.6×10^{-6} M; initial substrate concentration 5.14×10^{-5} M.

Abbreviated Proton Inventory: α-Chymotrypsin with *p*-Nitrophenyl Acetate. To examine whether the kind of proton-inventory picture presented for the acylation of α-lytic protease (Figure 2) might also hold for α-chymotrypsin, a determination was made at three points: pure H_2O, nearly pure D_2O, and the approximately equimolar mixture (n = 0.5). Here one relies on Albery's view[24] that the maximum dispersion of $k_n(n)$ from linearity will always occur at $n \approx 0.5$. The experiments were conducted by automated collection of spectrophotometric data over essentially the entire course of the disappearance of *p*-nitrophenyl acetate, beginning at concentrations in excess of the K_m value. These data were then smoothed and used to obtain a least-squares fit to the Michaelis–Menten equation. The resulting values of k_{ES} ($\equiv k_{cat}$), K_m and k_E ($\equiv k_{cat}/K_m$) are shown in Table I.

Both k_{ES} and K_m in H_2O agree very well with the recent values of Shimamoto and Fukutome[25] (4.91 ± 0.08 × 10^{-3} s^{-1} and 4.16 ± 0.24 × 10^{-6} M), but the value of K_m is about 2.7 times larger than that of Kézdy and Bender[26] (1.6 × 10^{-6} M). Our value of k_{cat} is the same as theirs (5.32 × 10^{-3} M^{-1} s^{-1}). Dupaix et al.[27] report a K_m of 3.5 × 10^{-6} M and a k_{cat} of 1.0 × 10^{-2} M^{-1} s^{-1}.

Discussion

pH and pD Dependence for Acylation of α-Lytic Protease by *p*-Nitrophenyl Acetate. There are two features of the k_E(pH), k_E(pD) profiles which are surprising: (1) the absolute values of the apparent pK_a values (which should, if actual pK_a values, be those for ionization of α-lytic protease) are much lower than the kinetic pK_a values for *N*-acetylvaline methyl ester[23] (6.70 in H_2O and 7.35 in D_2O), which agree in H_2O with NMR titrations,[9,10] (2) the magnitude of ΔpK_a = p$K_a(H_2O)$ – p$K_a(D_2O)$ = 0.68 (and the very similar value of 0.65 for *N*-acetylvaline methyl ester) is strikingly larger than $\Delta pK_a \approx 0.5$, which is expected for a simple ionization.[7,12] The interpretations of these points will be discussed in turn.

The most straightforward proposal to explain the "early" pK_a for *p*-nitrophenyl acetate (by about 0.8 pK unit) is that its rate-determining step changes[28] from one for which the transition state contains one less proton than the major form of α-lytic protease in acidic solution (EH) to one which has the same number of protons as EH. At the point of this change, the pH dependence of the rate will shift from one in which the rate increases with pH to one in which the rate is pH independent, generating an apparent titration curve. Now if this were a complete account, with the transition state which has the same number of protons as EH continuing to limit the rate even at high pH, then above the true pK_a of the enzyme, when it has become E, the rate would begin to *decrease* with higher pH. This does not happen, so we must add one further transition state, which has the same number

(22) When the rate constant k_E ($\equiv k_{cat}/K_m$) is measured, the initial reference state is free enzyme and free substrate and the effective transition state is that (or those) of the highest free energy leading through the first irreversible event (here the release of phenol product). This generally familiar point is treated in tedious detail in "Transition States of Biochemical Processes"; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; p 376.

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Table II. Rate Constants and Isotope Effects Estimated for the Model of Equation 3 for Acylation of α-Lytic Protease by *p*-Nitrophenyl Acetate[a]

rate constant	magnitude, M^{-1} s^{-1}	isotope effect, k_{H_2O}/k_{D_2O}
k_a	116 (H_2O)	
	48 (D_2O)	2.4
k_b	9.7×10^7 (H_2O)	
	1.9×10^8 (D_2O)	0.5
k_c	23 (H_2O)	
	11 (D_2O)	2

[a] Assuming that $K_1 = 2.0 \times 10^{-7}$ (H_2O), 4.5×10^{-8} (D_2O).

of protons as E. This species, in parallel with the other two serial transition states, must become the major transition state at around the enzyme pK_a of 6.7.

An algebraic form of this proposal can be developed and compared with the observed data. The steady-state kinetic law for the very generally stated mechanism of eq 3 is given by eq 4, where

$$EH \underset{H^+}{\overset{H^+}{\rightleftharpoons}} E + S \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} M \overset{k_b'[H^+]}{\underset{k_c'}{\longrightarrow}} \text{acetyl enzyme} + p\text{-nitrophenol} \qquad (3)$$

$$K_1$$

$$k_E = (K_1/[K_1 + a_H])(k_a[k_b a_H + k_c]/[k_a + k_b a_H + k_c]) \qquad (4)$$

K_1 is the true ionization constant of the enzyme, $k_b \equiv k_a k_b'/k_{-a}$ and $k_c \equiv k_a k_c'/k_{-a}$, and a_H is the hydrogen-ion activity expressed in molar units. In acidic solutions, such that $a_H \gg K_a$, $k_b a_H \gg k_c$, one has eq 5

$$k_E \approx K_1 k_a k_b/(k_a + k_b a_H) \qquad (5)$$

or eq 6

$$k_E \approx K_1 k_a/(k_a/k_b + a_H) \qquad (6)$$

Comparison of eq 6 and eq 2 then suggests that

$$k_a \approx k_E^{lim} K_a/K_1 \qquad (7)$$

$$k_b \approx k_a/K_a \qquad (8)$$

Similarly, in basic solutions where $a_H \ll K_a$, $k_b a_H \ll k_c$

$$k_E \approx k_a k_c/(k_a + k_c) \approx k_E^{lim} \qquad (9)$$

From the numerical fits to eq 2 (values under Results) and assuming $pK_1 = 6.70$ (H_2O), 7.35 (D_2O), we use eq 7–9 to obtain the estimated rate constants and isotope effects shown in Table II. These data will be employed in another section below to develop a mechanistic model for the process of eq 3.

The second feature of the pL–rate profiles (L = H or D) requiring notice is the unusual magnitude of ΔpK_a, which is 0.68 ± 0.03 for *p*-nitrophenyl acetate and 0.65 for *N*-acetyl-L-valine methyl ester[23] in spite of much different values for the individual pK values. This agreement suggests that the unusual ΔpK is actually a property of the enzymatic ionization (K_1 in eq 3) and not a result of kinetic involvement. If this is so, the isotope effect on K_1 can be used to obtain information about protonic states in E and EH. The isotope effect on K_1 is given[29] in terms of isotopic fractionation factors ϕ in eq 10, where l is the fractionation factor

$$K(H_2O)/K(D_2O) = 4.83 \pm 0.47 = \phi_{EL}/\phi_E l^3 \qquad (10)$$

for the aqueous hydronium ion (with a value[30] of 0.69 ± 0.02) and ϕ_E is an effective fractionation factor for the ionized enzyme and ϕ_{EL} for the protonated enzyme. These factors will be unity if the binding state of the protons in E or EL is the same as in bulk water (the standard for ϕ values), greater than unity if the binding is "stiffer" so that deuterium is preferred, and less than unity if the binding is "looser" so that protium is preferred.

Apparently,

$$\phi_{EL}/\phi_E = (4.83 \pm 0.47)(0.69 \pm 0.02)^3$$

$$\phi_{EL}/\phi_E = 1.59 \pm 0.17$$

Therefore, it must be that (a) there are protonic sites in EL considerably more "stiffly" bound than in water, while those in E are normal ($\phi_{EL} \approx 1.6$, $\phi_E \approx 1$), *or* (b) there are protonic sites in E considerably more "loosely" bound than in water, while those in EL are normal ($\phi_{EL} \approx 1$, $\phi_E \approx 0.6$), *or* (c) some combination of protonic sites in EL and E leads to the ratio of 1.6.

Which of these possibilities is correct is not certain, but there is reason to prefer b. This comes from the proton inventory experiment described in the next section.

Proton Inventory: α-Lytic Protease and *p*-Nitrophenyl Acetate. The proton inventory was carried out at pL 8.07 (H_2O) to 8.62 (D_2O), so that the enzyme is 95–96% ionized and the effective reactant state in all isotopic solvents is thus E. The transition states are strongly dominated (to about the level of 78–79% in both H_2O and D_2O, as can be calculated[31] from the data of Table II) by that for the step labeled k_c' in eq 3, corresponding to rate constant k_c in eq 4. Some contribution is also made by k_a (18–19%) and a nearly negligible contribution by k_b (3%). The solvent isotope effects thus refer roughly to the conversion of E and substrate to T_c, the transition state corresponding to k_c, with a small contribution from conversion to T_a, corresponding to k_a.

Figure 2 shows that the shape of the proton-inventory curve is "bulging up"[12] for which one interpretation[12] is that both reactant and transition states contribute subunit fractionation factors (i.e., have loosely bound protons). The simplest appropriate expression[12] is that of eq 11. The data can be very well fit to

$$k_E^n = k_E^0(1 - n + n\phi^T)/(1 - n + n\phi^R) \qquad (11)$$

this expression by a nonlinear least-squares technique, giving $\phi^T = 0.39 \pm 0.01$, $\phi^R = 0.82 \pm 0.01$.

The reactant-state fractionation factor $\phi_R \approx 0.8$, which emerges from this model for the proton inventory, can be compared with $\phi_R \approx 0.6$, which is suggested by $pK_a(H_2O) - pK_a(D_2O)$ if it is assumed that $\phi_{EH} \approx 1$ (see previous section). Possibly these numbers should be regarded as equal, although the estimated standard deviations indicate otherwise. If they are, then the ionized enzyme E may have a single protonic site with an unusually "loose" potential and $\phi \approx 0.6$–0.8. This protonic site would be involved in acid–base catalysis, with ϕ becoming ~0.4 in the transition state. Alternatively, it may be noted that $(0.8)^2 \approx 0.6$, so that there may be *two* protonic sites (each with $\phi \approx 0.8$) which are generated on conversion of EH to E. Only one of these sites then participates in acid–base catalysis, the other remaining unaltered in the transition state and therefore not appearing in eq 11. Further, more complex models involving more sites in both reactant and transition states can also be devised (the philosophy of comparing models of varying complexity with experimental data has been discussed elsewhere[7,12]). At this point, no choice can be made among these possibilities.

This form of proton inventory plot may also arise if introduction of deuterium to the solvent induces a change in rate-determining step.[12] This is discussed below in the section on "Heavy Atom Reorganization".

Mechanistic Model for Acylation of α-Lytic Protease by *p*-Nitrophenyl Acetate. It perhaps should go without saying that a large number of mechanistic models for such a complex reaction

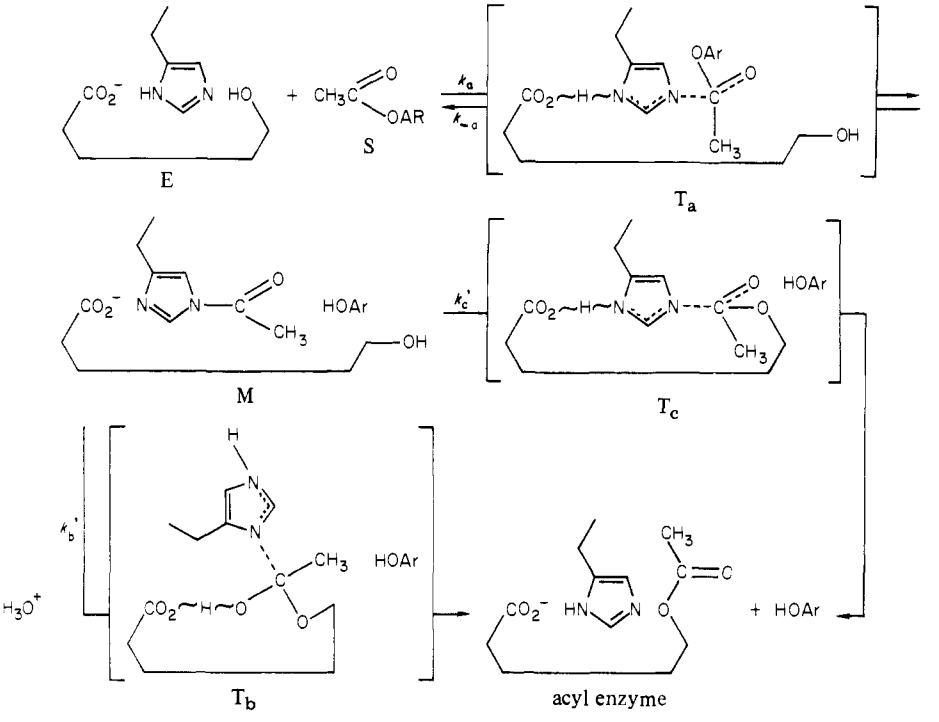
(29) Schowen, R. L. *Prog. Phys. Org. Chem.* **1972**, *9*, 275.
(30) Gold, V. *Adv. Phys. Org. Chem.* **1969**, *7*, 283.

(31) The quantity $k_E(K_1 + a_H)/K_1$, which relates all transition states to E as a common reactant state, can be dissected as follows (cf. eq 4):

$$[k_E(K_1 + a_H)/K_1]^{-1} = (k_a)^{-1} + (k_b a_H + k_c)^{-1}$$

The data of Table II show (at pH 8.07, pD 8.62) that the second term in this expression contributes 82% of the sum in H_2O and 81% in D_2O, and of this the k_b term contributes 3% in both cases, leaving k_c as the dominant rate-determining quantity (79% in H_2O, 78% in D_2O).

Scheme I



as that of α-lytic protease with *p*-nitrophenyl acetate could be matched with the primitive kinetic scheme of eq 3. Scheme I shows one of these models which we consider particularly compliant with the expected chemistry of the system.

Here the initial (k_a) process is taken to be acylation of the imidazole unit of the ABCE, rather than the serine hydroxyl. The proposed transition state T_a is one in which general-base catalysis of nucleophilic attack by imidazole is suggested. This is consistent with the solvent isotope effect of 2.4 (Table II), which indicates looser binding of at least one proton in T_a than in E. Acylation of imidazole in place of serine by nitrophenyl benzoates in attack on α-chymotrypsin was originally observed by Hubbard and Kirsch.[32] The situation there differed from this one in that solvent isotope effects were close to unity, indicating no protolytic catalysis of the sort shown in T_a. One of the generalizations that seems to emerge from such studies as those of Hubbard and Kirsch (uncatalyzed nucleophilic attack of α-chymotrypsin histidine on nitrophenyl benzoates), Dupaix et al.[27] (protolytically assisted attack of α-chymotrypsin *serine* on nitrophenyl *acetates*), and ourselves (protolytically assisted nucleophilic attack of α-lytic protease histidine on *p*-nitrophenyl acetate) is that serine proteases have a highly variable structure about the active site, which is strongly responsive (probably in structural, and surely in mechanistic, terms) to substrate structure and other conditions. Thus, the *exact* choice of enzyme, substrate, and probably reaction conditions, such as temperature, pH, salt concentration, etc., is very important in selecting from a panoply of possible pathways and rate-determining steps. Other work indicating variation of rate-determining step of serine-protease action with conditions has been reviewed.[2] Doubtless as the physiological substrate structure is approached, the strong transition-state complementarity of enzyme and substrate will narrow the accessible pathways to a single one.

The intermediate compound M is shown in Scheme I as the *N*-acyl enzyme, with the product phenol still bound. Protonation of phenoxide and deprotonation of carboxyl in the course of reaching M may have occurred directly, with solvent mediation, or in independent processes. The phenol product must, in fact, be considered still bound to the enzyme at this stage and through the next rearrangement stages because once it is released an effectively irreversible point will have been passed. The measured rate constant k_E cannot be determined in any part by events subsequent to phenol release.[22] One possible reason for delayed release of the phenol might be that it lies in the side-chain binding region of the active site so that its departure is blocked by the acylimidazole unit.

Two parallel pathways are portrayed for the N → O rearrangement which carries M into *O*-acyl enzyme. One, through transition state T_b, is acid catalyzed. If the imidazole leaving group had been completely protonated prior to T_b, and no protolytic assistance were being rendered, the expected solvent isotope effect[29] would just be the inverse of that for acid ionization, or $k_D/k_H \approx 3$. The observed effect for k_b (Table II) is indeed inverse but of smaller magnitude, $k_b(D)/k_b(H) \approx 2$. This leads to the proposal of protolytic assistance, with an isotope-effect contribution of $k_H/k_D \approx 1.5$.

The "neutral" pathway for the N → O acyl shift, through T_c, is suggested to have a transition state strongly reminiscent of T_a. Because $k_c(H)/k_c(D) \approx 2$ (Table II), protolytic assistance of imidazole departure also seems indicated. In both the acid-catalyzed and neutral pathways, various protonation–deprotonation reactions are involved and, here again, these may occur directly between the groups or with solvent mediation or by independent reaction pathways.

The model of Scheme I, consisting of an initial, protolytically assisted formation of *N*-acyl enzyme followed by parallel "neutral" and specific-acid-catalyzed routes of N → O acyl shift (each route subject to protolytic general-base catalysis), is supported by studies of the chemistry of related, nonenzymatic systems, as summarized in the following points.

(1) The reactions of ambident, alcohol–imidazole nucleophilic entities with esters under a variety of conditions, including cases in which the nucleophilic entity is included in polymer and micelle structures, show a variable regiospecificity with alcohol acylated under some conditions, imidazole under some conditions, and with intramolecular acyl migration often following the initial reaction. Some recent work has been reviewed by Kirby[33a] and Fife[33b] and their references provide an entry to the literature. This agrees

(32) Hubbard, C.; Kirsch, J. F. *Biochemistry* **1972**, *11*, 2483.

(33) (a) Kirby, A. J. In "Organic Reaction Mechanisms, 1977"; Knipe, A. C., Watts, W. E., Eds.; Wiley: New York, 1978; Chapter 2. (b) Fife, T. H. *Adv. Phys. Org. Chem.* **1975**, *11*, 1-122.

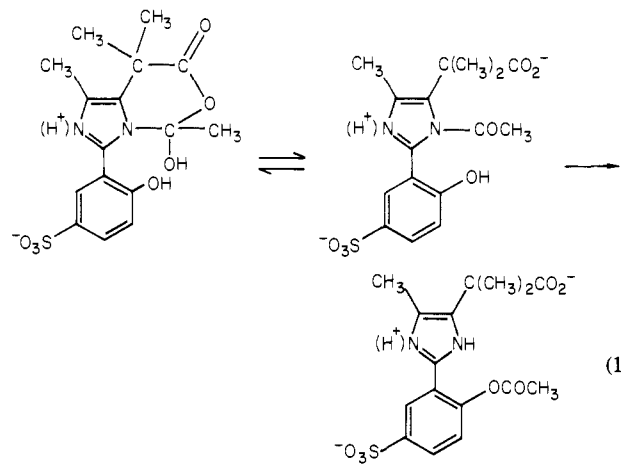
with the observations of N-acylation here and by Hubbard and Kirsch with α-chymotrypsin[32] and of O-acylation with α-chymotrypsin by Dupaix et al.[27]

(2) The attack of imidazole on less activated aryl acetates, such as *p*-tolyl acetate, is accelerated by general bases, including imidazole itself,[34] as shown in T_a. The termolecular reaction of two imidazoles with *p*-tolyl acetate exhibits a solvent isotope effect of 2.2 at 34 °C, strongly reminiscent of the 2.4 seen for T_a at 25 °C. No termolecular term with imidazole was measured for *p*-nitrophenyl acetate itself, but with α-lytic protease, general-base catalysis will be much favored by prior assembly and orientation of the functional groups in the enzyme active site. Extrapolation using the activation parameters and Hammett ρ values of Bruice and Benkovic[34] generates an expected value of the rate constant for imidazole catalysis of imidazole attack on *p*-nitrophenyl acetate at 25 °C of 3.9×10^{-3} M^{-2} s^{-1}. Since the value of k_a is 116 M^{-1} s^{-1}, an effective molarity of 3×10^4 M is obtained for catalysis in the acylation of α-lytic protease. This is compounded of the proximity-effect contribution and any difference in basicity between the imidazole dimer in free solution and the enzymatic ABCE.

(3) Nucleophilic attack on acylimidazoles is accelerated by general catalysis involving the partial protonation of the leaving group (as in T_c) is some cases[35] and by general-base catalysis with prior protonation of the leaving group in other cases,[35,36] as portrayed in T_b. Prior protonation of imidazole is revealed by the fact that rate constants calculated on this assumption agree with those for reaction of nucleophiles with the similar *N*-methylimidazolium substrate.[36] Reactions in this category include nucleophilic attack by weakly basis amines (e.g., $CF_3CH_2NH_2$ and CH_3ONH_2) and water.[35] Hogg, Phillips, and Jergens[37] have conducted a proton inventory of the hydrolysis of acetylimidazolium ion, showing the solvent isotope effect to be quantitatively consistent with general-base catalysis by a second water (the transition-state fractionation factors for the catalytic water being correctly predicted by the previously determined[36,38] Brønsted β of 0.34). They found the bridging proton to generate $k_H/k_D \approx 1.9$, which is not too far from the value of 1.5 estimated for T_b above. When the attacking nucleophile is more reactive (NH_3 or oxyanions, for example), the reactions of *N*-methylimidazolium substrates no longer simulate those of the acylimidazole.[38] Oakenfull, Salvesen, and Jencks[38] suggest that protonation of the leaving group is now incomplete, with a general acid functioning as in T_c. Patterson, Huskey, Venkatasubban, and Hogg[39] have shown that in a typical case (imidazole-catalyzed hydrolysis of acetylimidazole), a single proton generates the solvent isotope effect of 3.3. This differs substantially from the value of 2 estimated above (Table II) for T_c, but the fractionation factors may be fairly strongly dependent on environmental conditions.[40]

(4) Essentially all of this catalytic chemistry, which is based on intermolecular reactions, carries over into the rearrangement of acetyl groups from imidazole to hydroxyl in the intricately designed, highly elegant intramolecular system studied by Rogers and Bruice[41] (eq 12).

(5) The hydrolysis of acylimidazoles such as M is accelerated by hydroxide ion.[35,38] This may be the explanation of the rise in rate above pD 9 which can be seen in Figure 1, on the curve for deuterated solvent. This rise may begin at lower pD (in D_2O) than it does in H_2O, which is consistent with the expectation that



(12)

a base-catalyzed reaction would proceed faster in D_2O.

Heavy-Atom Reorganization in the Acylation of α-Lytic Protease by *p*-Nitrophenyl Acetate. To ascertain whether the internal force field of the substrate had been changed in formation of the acylation transition state, the β-deuterium isotope effects were measured at pH 7.97. Near pH 8, the acylation rate constant is predominantly governed[31] by the k_c process of eq 4 (transition state T_c of Scheme I). The magnitude of k_{3H}/k_{3D} (about 0.98) is inverse, consistent with a dominance of nucleophilic interaction at carbonyl in T_c, leading to decreased hyperconjugation from the β-CH bonds and thus an inverse isotope effect.[42,43] It is, however, small in comparison to the effect K_{3H}/K_{3D} of 0.87 estimated for complete addition to carbonyl. It is similar in magnitude to effects observed[43] for basis hydrolysis and methanolysis of phenyl acetate (0.98 in both cases) and basic methanolysis of *p*-methoxyphenyl acetate (0.96).

The finding is consistent with the structure suggested for T_c if (a) the N–C bond is very weak so that the carbonyl structure is closer to trigonal than tetrahedral or (b) the N–C bond is strong but some other transition state with trigonal or near-trigonal carbonyl (e.g., a reactant-like transition state for nucleophilic attack by serine hydroxyl) also participates in determining the rate along with T_c. If the latter is the case, then the balance between the two transition states must not be shifted by deuteration of the solvent, for k_{3H}/k_{3D} is not different in H_2O and D_2O. This would necessitate similar solvent isotope effects for the two transition states. If the curvature of the proton inventory plot of Figure 2 arose from a shift among rate-determining transition states (an in principle possibility, as noted above), it would also be surprising to find equal β-deuterium isotope effects in light and heavy water.

Acylation of α-Chymotrypsin by *p*-Nitrophenyl Acetate. The data given in Table I tend both (a) to confirm our previous report[17] that the proton inventory for deacetylation of acetyl-α-chymotrypsin is linear ("one-proton catalysis") and (b) to indicate that acetylation of α-chymotrypsin by *p*-nitrophenyl acetate gives the same kind of "bulging up" proton-inventory plot as does acetylation of α-lytic protease.

If a linear proton-inventory relationship were obeyed, then at any value of *n* the atom fraction of deuterium, eq 13, would be

$$k_n = k_0(1 - n + n\phi) \tag{13}$$

obeyed. For two values of *n*, say n_1 and n_2, eq 14 should hold.

$$(k_{n_1}/k_0) = (n_1/n_2)[(k_{n_2}/k_0) - (1 - n_2)] + (1 - n_1) \tag{14}$$

If one predicts a value of k_{n_1}/k_0 from the right-hand side of eq 14, then agreement with experiment indicates a linear proton inventory, while an experimental value exceeding the prediction indicates a "bulging up" plot and an experimental value below

(34) Bruice, T. C.; Benkovic, S. J. *J. Am. Chem. Soc.* **1964**, *86*, 418–426.
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(37) Hogg, J. L.; Phillips, M. K.; Jergens, D. E. *J. Org. Chem.* **1977**, *42*, 2459–2461.
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(42) Hogg, J. L.; Rodgers, J.; Kovach, I. M.; Schowen, R. L., *J. Am. Chem. Soc.* **1980**, *102*, 79.
(43) Kovach, I. M.; Hogg, J. L.; Raben, T.; Halbert, K.; Rodgers, J.; Schowen, R. L., *J. Am. Chem. Soc.* **1980**, *102*, 1991.

the prediction means a "bulging down" plot. From Table I, one has 10^5k_{ES} = 523 ± 10 (n = 0.000), 374 ± 9 (n = 0.473), 217 ± 5 (n = 0.946) s^{-1}. This leads to a predicted value of $10^5k_{0.473}$ of 370 ± 8 s^{-1} on the linear model, which agrees well with 374 ± 9. This is consistent with the previous report[17] of a linear proton inventory for deacetylation, which is rate limiting for k_{ES}. Also in Table I are given values of k_E = 1208 ± 36 (n = 0.000), 1024 ± 26 (n = 0.473), 685 ± 23 (n = 0.946) M^{-1} s^{-1}. If $k_E(n)$ were linear, the value of $k_{0.473}$ should be 946 ± 16 M^{-1} s^{-1}. The observed mean value of $k_{0.473}$ exceeds this by 4.9 standard deviations; alternatively stated, the predicted value on the linear model is lower than the observed value by 3 standard deviations. The proton inventory for acylation of α-chymotrypsin by p-nitrophenyl acetate can therefore be taken as "bulging up" like that for α-lytic protease in Figure 2, at something like the 99% confidence level.

The simplest explanation of this would be that there is a reactant-state protonic site in α-chymotrypsin, like that for α-lytic protease, which has a "loosely bound" proton with a subunit fractionation factor. If this proton becomes still more loosely bound, as in a catalytic bridge, in the acylation transition state, then—just as with α-lytic protease—the shape of the curve is accounted for. If the analogy of α-chymotrypsin and α-lytic protease were complete, an abnormally large value of $pK_a(D_2O) - pK_a(H_2O)$ would be expected for ionization of the ABCE of α-chymotrypsin (like the value of 0.65 for α-lytic protease). The measurement of this quantity for α-chymotrypsin is not so easy as for α-lytic protease because a second acid–base process produces a bell-shaped pH–rate profile.[3] Thus, the ΔpK values tabulated by Bender, Clement, Kẽzdy, and Heck[44] fall in the range 0.5 ± 0.2, but this includes possible abnormal values. The most relevant case is that of k_{cat}/K_m for N-acetyl-L-tryptophan ethyl ester, for which Bender et al. calculated a pK_1 of 6.77 (H_2O) and 7.49 (D_2O), generating ΔpK = 0.72 in agreement with our hypothesis. To exemplify the difficulty, however, an independent refit[2] of the same data gave 6.8 and 7.1, $\Delta pK \approx 0.3$, an abnormally *small* value. For acylation of α-chymotrypsin by 2,4-dinitrophenyl benzoate, Hubbard and Kirsch measured pK_1 values of 6.76 (H_2O) and 7.23 (D_2O); thus, ΔpK = 0.47, a normal value not indicating abnormal sites in the unprotonated enzyme. It is, however, entirely possible that abnormal protonic sites exist in *both* protonated and unprotonated enzyme. Then the magnitude of the ΔpK cannot detect them. If one of these sites is involved in catalysis of acylation, the proton-inventory behavior observed ("bulging up" curve) is expected.

If it is provisionally assumed, as with α-lytic protease, that the solvent isotope effect for acylation of α-chymotrypsin arises from a combination of a loosely bound reactant proton and a still more loosely bound proton in the transition state (presumably a catalytic bridge), the appropriate fractionation factors can be estimated from the data of Table I. The resulting values are for the reactant-state site ϕ_R = 0.68 ± 0.02 [cf. $\phi_E \approx 0.63$ estimated from ΔpK for α-lytic protease and $\phi_R \approx 0.82$ (possibly $[0.63]^2$) estimated from the proton inventory for α-lytic protease] and for the transition-state site ϕ_T = 0.36 ± 0.02. The latter could represent a transition-state catalytic bridge with an isotope effect corresponding to $k_H/k_D \approx 2.8$. But because the reactant-state site is already loosely bound, the observed value of $k_E(H_2O)/k_E(D_2O)$ is only 1.76.

Acylation of Elastase by N^α-(Carbobenzyloxy)-L-alanine p-Nitrophenyl Ester. This substrate is one which begins the progression from the "unnatural" structure of p-nitrophenyl acetate toward the more nearly "natural" structure of the oligopeptide analogue Ac-L-Ala-L-Pro-L-Ala p-nitroanilide studied by Hunkapiller, Forgac, and Richards.[16] As Figure 3 shows, the proton inventory is linear, consistent with a single transition-state protonic bridge generating the solvent isotope effect $k_E(H_2O)/k_E(D_2O)$ = 1.75. The data are linear at the 99.9% confidence level with an added quadratic term significant only below the 80% confidence level.[12]

Elastase[45] has been reported to exhibit a normal ΔpK in H_2O and D_2O, but the data refer to a k_{cat} measurement and are not strictly relevant, since the free enzyme might behave differently from a complex. If it should emerge that free elastase has a normal pK, this would indicate, when combined with the linear proton inventory, either (a) that no abnormal protonic site is present in unprotonated elastase (thus, reactant fractionation factors are around unity and one changes to 1.75^{-1} upon formation of the acylation transition state) or (b) an abnormal site is present in both protonated and unprotonated elastase (as may be true for α-chymotrypsin, as noted above) and a *different*, reactant-state proton with unit fractionation factor is activated to a catalytic bridge in the acylation transition state. In the second case, the abnormal protonic site remains "silent" in both reactant-state and transition-state behavior.

Conclusions

These findings suggest a notable variability in the response of the active site of these serine proteases, both in site of nucleophilic attack and in the involvement of protonic catalysis, when the substrate structures for the acylation process do not approach the natural polypeptide character for which the catalytic machinery of the enzymes has been developed through the course of biological evolution. This is consistent with the view that the full catalytic capability of the enzymes can only be called into action through strong mutual interaction at many points between natural substrate and enzyme functions in the transition state for acylation, a point eloquently made by Hunkapiller, Forgac, and Richards.[16] The enzymes can make use of a part of their capacities for catalysis, as exemplified by a fairly large effective molarity of about 10^4 M for the apparent protolytically assisted attack of the imidazole of α-lytic protease upon p-nitrophenyl acetate, but the inherent potential for two-proton catalysis[16,21] is not aroused by such "incomplete" substrates as acetyl or N-acylalanyl esters.

Experimental Section

Materials and Solutions. p-Nitrophenyl acetate was prepared from p-nitrophenol and acetyl chloride and was purified by vacuum sublimation (mp 77.5–77.8 °C; lit.[46] mp 81–82 °C). The triply deuterated material was obtained from 99.5% CD_3CO_2D (Diaprep) as previously described[43] and contained >98% D (NMR). N^α-(Carbobenzyloxy)-L-alanine p-nitrophenyl ester, obtained from Sigma Chemical Co., had melting point 78–80 °C and the expected elemental analysis. Anal. Calcd: C, 59.30; H, 4.68; N, 8.13. Found: C, 59.27; H, 4.61; N, 8.05. It was used without further purification. Protium oxide used for buffer preparation was once distilled and deionized by passing through a mixed bed ion-exchange column (Barnstead, Sybron Corp.). Deuterium oxide, obtained from Stohler Isotope Chemicals, was distilled and stored under nitrogen. The isotopic purity of each bottle of deuterium oxide was measured by proton NMR, using CH_3CN as an internal standard. Tris-HCl (Sigma Chemical Co.) and sodium acetate (Mallinckrodt Chemical Works) were desiccated before use. Concentrated HCl and NaOH pellets were obtained from Fisher Scientific Co. and Mallinckrodt Chemical Works, respectively. Various buffered solutions of protium and deuterium oxides were prepared by mixing the appropriated amounts of stock solutions of sodium acetate, Tris-LCl, NaOL, and LCl (L = H or D).

Enzymes. Bovine pancreatic α-chymotrypsin was obtained from Worthington Biochemical Corp. as the once-crystallized dialyzed salt-free powder and had an activity of 50.5 units/mg, where a unit is the quantity of enzyme that will hydrolyze 1.0 mol of N^α-benzoyl-L-tyrosine ethyl ester per minute at pH 7.8, 25 °C. Porcine pancreatic elastase was obtained from Sigma Chemical Co. as a chromatographically purified, lyophilized powder with an activity of 50 units/mg, where a unit is the quantity of enzyme that will solubilize 1 mg of elastin in 20 min at pH 8.8, 37 °C. α-Lytic protease from Myxobacter 495 was the kind gift of Drs. J. H. Richards and M. W. Hunkapiller.

Kinetics. Enzyme-catalyzed hydrolyses of p-nitrophenyl acetate and of N^α-(carbobenzyloxy)-L-alanine p-nitrophenyl ester were monitored by following the increase in optical density at 400 nm, resulting from production of p-nitrophenoxide, on a Cary 16 UV–visible spectrophotometer.

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(45) Marshall, T. H., unpublished data; cited in Stoops, J. K., Ph.D. Dissertation, Northwestern University, 1966, p 300.

(46) "Handbook of Chemistry and Physics", 53rd ed; Chemical Rubber Co.: Cleveland, Ohio, 1972–1973; p C-81.

Hydrolysis of *p*-nitrophenyl acetate at lower values of pH and pD was followed by monitoring the change in optical density at 270 nm. Data acquisition and analysis were computer controlled; the details of the acquisition system have been previously described.[47] Typical kinetic time courses consisted of 1000 observations of optical density at equal time intervals. First-order enzymatic rate constants V/K were calculated by weighted, nonlinear least squares; reactions were followed for more than three half-lives. In instances where buffer-catalyzed hydrolysis contributed significantly to the overall rate, i.e.,

$$k_{obsd,total} = V/K + k_{background}$$

the observed rate constant was corrected for the background contribution to give the "enzyme-only" rate constant by determining $k_{background}$ in the absence of enzyme. This was done by measuring the initial velocity of the background reaction and calculating the rate constant from

$$k_{background} = \frac{v_{initial}}{\Delta\epsilon \cdot S_0}$$

where $\Delta\epsilon$ is the absorptivity constant change for the reaction and S_0 is the initial substrate concentration.

Values for V and V/K of α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate were determined by using initial substrate concentrations of ~5–10 K_m and following the reactions to near completion by monitoring optical density changes at 400 nm. The first derivatives of the mixed-order time courses were calculated according to the methods of Savitzky and Galay.[48] The resulting table of instantaneous velocities was used to calculate V_m and K_m by hyperbolic least squares, as described by Cleland.[49]

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Protonic Reorganization and Substrate Structure in Catalysis by Amidohydrolases[1]

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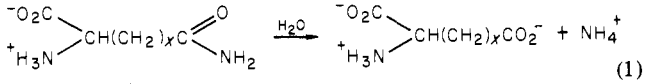
Abstract: Asparaginases, catalyzing the hydrolysis of asparagine, and glutaminase, catalyzing the hydrolysis of glutamine, exhibit modest solvent isotope effects which seem to arise from transition-state reorganization at two (or perhaps more) protonic sites. Thus, asparaginases from *Escherichia coli*, *Erwinia carotovora*, and *Proteus vulgaris* show V_{H_2O}/V_{D_2O} (37 °C, pH 7.1–7.2) of 2.93, 2.62, and 3.31, respectively, when catalyzing asparagine hydrolysis. Proton inventory experiments are consistent with these effects as arising from two sites, each contributing 1.75, 1.62, and 1.82, respectively. Similarly, a glutaminase of *Escherichia coli*, catalyzing glutamine hydrolysis, has $V_{H_2O}/V_{D_2O} = 1.80$, apparently from two contributions of 1.32. When the substrate structure is truncated so that a good transition-state fit of enzyme and substrate remains possible, little change occurs [thus, succinamate ion with *Erwinia* asparaginase produces $V_{H_2O}/V_{D_2O} = 2.27 \sim (1.51)^2$]. When the capacity for such a fit is removed, the mechanism becomes variable and degrades to one-proton catalysis. Thus *Erwinia* asparaginase with glutamine substrate has $V_{H_2O}/V_{D_2O} = 1.69$ from a single site. *Escherichia* asparaginase with glutamine substrate has a single transition-state site generating an effect of 2.4, with an inverse contribution of about 1.25, either from a transition-state site (perhaps the HO group of a tetrahedral intermediate), a loose reactant-state site, or perhaps from changes in solvent and protein-structural sites.

The amidohydrolases are enzymes which catalyze the appealingly simple reaction of eq 1. Asparaginases[2-4] catalyze the



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hydrolysis of asparagine (**1**, $x = 1$) and glutaminases[5] the hydrolysis of glutamine (**1**, $x = 2$). These enzymes have not been studied so thoroughly as have the serine proteases but, on the basis of currently available information, there seems to be a good deal of mechanistic similarity. It appears that the amidohydrolases react by way of an acyl-enzyme intermediate (eq 2), with the

$$ACH_2CONH_2 + EOH \rightarrow ACH_2COOE + NH_4^+ \quad (2a)$$

$$ACH_2COOE + H_2O \rightarrow ACH_2CO_2^- + EOH \quad (2b)$$

enzymatic nucleophile a serine. Like the serine proteases, the amidohydrolases exhibit a rate-depression in deuterium oxide solution, suggesting a role for acid–base catalysis by enzyme functional groups. There is no information at the present time about the structure or composition of the active-site catalytic entity of the amidohydrolases.

In contrast, it is known from crystallographic studies that the serine protease active sites contain an acid–base catalytic entity consisting of a chain of hydrogen bonds (the "charge-relay system"). Their catalytic transition states might therefore involve multiple-proton catalytic bridging.[6] Proton-inventory experiments[7] (in which the rate effect of gradual introduction of deuterium to the solvent is examined) show that oligopeptide analogues (approximating the structure of the natural substrate) indeed show a roughly quadratic dependence on the atom fraction of deuterium in the solvent.[8,9] This suggests full function of the charge-relay

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