anti isomerization in 2′-deoxyadenosine decreases on transferral of the nucleoside from a self-stacked environment to one involving heterostacking to either a tryptophan derivative (indole-3-acetic acid) or ethidium bromide. Obviously, one is tempted to speculate on the significance of such results. In the binding of intercalators to DNA the flexibility of the glycosyl dihedral angle must be severely restricted in any case. It is well established that intercalculation causes unwinding of DNA.[24] If the intercalator were to be located between the penultimate and terminal residues, ease of glycosyl bond rotation for the terminal residue is suggested. Equally interesting is the suggestion from our results that, when the purine nucleoside is bound to some receptor (typically a protein), the barrier to glycosyl conformational rotation is smaller than for the free (mostly self-stacked) nucleoside. Conformational changes around glycosyl C–N bonds upon binding to enzymes have already been suggested.[25]

References and Notes

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Protonic Reorganization and Substrate Structure in Catalysis by Serine Proteases[1]

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Abstract: Proton inventories (rate measurements in binary mixtures of protium and deuterium oxides) have been used to estimate the number of protons involved in hydrolytic catalysis by serine proteases with various substrates. Trypsin with the oligopeptide analogue BzPhe-Val-Arg *p*-nitroanilide as substrate produces an overall solvent isotope effect V_{H_2O}/V_{D_2O} of 4.30, and a proton inventory consistent with two-proton catalysis, with each proton generating an isotope effect of about 2.1. α-Chymotrypsin with the truncated substrates $AcTrpNH_2$ (acylation rate limiting) and 4-nitrophenyl 3-phenylpropanoate (deacylation rate determining) also produces apparent two-proton catalysis but with smaller overall isotope effects and skewed contributions from the two sites ($V_{H_2O}/V_{D_2O} = 1.90 \sim 1.69 \times 1.14$ for $AcTrpNH_2$ and $V_{H_2O}/V_{D_2O} = 2.85 \sim 1.85 \times 1.54$ for 4-nitrophenyl 3-phenylpropanoate). On the other hand, trypsin with the similar substrate BzArgOEt ($V_{H_2O}/V_{D_2O} = 3.03$) and thrombin with BzArgOEt ($V_{H_2O}/V_{D_2O} = 2.92$) give one-proton results, with deacylation presumably rate limiting in both cases. The minimal substrate *p*-nitrophenyl acetate with α-chymotrypsin ($V_{H_2O}/V_{D_2O} = 2.40$) and elastase ($V_{H_2O}/V_{D_2O} = 2.92$) shows one-proton catalysis for rate-determining deacetylation, while with trypsin the overall effect ($V_{H_2O}/V_{D_2O} = 1.38$) is too small to resolve the question of the number of active protons. Apparently, oligopeptide structure, leading to enzyme–substrate interactions at remote subsites as well as at the catalytic site in the catalytic transition state, is required to bring into action the full evolutionarily developed acid–base machinery of the serine proteases. If the structure is reduced to the point where only catalytic site interactions occur, the reliability of the acid–base machinery is much impaired, while, with minimal substrates, the enzyme acts either as a simple general catalyst or perhaps even as a nucleophilic catalyst. Compression of the distance across the catalytic hydrogen-bond chain of the active site as a consequence of remote-site interactions in the transition state, with relaxation of the enzyme structure occurring in their absence, is a reasonable mechanism for the coupling and decoupling of the protonic interactions by substrate structure.

The serine proteases[2-4] are enzymes, found at all levels of biological development, which have arrived at a common chemical solution to the catalytic problem of accelerating the hydrolysis of polypeptides to amino acids. They employ a double-displacement mechanism (eq 1), in which the eponymous serine is first acylated with expulsion of the amine leaving group (eq 1a) to form the acyl-enzyme intermediate; the acyl-enzyme then hydrolyzes in the second step (eq 1b) to give the carboxylate product.

$$ECH_2OH + R_1CONHR_2 \rightarrow ECH_2OCOR_1 + R_2NH_3^+ \quad (1a)$$

$$ECH_2OCOR_1 + H_2O \rightarrow ECH_2OH + R_1CO_2^- \quad (1b)$$

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Beyond this general mechanistic similarity, the serine proteases also share a common acid–base catalytic entity (ABCE) at the active site.[5] In this entity (the "charge-relay system"), the nucleophilic serine hydroxyl is hydrogen bonded to a histidine imidazole, which is similarly linked to a carboxylate, which can be further hydrogen bonded. Such an array of hydrogen bonds might conceivably be of extraordinary catalytic potential. Up to the present time, studies of the protolytic behavior of stable enzyme species (as opposed to the transition states which must of course govern the dynamics of catalysis) are conflicting,[6,7] and model-reaction investigations have given negative results.[8]

Although structural investigations clearly show the evolutionary conservation of this entity,[9] and the solvent isotope effects for serine proteases indicate some kind of protonic reorganization in catalysis,[10] the question remains: under what circumstances is the potential of this machinery actually realized in catalysis? To illuminate this point, we have conducted *proton inventories* for several serine proteases with various substrates. Proton inventories[10,11] are rate measurements in mixtures of light and heavy isotopic solvents (here H_2O and D_2O, $n \equiv$ atom fraction of deuterium). The velocity at n, V_n, is given by:

$$V_n = V_0 \frac{\prod_i^{\nu_T} (1 - n + n\phi_{Ti})}{\prod_j^{\nu_R} (1 - n + n\phi_{Rj})} \quad (2)$$

where the ϕ_{Ti} are transition-state deuterium fractionation factors and the ϕ_{Rj} are reactant-state deuterium fractionation factors, relative to bulk water (thus $\phi_i \equiv (X_{Di}/X_{Hi})/(n/[1 - n])$ where the Xs are mole fractions). If all reactant-state sites had $\phi_{Rj} = 1$ (see below for other possibilities), the simplest forms that could be observed for eq 2 would be those of eq 3–5.

$$V_n = V_0(1 - n + n\phi_T) \quad (3)$$

$$V_n = V_0(1 - n + n\phi_{T1})(1 - n + n\phi_{T2}) \quad (4a)$$

$$V_n = V_0(1 - n + n\phi_T)^2 \quad (4b)$$

$$V_n = V_0(1 - n + n\phi_T)^{\nu_T} \quad (5a)$$

$$V_n = V_0(1 + n[\phi_T - 1])^{\nu_T} \quad (5b)$$

$$V_n \simeq V_0(e^{\nu_T[\phi_T - 1]})^n = V_0 e^{zn} \quad (5c)$$

Equation 3 would be observed if the entire solvent isotope effect were generated at a single hydrogenic site in the transition state ("one-proton catalysis"); it predicts $V_n(n)$ to be linear. Equations 4a and 4b would be expected if two transition-state sites contributed ("two-proton catalysis"), with the two generating different isotope effects (eq 4a) or the same isotope effect (eq 4b). In these cases, $V_n(n)$ is a quadratic function, with $\sqrt{V_n(n)}$ becoming linear in n for the special case of equal isotope effects. Corresponding cubic equations for "three-proton catalysis", quartic equations for "four-proton catalysis", etc., can be written, but these are exceedingly difficult to distinguish from each other unless the overall isotope effect is very large or the data are of unusual precision. The limiting case[12] is that of eq 5a–c, which envision a large number ν_T of contributing sites, each giving a very small isotope effect so that ϕ_T is nearly unity and $\phi_T - 1$ is a small (positive or negative) number. Then, since $1 + x \sim e^x$ for small x, eq 5b can be rewritten as eq 5c, suggesting an exponential dependence on n. This is the result expected, for example, if widespread small changes in the status of solvating waters or protein structural hydrogens account for the isotope effect.[13] No change in the algebraic form of eq 5c arises if numerous reactant-state sites are also included; instead of $z = \nu_T[\phi_T - 1]$, we then have $z = \{\nu_T[\phi_T - 1] - \nu_R[\phi_R - 1]\}$. Thus, reactant-state sites in this class are included in the treatment. Reactant-state sites with larger fractionation factors not cancelled in the transition state can produce a systematic "bowing upward" of the plot of $V_n(n)$ and can be detected by such an observation.[11]

The simplest expectations for proton inventories of the serine proteases are thus: $V_n(n)$ linear, one-proton catalysis as in the familiar general acid–base catalysis of model systems; $V_n(n)$ polynomial, multiproton catalysis as expected for involvement of the ABCE hydrogen-bond array; $V_n(n)$ exponential, isotope effect arising merely from solvation changes on formation of the transition state.

Results

Table I shows the ratio V_n/V_1 (velocity in mixture of H_2O and D_2O with atom fraction of deuterium n divided by the velocity in pure D_2O: a "partial solvent isotope effect") for eight examples of serine protease action. The values of V_1 were obtained by extrapolation to pure D_2O and are given in the leftmost column of the table, so that the original velocities can be calculated if desired. The leftmost column also gives enzyme, substrate, and reaction conditions. The data were quite reproducible, with the best case being the elastase reaction (no. 7) with an average *range* of ±2.5‰ at any value of n, and the worst case being a trypsin reaction (no. 3) with an average *range* of ±20‰. For the entire set of eight reactions, the average range of the data at a single value of n was ±8‰, slightly less than 1%. Standard deviations and other customary measures of dispersion will of course be smaller than this.

A useful method for surveying the results of proton-inventory experiments is related to the γ method of Albery.[12] Albery shows that for simple cases, the maximum deviation between theoretical proton-inventory curves $V_n(n)$ for different mechanistic models occurs at the midpoint of the binary isotopic solvent mixture, $n = 0.5$. This suggests comparing the observed *midpoint partial solvent isotope effect*, V_m/V_1, with predictions made on various models. Since measurements may not have been made at exactly $n = 0.500$, we use the more general equations 6–8, which are rearranged from eq 3, 4b, and 5c, respectively. Table II shows a comparison of observed and predicted values of V_m/V_1 for the eight reactions studied. The following comments may be made.

one-proton catalysis:

$$V_m/V_1 = (1 - n_m)(V_0/V_1) + n_m \quad (6)$$

two-proton catalysis (equal isotope effects):

$$(V_m/V_1) = [(1 - n_m)[V_0/V_1]^{1/2} + n_m]^2 \quad (7)$$

generalized solvation changes:

$$(V_m/V_1) = [V_0/V_1]^{(1-n_m)} \quad (8)$$

(1) The overall isotope effect for trypsin with PNPA (reaction no. 1) is so small (1.38) that no effective choice among models is possible, although the result probably excludes generalized solvation changes. Clearly, however, whether one, two, or more protons are involved, the isotope effect for each is quite small.

(2) For trypsin with BAEE (reaction no. 2), the isotope effect is much larger (3.03) and substantially more consistent with one-proton catalysis than with any other model. The same is true for α-chymotrypsin with PNPA (reaction no. 4), elastase with PNPA (reaction no. 7), and thrombin with BAEE (reaction no. 8).

(3) Trypsin with the oligopeptide analog BzPhe-Val-Arg *p*-nitroanilide (reaction no. 3) exhibits a quite large overall effect of 4.19, giving fairly good agreement with a model for two-proton catalysis with equal isotope effects at the two sites. There is perhaps even room for involvement of further catalytic

Table I. Proton Inventories for the Action of Serine Proteases on Various Substrates

reaction and condition[a]	atom fraction of deuterium [V_n/V_1]
1. trypsin, 2 mg mL^{-1}, pH 7.50 and equiv, 25.00 ± 0.03 °C; *p*-nitrophenyl acetate, 2.42 × 10^{-3} M, 3.1% acetonitrile; V_1 = 1.190 × 10^{-7} M s^{-1}	0.016 [1.382, 1.374]; 0.113 [1.353, 1.345]; 0.220 [1.304, 1.325]; 0.308 [1.258, 1.248]; 0.401 [1.247, 1.224]; 0.498 [1.187, 1.183]; 0.599 [1.154, 1.163]; 0.692 [1.120, 1.115]; 0.790 [1.081, 1.098]; 0.887 [1.059, 1.068]; 0.998 [0.982, 0.972]
2. trypsin, 5 mg mL^{-1}, pH 8.00 and equiv, 25.00 ± 0.03 °C; *N*-benzoyl-L-arginine ethyl ester, 4.76 × 10^{-4} M; V_1 = 4.521 × 10^{-7} M s^{-1}	0.008 [3.022, 3.046]; 0.105 [2.839, 2.880]; 0.204 [2.700, 2.663]; 0.304 [2.417, 2.425]; 0.401 [2.241, 2.204]; 0.500 [2.062, 2.028]; 0.598 [1.923, 1.836]; 0.695 [1.626, 1.664]; 0.795 [1.513, 1.437]; 0.894 [1.238, 1.195]; 0.989 [1.066, 1.017]
3. trypsin, 0.3 mg mL^{-1}, pH 7.44 and equiv, 25.00 ± 0.02 °C; *N*-benzoyl-L-phenylalanyl-*L*-valyl-L-arginine *p*-nitroanilide, 2.54 × 10^{-4} M; V_1 = 1.570 × 10^{-4} M s^{-1}	0.017 [4.25, 4.08, 4.24]; 0.108 [3.81, 4.04]; 0.205 [3.33, 3.16]; 0.301 [3.14, 2.90]; 0.399 [2.71, 2.69]; 0.496 [2.31, 2.20]; 0.592 [1.87, 1.92]; 0.738 [1.50, 1.53]; 0.883 [1.19, 1.24]; 0.980 [1.07, 1.08]
4. α-chymotrypsin, 0.2 mg mL^{-1}, pH 7.43 and equiv, 25.00 ± 0.03 °C; *p*-nitrophenyl acetate, 7.5 × 10^{-5} M, 1.61% acetonitrile; V_1 = 1.10 × 10^{-8} M s^{-1}	0.000 [2.406, 2.401]; 0.175 [2.201, 2.200]; 0.261 [2.000, 2.022, 2.074]; 0.398 [1.945, 1.924, 1.920]; 0.485 [1.695, 1.707, 1.711]; 0.583 [1.637, 1.605, 1.632]; 0.745 [1.324, 1.344, 1.358]; 0.765 [1.337, 1.314, 1.320]; 0.995 [1.005, 1.044, 0.943, 1.002, 1.009]
5. α-chymotrypsin, 1.7 mg mL^{-1}, pH 8.10 and equiv, 25.00 ± 0.05 °C; *N*-acetyl-L-tryptophanamide, 2.5 × 10^{-3} M; V_1 = 7.1 × 10^{-7} M s^{-1}	0.000 [1.903, 1.900, 1.905, 1.873]; 0.099 [1.809, 1.798]; 0.196 [1.720, 1.694, 1.719]; 0.296 [1.640, 1.580]; 0.395 [1.538, 1.550]; 0.492 [1.416, 1.408, 1.408]; 0.595 [1.347, 1.347, 1.341]; 0.795 [1.146, 1.196, 1.139]; 0.894 [1.079, 1.083, 1.079]; 0.995 [0.988, 1.035, 1.010]
6. α-chymotrypsin, 43 mg mL^{-1}, pH 7.60 and equiv, 25.00 ± 0.03 °C; 4-nitrophenyl 3-phenylpropanoate, 3.91 × 10^{-5} M, 3.2% acetonitrile; V_1 = 7.18 × 10^{-8} M s^{-1}	0.005 [2.873, 2.832]; 0.104 [2.622, 2.620]; 0.203 [2.424, 2.396]; 0.302 [2.153, 2.152]; 0.400 [2.043, 2.039]; 0.499 [1.839, 1.830]; 0.598 [1.674, 1.662]; 0.697 [1.505, 1.508]; 0.796 [1.332, 1.329]; 0.896 [1.168, 1.177]; 0.993 [1.020, 1.024]
7. elastase, 0.3 mg mL^{-1}, pH 7.50 and equiv, 25.00 ± 0.03 °C; *p*-nitrophenyl acetate, 4.31 × 10^{-4} M, 3.1% acetonitrile; V_1 = 2.67 × 10^{-8} M s^{-1}	0.016 [2.445, 2.453]; 0.106 [2.313, 2.309]; 0.209 [2.189, 2.182]; 0.306 [2.064, 2.048]; 0.403 [1.883, 1.900]; 0.501 [1.766, 1.773]; 0.595 [1.621, 1.623]; 0.693 [1.493, 1.475]; 0.789 [1.359, 1.350]; 0.886 [1.215, 1.200]; 0.981 [1.044, 1.041]
8. thrombin, 17 mg mL^{-1}, pH 8.00 and equiv, 25.00 ± 0.03 °C; *N*-benzoyl-L-arginine ethyl ester, 4.76 × 10^{-4} M; V_1 = 3.39 × 10^{-7} M s^{-1}	0.016 [2.940, 2.901]; 0.108 [2.733, 2.683]; 0.202 [2.535, 2.534]; 0.307 [2.365, 2.307]; 0.402 [2.211, 2.114]; 0.500 [1.917, 1.953]; 0.596 [1.750, 1.732]; 0.692 [1.626, 1.607]; 0.790 [1.383, 1.362]; 0.887 [1.237, 1.255]; 0.949 [1.134, 1.118]

[a] Equivalent pH refers to a pL in mixed isotopic solvents obtained by maintaining a constant ratio of buffer components, so that the rate is measured in each mixture at a corresponding point on the rate/pL profile[11] (see Experimental Section). All reactions were carried out in Tris/Tris-HCl buffers with a total concentration of buffer components of 0.100 M (case 5), 0.050 M (cases 2 and 8), 0.030 M (cases 1, 4, 6, and 7), or 0.0125 M (case 3).

Table II. Comparison of Midpoint Partial Solvent Isotope Effects with Predictions for One-Proton Catalysis, Two-Proton Catalysis, and Generalized Solvation Changes

enzyme, substrate (average range of data at constant *n*, ‰)	V_0/V_1	midpoint partial solvent isotope effect, V_m/V_1 (n_m)	V_m/V_1 predicted for (deviations from obsd V_m/V_1, ‰)		
			one-proton catalysis	two-proton catalysis	generalized solvation changes
1. trypsin, PNPA (±4)	1.378	1.185 (0.498)	1.190 (+4)	1.182 (−3)	1.175 (−8)
2. trypsin, BAEE (±13)	3.034	2.045 (0.500)	2.017 (−14)	1.879 (−81)	1.742 (−148)
3. trypsin, BzPhe-Val-Arg *p*-nitroanilide (±20)	4.30	2.26 (0.496)	2.66 (+180)	2.38 (+53)	2.06 (−880)
4. α-chymotrypsin, PNPA (±6)	2.404	1.704 (0.485)	1.723 (+11)	1.647 (−33)	1.571 (−783)
5. α-chymotrypsin, AcTrpNH$_2$ (±9)	1.895	1.410 (0.492)	1.454 (+31)	1.419 (+6)	1.384 (−18)
6. α-chymotrypsin, 4-nitrophenyl 3-phenylpropanoate (±4)	2.852	1.835 (1.499)	1.928 (+51)	1.809 (−14)	1.691 (−78)
7. elastase, PNPA (±3)	2.449	1.770 (0.501)	1.723 (−26)	1.643 (−71)	1.564 (−116)
8. thrombin, BAEE (±7)	2.921	1.935 (0.500)	1.961 (+13)	1.835 (−52)	1.709 (−117)

protons with smaller isotope effect or of some solvation changes. α-Chymotrypsin with AcTrpNH$_2$ (reaction no. 5), a substrate having some features of natural substrate structure, shows a modest isotope effect (1.90) which agrees well with the two-proton prediction. The same enzyme with 4-nitrophenyl 3-phenylpropanoate (reaction no. 6; this substrate should fill the hydrophobic pocket but not occupy the *N*-acyl site) exhibits a larger overall effect, 2.85, with a midpoint effect which falls between the one-proton and two-proton (equal isotope effects) predictions.

Figures 1–3 present graphical illustrations of three typical data sets.

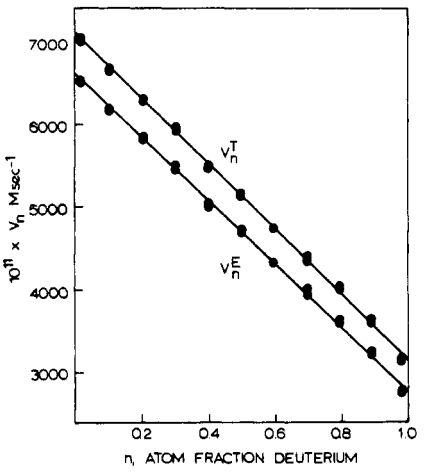

Figure 1. Variation with atom fraction of deuterium n of zero-order rate constants for the elastase-catalyzed hydrolysis of p-nitrophenyl acetate in binary mixtures of protium and deuterium oxides. V_n^T is the total velocity of hydrolysis and V_n^E is the velocity of enzymic hydrolysis after correction for the background reaction. The solid lines were calculated by a least-squares fit.

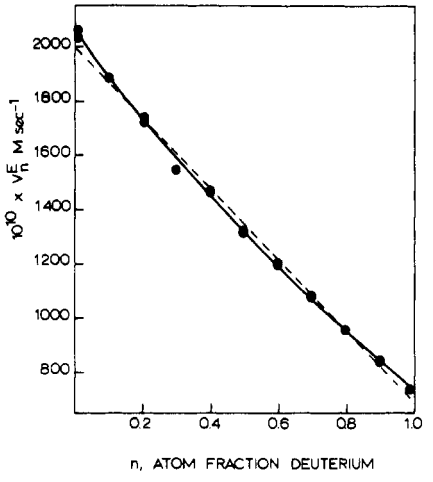

Figure 2. Variation with atom fraction of deuterium n of zero-order rate constants for the α-chymotrypsin-catalyzed hydrolysis of 4-nitrophenyl 3-phenylpropanoate in binary mixtures of protium and deuterium oxides. The dashed line is the best linear fit to the data and the solid line is the nonlinear least-squares fit, generating the expression $V_n = V_0(1 - n + n[0.54])(1 - n + n[0.65])$.

Discussion

The substrate most closely approaching the polypeptide structure of the natural substrates of the serine proteases, BzPhe-Val-Arg p-nitroanilide, produces with trypsin a nonlinear proton inventory curve (Figure 3) which is consistent with at least two protonic sites catalytically active in the transition state, each generating an isotope effect k_H/k_D of about 2.1. Each of these effects of 2.1 is similar to those for protons involved in catalytic bridging in model-reaction transition states where protonic interaction at an electronegative center is assisting in bond formation or fission.[11] Two-proton catalysis has also been found by Hunkapiller, Forgac, and Richards[14] for the very similar substrate AcAla-Pro-Ala p-nitroanilide with α-lytic protease and elastase, the reaction catalyzed by the α-lytic protease generating an isotope effect of about 1.7 for each of two protons and that catalyzed by elastase generating about 1.5 for each of two protons. These authors showed that there accumulated with elastase a species likely to be the tetrahedral adduct of the substrate carbonyl function with the enzymatic serine hydroxyl, so that the

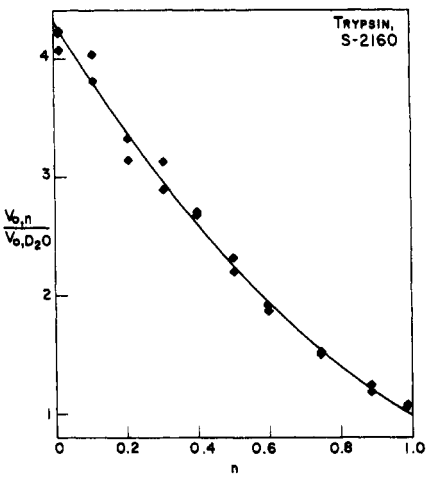

Figure 3. Partial solvent isotope effect as a function of atom fraction of deuterium n for the trypsin-catalyzed hydrolysis of BzPhe-Val-Arg p-nitroanilide ("S 2160") in binary mixtures of protium and deuterium oxides. The solid line is the best-fit polynomial representation of the data: $(V_n/V_1) = 4.30 - 4.89n + 1.59n^2$.

transition-state protonic bridging is very probably to the anilide nitrogen, as it leaves the tetrahedral intermediate. We have not done such experiments with trypsin and our substrate, but the bridging may well be to this site in our case as well.

When the substrate structure is reduced to the point where only some of the catalytic site interactions of the natural substrate and none of the interactions at remote sites on the enzyme are simulated, the two-proton catalysis can no longer be relied upon. In some cases, it is observed but with smaller isotope effects, or with one of the protons generating only a modest effect. Thus, α-chymotrypsin with $AcTrpNH_2$ generates a proton inventory seemingly consistent with two-proton catalysis with $k_H/k_D \sim 1.4$ for each proton (Table II, reaction no. 5), but the best nonlinear least-squares fit of the entire data set[15] to eq 4a yields $\phi_{T1} = 0.88 \pm 0.07$, $\phi_{T2} = 0.59 \pm 0.04$. This corresponds to isotope effects k_H/k_D of 1.69 ± 0.11 and 1.14 ± 0.09. This is essentially one-proton catalysis ($k_H/k_D = 1.69$) with a small effect (1.14) either from a second proton or from some larger number of protons, each generating very small effects (as in changes of status for protein structural or solvation sites).[13] Similarly, α-chymotrypsin with 4-nitrophenyl 3-phenylpropanoate (Table II, reaction no. 6; Figure 2) produces effects consistent with two-proton catalysis, with the best nonlinear least-squares fit to eq 4a giving $\phi_{T1} = 0.54$ ($k_H/k_D = 1.85$), $\phi_{T2} = 0.65$ ($k_H/k_D = 1.54$). These effects are about the same as those found for the oligopeptide analogue with elastase and α-lytic protease by Hunkapiller, Forgac, and Richards,[14] although with the 4-nitrophenol substrate the deacylation of the acyl-enzyme is almost surely rate limiting. In two cases with substrates involving catalytic site interactions only and having the deacylation process rate limiting (trypsin with BAEE, reaction no. 2 of of Table II; thrombin with BAEE, reaction no. 8 of Table II), there is strong preference for one-proton catalysis but with quite large isotope effects (3.03 for trypsin, 2.92 for thrombin).[16]

Finally, with the constriction of the substrate structure to the point where evolutionarily developed interactions with enzyme have been eliminated, as in the hydrolysis of p-nitrophenyl acetate catalyzed by α-chymotrypsin and elastase (reactions no.'s 4 and 7, Table II), one-proton catalysis becomes the rule. The isotope effects are substantial ($k_H/k_D = 2.40$ and 2.45, respectively), similar to those for general catalysis in model systems.[11] These are reactions where deacetylation of acetyl-enzyme is rate limiting. In the case of p-nitrophenyl acetate with trypsin, the overall isotope effect is only 1.39, which is smaller than expected even for a one-proton

solvation bridge, as seen in some model systems.[11] One wonders whether acetyltrypsin may not be undergoing deacetylation at least in part by rate-determining rearrangement to the *N*-acetylhistidine form, followed by fast hydrolysis, in a process related to that proposed by Hubbard and Kirsch[17] for acylation of α-chymotrypsin by reactive esters.[18]

The general conclusion would seem to be that the serine proteases require evolutionarily determined interactions in the catalytic transition state with substrate functions at several remote sites in addition to interactions at the catalytic site before the full potential of the enzyme for multiproton catalysis is called into action. When only some of the catalytic site interactions are present, and no remote interactions, then either one- or two-proton catalysis may be observed, perhaps depending on the exact structural results of particular interactions or even details of reaction conditions. For minimal substrates, simple general catalysis with a one-proton bridge (or conceivably nucleophilic catalysis), as in model systems, is found.

Two questions of some interest remain: (1) What is the nature of protonic bridging in the one-proton and two-proton modes of catalysis? (2) What is the means by which substrate structure affects the choice between these? The magnitudes of the isotope effects at a single protonic site, which we observe here in both one- and two-proton modes, are smaller than the effects k_H/k_D of approximately 3–7 reported for cases in which a simple "proton switch" between electronegative atoms, not concerted with heavy-atom reorganization, is believed to determine the rate.[19-21] It therefore seems unlikely that such a proton switch is purely rate limiting in either catalytic mode. If the reorganization of the heavy-atom framework of the substrate were the chief component of the critical coordinate, the hydrogen bridges might be of the type involved in "solvation catalysis[22]". The magnitudes of the isotope effects are similar to those seen for single protonic sites in model reactions in this category.[10,11,21-25] A third possibility is that there is a substantial component of both proton and heavy-atom motions in the critical coordinate ("concerted" catalysis). If it should emerge that the histidine of the charge-relay system is more basic than the aspartate, as has recently been argued,[7] and if this relative basicity carries into the catalytic transition state, then two-proton catalysis in a concerted mode would offer no advantage. Our view is that solvation catalysis is the most likely mode, although the distinction from other hypotheses is not strong.

It is reasonable to suppose that a compression of the enzyme structure resulting from remote binding of oligopeptide units is the structural effect which activates the multiproton catalytic machinery. Quantum chemical calculations have shown that compression across hydrogen-bond chains favors correlated, or coupled, changes in state of the protons in the component hydrogen bonds.[26] As the substrate is reduced in size, the loss of these compressing interactions in the transition state may lead to a relaxation across the hydrogen bonds and a decoupling of the protonic changes in state. Enzyme compression might also be expected to produce isotope effects of the magnitudes observed in this work for single protonic sites in both one- and two-proton catalytic modes. Kreevoy, Liang, and Chang[27] have observed isotope effects of this size for what appear to be short, strong hydrogen bonds. These bonds have been suggested to be stable-state analogues of transition-state catalytic bridges[2,28] in reactions subject to solvation catalysis.[23]

Conclusion

Interactions at remote subsites, in addition to specific interactions in the catalytic site, are required in the transition state for catalytic action by the serine proteases in order to bring into function the full multiproton catalytic capability of the ABCE, which has been developed by molecular evolution and inferred from structural studies. As the substrate structure is truncated, the reliability of this function decays until, with minimal substrates, the enzyme functions as a simple one-proton general catalyst (and perhaps sometimes as a nucleophilic catalyst).

Experimental Section

Materials. All salts and organic reagents were obtained as analytical or reagent grade materials and used without purification or after simple drying, distillation, or recrystallization. Water (protium oxide) was copper distilled, glass distilled, and deionized on a mixed-bed ion-exchange column (Barnstead Ultrapure Catalogue No. D8902, Sybron Corp.). Heavy water (deuterium oxide) was commonly obtained from Stohler Isotope Chemicals, "99.8% D", and was glass distilled before use. The distilled material showed $n = 0.9974 \pm 0.0002$ by NMR with acetonitrile as internal standard. Experiments were also done with deuterium oxide from Diaprep; this material had $n = 0.9954 \pm 0.0012$ by NMR. In some experiments, undistilled deuterium oxide was also employed and no differences resulting from purification were ever noted.

Substrates. *p*-Nitrophenyl acetate was prepared from *p*-nitrophenol (Matheson, Coleman and Bell) and acetic anhydride (Baker) with pyridine in benzene under reflux for 1 h, followed by recrystallization from hot petroleum ether (bp 60–110 °C) and sublimation (mp 77.5–77.8 °C, lit.[29] 79.5–80.0 °C; lit.[30] 77.5–78.0 °C). 4-Nitrophenyl 3-phenylpropanoate resulted from reaction of *p*-nitrophenol and pyridine in diethyl ether with 3-phenylpropanoyl chloride, added slowly in diethyl ether. The acid chloride had been made from the acid (Aldrich; recrystallized from hexane) by treatment with thionyl chloride. After several hours of stirring, the reaction mixture was washed with 0.1 M HCl, 0.1 M $NaCO_3$, and water and dried with $MgSO_4$. Ether was evaporated and the ester recrystallized to give clear needles, mp 98–98.1 °C. Anal. Calcd for $C_{15}H_{13}NO_4$: C, 66.41; H, 4.83; N, 5.16. Found: C, 66.55; H, 4.79; N, 4.99. *N*-Acetyl-*L*-tryptophanamide (Sigma Chemical Co.) had mp 192–193 °C (lit.[31] 192–193 °C), showed no impurities on TLC, and was used as supplied. *N*-Benzoyl-L-arginine ethyl ester (BAEE) (Sigma Chemical Co.) had mp 130–133 °C (lit.[31] 128–133 °C) and was dried over phosphorus pentoxide in a vacuum desiccator before use. *N*-Benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide ("S2160') was purchased from AB Bofors, Nobel Division, Peptide Research, P.O. Box 156, S-431 22 Mölndal, Sweden. It was used as supplied.

Enzymes. Bovine pancreatic trypsin (EC 3.4.21.4, Sigma Chemical Co., dialyzed, once-recrystallized, salt-free powder with an activity of 7500 BAEE units/mg) was used after treatment as described below to obtain autolytically stable solutions; with poorer substrates, chymotryptic activity was removed by treatment with diphenylcarbamoyl chloride. Bovine pancreatic α-chymotrypsin (EC 3.4.21.1) was obtained from Sigma Chemical Co. (salt-free, lyophilized, three times recrystallized powder with an activity of 52 BTEE units/mg) or from Worthington Biochemical Corporation (dialyzed, salt-free, once-recrystallized powder with an activity of 51 BTEE units/mg). Elastase (Pancreatopeptidase E, EC 3.4.21.11, from hog pancreas, chromatographically purified, lyophilized, water-soluble powder with an activity of 50–51 elastin units/mg) and thrombin (EC 3.4.21.5 from bovine plasma, lyophilized powder containing about 30% (w/w) buffer salts such as glycine, $CaCl_2$, NaCl, benzethonium chloride; activity 150 NIH (fibrinogen) units/mg) were both obtained from Sigma Chemical Co.

Solutions and Reaction Conditions. Mixtures of protium and deuterium oxides were prepared gravimetrically as a rule (occasionally volumetrically) under a nitrogen atmosphere, buffer components being added similarly to avoid exposure to atmospheric moisture insofar as possible. Deuterium contents were determined by NMR using acetonitrile as internal standard and are reliable at levels from several percent at low n to several parts in ten thousand at high n. In some cases, low-n solutions were prepared by quantitative dilution of high-n solutions. pL (−log [lyonium ion activity]) measurements were made with a GK 2322C combination glass and calomel electrode with a Radiometer 26 pH meter. The meter readings (MR) were converted to pL according to[32] $pL = MR + 0.3314n + 0.076n^2$ or other equivalent empirical relationships.[11] These enzymes appear[11,33,34] to behave as "normal acids", i.e., the isotope effect on the pKs of the rate/pL profile is $\Delta pK \sim 0.50$, deriving from formation of the lyonium ion,

as does the isotope effect on the buffer pK. The reactions therefore were carried out in solutions of *equivalent* or *corresponding* pL, i.e., at the corresponding point on the rate/pL profile, by maintaining the acid–base ratio of the buffer constant through all isotopic mixtures.[11] pL readings were made before and after all kinetic runs and in some cases during runs, and were constant for all accepted experiments. Enzyme solutions were prepared gravimetrically, commonly in solvents of $n \sim 0.5$ but sometimes in solvents of $n = 0$ or $n \sim 1$ with no difference in behavior observable. Concentrated solutions (>1 mg mL^{-1}) were centrifuged at 15 000 rpm and 4 °C for 30 min. All solutions, except those of trypsin, were made immediately before use and were stored in the cold until use. Autolytically stable solutions of trypsin were prepared in a solvent of $n \sim 0.5$ with 10^{-3} M hydrochloric acid by, first, dissolving at a level of 0.5 mg mL^{-1}, then allowing the solution to remain at room temperature for 2 h, then at 5 °C, and then centrifuging (we are pleased to thank Professor C. Ghiron for this procedure).

Kinetic Procedures. All reaction rates were measured spectrophotometrically under zero-order conditions. 4-Nitrophenyl ester reactions were monitored at 400 nm. The extinction coefficients of 4-nitrophenoxide ion at 400 nm were determined from Beer's law plots for solutions 0.1 M in NaOH in H_2O and 0.1 M in NaOD in D_2O to be 17 074 ± 36 M^{-1} cm^{-1} (H_2O) and 17 120 ± 22 M^{-1} cm^{-1} (D_2O), and thus identical to within 3‰. A value of $\epsilon = 17\,097 \pm 42$ M^{-1} cm^{-1} was used for both solvents and all mixtures. Although the pK of 4-nitrophenol varies with n, the fraction ionized will not vary at constant buffer ratio if 4-nitrophenol behaves as a "normal acid", i.e., if the entire isotope effect on ionization results from lyonium ion formation.[11] This would produce[11] $\Delta pK = \log (0.69)^{-3} = 0.48$. We find under our conditions that for 4-nitrophenol, $pK = 6.98 \pm 0.04$ ($n = 0$), 7.48 ± 0.08 ($n = 1$), yielding $\Delta pK = 0.50 \pm 0.09$. Combining this finding with the demonstration by Pentz and Thornton[35] that the pKs of both 2-nitrophenol and 2,4-dinitrophenol respond normally to n generates reasonable confidence that the fraction of ionized 4-nitrophenol is independent of n under these conditions. The hydrolysis of *N*-acetyl-L-tryptophanamide was followed at 306 nm ($\Delta\epsilon = 73.6$ M^{-1} cm^{-1} in agreement with a previous report[26]), and that on *N*-benzoyl-L-arginine ethyl ester was followed at 260.5 nm ($\Delta\epsilon = 533$ M^{-1} cm^{-1}). The hydrolysis rate of *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide was measured at a wavelength of 400 nm ($\Delta\epsilon = 11\,520$ M^{-1} cm^{-1}). In a typical experiment, a cuvette containing all components of the reaction mixture except enzyme was brought to thermal equilibrium in a jacketed holder in the cell compartment of a Cary 16 or 118 spectrophotometer. The temperature was maintained by water circulated from a Lauda 4KR bath and was measured by a glass-covered thermistor probe in the reaction cell or by a probe or thermometer in an adjacent cell. Injection of 100 mL or less enzyme solution from a micropipet initiated the reaction. Absorbances were continuously measured, digitized, averaged, and stored in a Hewlett-Packard 2100A computer by a means elsewhere detailed.[36] The zero-order data were fit to a linear dependence on time by linear least squares.

Background Corrections. In reactions with *p*-nitrophenyl acetate as substrate, the reaction of the ester with solvent, lyoxide ion, and buffer sometimes contributed to the observed rate. A correction was applied in the following way. Assuming the background reaction to be first order in substrate, the total velocity in a particular isotopic solvent mixture, V_n^T, would be a sum of enzymic (V_n^E) and nonenzymic (V_n^N) contributions defined by:

$$V_n^T = V_n^E + V_n^N = V_n^E + k_{obsd}^n[S]$$

Values of k_{obsd}^n were determined in the absence of enzyme and under conditions otherwise identical with those of the enzymatic reactions of interest. In 0.03 M Tris buffer, pH 7.50 and equivalent, 25.00 ± 0.03, the best-fit least-squares third-order polynomial representation of k_{obsd}^n is:

$$10^8 k_{obsd}^n(s^{-1}) = 1174.276 - 463.764n + 297.927n^2 - 143.244n^3$$

This equation was used to generate values of k_{obsd}^n which agreed to within ±3% with observed values; corrections were then made to enzymatic values. The magnitude of the correction never exceeded 15% of the total rate.

References and Notes

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