Solvent Isotope Effects in H_2O-D_2O Mixtures (Proton Inventories) on Serine-Protease-Catalyzed Hydrolysis Reactions. Influence of Oxyanion Hole Interactions and Medium Effects

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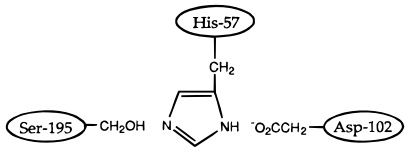
Abstract: Rates of hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide catalyzed by subtilisin and its N155G mutant were measured in H_2O, D_2O, and "HDO" (1:1 H_2O:D_2O). The solvent isotope effects (proton inventories) determined by these data showed no differences between the wild-type and mutant enzymes, despite the fact that the mutation removes a hydrogen-bonding interaction in the oxyanion hole of the enzyme worth two orders of magnitude in reaction rate. This suggests that curvature previously observed (ref 10) in the proton inventory for a reaction catalyzed by methyl chymotrypsin is also not due to oxyanion hole interactions, and this curvature can in fact be accounted for by a medium effect. Proton inventory analysis of the isotope effects for subtilisin and its mutant also indicate the presence of strong medium effects in those systems.

Solvent isotope effects on reaction rates in H_2O-D_2O mixtures are commonly analyzed using the formalism represented by eq 1,[3,4] in which k_x is the rate constant

$$\frac{k_x}{k_H} = \frac{\Pi_i(1 - x + x\phi_i^{\ddagger})}{\Pi_j(1 - x + x\phi_j^{IS})} \qquad (1)$$

determined in an H_2O-D_2O mixture of deuterium atom fraction x, k_H is the corresponding rate constant determined in H_2O, and $\phi^{\ddagger}$ and ϕ^{IS} are fractionation factors for hydrogenic sites in the transition state and the initial state, respectively. Fractionation factors are partial isotope effects expressed as D:H ratios at particular sites relative to the D:H ratio of the solvent with which they are equilibrated. They reflect the tightness of bonding to the hydrogens they represent: fractionation factors are significantly different from unity for hydrogens being transferred, where they represent primary isotope effects, and also for hydrogens in strong hydrogen bonds, whose overall bonding is loose.[7] Analyses using this fractionation factor formalism are often called proton inventories, especially when applied to enzyme catalyzed reactions.[8]

Scheme 1



The right side of eq 1 must include terms of the form $(1 - x + x\phi)$ for all exchangeable sites in both the transition state and the initial state. When applied to enzymatic reactions, however, an abbreviated version of eq 1 retaining only transition state terms is often used, under the assumption that initial state fractionation factors will either be unity ($\phi^{IS} = 1.00$) or will not change between initial state and transition state ($\phi^{IS} = \phi^{\ddagger}$) and will therefore drop out of the full expression. In this circumstance, eq 1 will reduce to a linear function in x if only one transition state fractionation factor is significantly different from unity; to a quadratic function if two $\phi^{\ddagger}$ differ from unity; to a cubic function if three $\phi^{\ddagger}$ differ from unity; etc. In practice, while it is usually possible to determine whether an experimentally derived relationship is linear or curved, because departures from linearity are ordinarily quite small, it is generally difficult to tell whether the curvature is quadratic, cubic, or of some higher order.

A number of proton inventories for serine protease catlayzed hydrolyses of ester and amide subtrates have been determined, and some have been found to be linear and some curved.[9] This difference has been interpreted in terms of a reaction mechanism involving the catalytic triad found in the active site of all serine proteases, consisting of Ser-195, His-57, and Asp-102 (chymotrypsin numbering), shown in Scheme 1. As the carbonyl carbon atom of the hydrolysis substrate is attacked by the hydroxyl group of Ser-195, the nucleophilicity of this hydroxyl group is increased by transfer of its proton to the imidazole

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(4) This relationship is often called the Gross−Butler equation, after pioneering work done by Gross[5] and Butler[6] on phenomena in H_2O-D_2O mixtures. Gross and Butler, however, derived only a special case of eq 1, that pertaining to the ionization of a monobasic acid, and the completely general relationship of eq 1 was not available until it first appeared in ref 3.

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(6) Hornell, J. C.; Butler, J. A. V. *J. Chem. Soc.* **1936**, 1361−1366. Orr, W. J. C.; Butler, J. A. V. *J. Chem. Soc.* **1937**, 330−335. Nelson, W. E.; Butler, J. A. V. *J. Chem. Soc.* **1938**, 957−962.

(7) Kreevoy, M. M.; Liang, T. M. *J. Am. Chem. Soc.* **1980**, *102*, 3315−3322. Cleland, W. W. *Biochemistry* **1992**, *31*, 317−319. Cleland, W. W.; Kreevoy, M. M. *Science*, **1994**, *264*, 1887−1890. Frey, P. A.; Whitt, S. A.; Tobin, J. B. *Science* **1994**, *264*, 1927−1930.

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(9) For a review, see: Venkatasubban, K. S.; Schowen, R. L. *CRC Crit. Rev. Biochem.* **1984**, *17*, 1−44.

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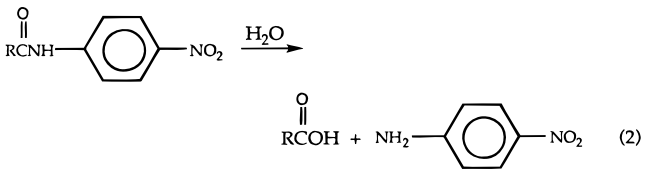
side chain of His-57, and this may or may not be accompanied by a second proton transfer from the back side of the forming imidazolium ion to the carboxylate group of Asp-102. Linear proton inventories have been taken as evidence that only the first proton transfer, that from serine to histidine, is taking place, whereas curved proton inventories have been taken to mean that the second proton transfer, that from histidine to aspartate, is occurring as well.

Several years ago, a critical experiment was performed whose outcome cast doubt upon this interpretation.[10] It was found that a hydrolysis reaction catalyzed by a chymotrypsin derivative whose His-57 imidazole hydrogen had been replaced by a methyl group, and a second proton transfer to Asp-102 was consequently impossible, still gave a curved proton inventory. This unexpected result was rationalized by proposing that the curvature in this case was caused by strengthened hydrogen bonding interactions between the substrate and groups situated in the oxyanion hole of the enzyme. This experiment, however, nevertheless showed that curved proton inventories do not necessarily signify occurrence of a second proton transfer.

In order to determine whether oxyanion hole interactions can in fact influence proton inventories, we have examined a hydrolysis reaction catalyzed by an enzyme in which this interaction has been impaired by site-directed mutagenesis. Such an experiment cannot be performed with chymotrypsin, for the hydrogen bonding interactions in the oxyanion hole of this enzyme are provided by main-chain amide groups, and removing them would destroy the enzyme. In subtilisin, however, only one of the oxyanion hole interactions is provided by a main chain amide—two others are furnished by the side chains of Asn-155 and Thr-220. Site-directed mutagenesis has shown that the interaction with Asn-155 is by far the stronger of these two,[11] and an X-ray structure determination indicates that the third interaction, that with the main chain amide group, must operate over a longer distance and is consequently weak.[12] We have therefore performed our study using the N155G mutant of subtilisin in which Asn-155 is replaced by glycine, a residue with no side-chain capable of providing a hydrogen bonding interaction.

The substrate we have used is succinyl-Ala-Ala-Pro-Phe-nitroanilide,[13] and the reaction we have studied is consequently the amide hydrolysis shown in eq 2. We



(2)

constructed proton inventories using both V_m (maximum velocity) and V_m/K_M (first-order) rate constants, and by taking the ratios $(V_m/K_M):V_m$, we also obtained proton inventories based upon $1/K_M$ values. For anilide substrates such as the one we used, acyl-enzyme formation is believed to be rate-determining,[14] and $1/K_M$ then takes on the form of an enzyme-substrate association constant. Because we found there to be an appreciable solvent isotope effect on $1/K_M$, we also investigated a much simpler system which nevertheless still retains many of the features of enzyme−substrate association: complex formation between α-cyclodextrin and *p*-nitrophenoxide ion.

It is the degree of curvature that carries the mechanistic information in a proton inventory, and curvature is maximized at the midpoint near $x = 0.5$. Following Albery,[15] we have consequently concentrated our measurements in this region, dubbed "HDO", in addition to $x = 0$ and $x = 1$.

A portion of the present work has been published in preliminary form.[16]

Experimental Section

Materials. Wild-type subtilisin BPN′ and its N155G mutant were prepared and purified as has already been described.[17] All other materials were best available commercial grades.

Enzyme Kinetics. Rates of the enzyme-catalyzed hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were determined by monitoring the appearance of *p*-nitroaniline through its absorbance at $\lambda = 410$ nm. Measurements were performed using Cary 118 and 2200 spectrometers whose cell compartments were thermostatted at 25.0 ± 0.05 °C.

Zero-order initial rates of reaction, v, for fitting of the Michaelis−Menten expression, eq 3, were determined at different substrate concentrations, [s], by adding

$$v = \frac{V_m\,[s]}{K_M + [s]} \tag{3}$$

successive amounts of substrate to the same reaction mixture. Enyzme plus buffer solution, made by adding 30 μL of enzyme stock solution (H_2O, HDO, or D_2O solvent) to 1.00 mL buffer contained in a low-volume cuvette, was first allowed to come to temperature equilibrium with the spectrometer cell compartment. Two μL of substrate stock solution (DMSO solvent) was then added, and the change in absorbance was recorded for 2−4 min. A second 2-μL portion of substrate stock solution was then added, and the change in absorbance was recorded again. This process was usually repeated for six substrate additions, after which another set of six determinations was performed using substrate stock solution of a different concentration. Four to six such sets of six measurements were usually made, and in this way a number of reaction velocities, v, at different substrate concentrations distributed about K_M, $[s] = (0.05-15)\,K_M$, were supplied. The extent of reaction during the 2−4 min over which the zero-order rate measurements were made varied from just under 10% to less than 1%. The absorbance versus time records were accurately linear; their slopes were determined by eye, and these were then converted to rates of change of substrate concentration using the extinction coefficient for *p*-nitroaniline $\epsilon = 8480\ M^{-1}\ cm^{-1}$.[13] Substrate concentrations used for fitting of eq 3 were average values for the period over which absorbances were recorded; these were calculated from knowledge of the substrate stock solution concentrations, the rate of reaction, and the times over which absorbance was measured plus the intervals between successive additions. A representative Michaelis−Menten plot is shown in Figure 1; it may be seen that the data conform to this rate law well.

First-order rates of reaction were determined at initial substrate concentrations much less than K_M, $[s]_o = (0.004-0.02)\,K_M$, where eq 3 reduces to the first-order expression $v = (V_m/K_M)\,[s]$. These reactions were allowed to run essentially to completion (5 half-lives or longer). The data obeyed the first-order rate law well, and rate constants were calculated by nonlinear least squares fitting of an exponential expression.

In all cases, groups of measurements in H_2O, HDO, and D_2O solutions were made on the same day using the same enzyme stock

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solution. Replicate measurements made at the beginning and end of the day showed that the enzyme solutions were stable for this period of time.

Cyclodextrin Complex Formation. Equilibrium constants for complex formation between α-cyclodextrin and *p*-nitrophenoxide ion were determined using the shift to longer wavelengths that the absorption band of this ion at $\lambda_{max} = 400$ nm undergoes upon inclusion into the cyclodextrin. The change in absorbance accompanying complex formation is greatest at $\lambda = 370$ nm, and measurements were consequently made at this wavelength, using a Cary 2200 spectrometer with cell compartment thermostatted at 25.0 ± 0.05 °C. Absorption measurements were made on a series of solutions of fixed *p*-nitrophenoxide concentration (*ca*. 10^{-4} *M*) but increasing α-cyclodextrin concentration. This was accomplished by making successive additions of a solution containing α-cyclodextrin and *p*-nitrophenol to a buffer solution (0.1 M HCO^-_3/CO^{2-}_3, pH = 10.0) containing *p*-nitrophenol at exactly the same concentration as the cyclodextrin solution. The data were analyzed by nonlinear least squares fitting of eq 4, in which *A* is

$$A = \epsilon_p b + (\epsilon_c - \epsilon_p)\left\{\frac{(a + b + 1/K) - \sqrt{(a + b + 1/K) - 4ab}}{2}\right\} \quad (4)$$

absorbance, ϵ_p and ϵ_c are extinction coefficients of free and complexed *p*-nitrophenoxide ion respectively, *a* and *b* are stoichiometric concentrations of α-cyclodextrin and *p*-nitrophenoxide ion, respectively, and *K* is the complex association constant. The value of *b* for each series of solutions was supplied whereas ϵ_p, ϵ_c, and *K* were parameters determined by the fitting procedure. Figure 2 shows a representative set of data and the line produced by the fit; it may be seen that the relationship of eq 4 is obeyed quite well.

Results

Enzyme Kinetics. Zero-order initial rates of hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide catalyzed by wild-type subtilisin and its N115G mutant were determined in H_2O, HDO, and D_2O solution at substrate concentrations in the vicinity of K_M ($[s]_o = (0.5-15)\ K_M$). The data so obtained were analyzed by fitting of the Northrop version of the Michaelis−Menten equation,[18] which gives maximum velocities, V_m, and first-order rate constants, V_m/K_M, directly. The measurements were made in sets using the same enzyme stock solution for all three solvents; this ensured that enzyme concentrations were exactly the same in all three solvents and that the isotope effects were consequently being evaluated at the same enzyme concentration. Each Michaelis−Menten plot was based on 20−40 initial rate values, and 10 such plots were constructed with the wild-type enzyme and 14 with the mutant. The parameters obtained from these analyses were formulated into HDO/H_2O and D_2O/H_2O isotope effects on V_m and V_m/K_M by taking appropriate ratios. The results are summarized in Table S1[19] and are displayed in Figure 3.

Rates of enyzmatic hydrolysis were also determined at low substrate concentrations ($[s]_o = 0.004-0.02\ K_M$) where the enzyme-catalyzed reactions become a first-order process with rate constant equal to V_m/K_M. Measurements in H_2O, HDO, and D_2O were again made in sets using the same enzyme stock solution, and replicate determinations (3−8) were made for each solvent within a given set. Nine such sets of measurements were made with the wild-type enzyme and three with the mutant. The isotope effects obtained are listed in Table S2[19] and are also displayed in Figure 3.

(18) Northrop, D. B. In *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, MD, 1977; pp 122−152.

(19) Supporting information; see paragraph at the end of this paper regarding availability.

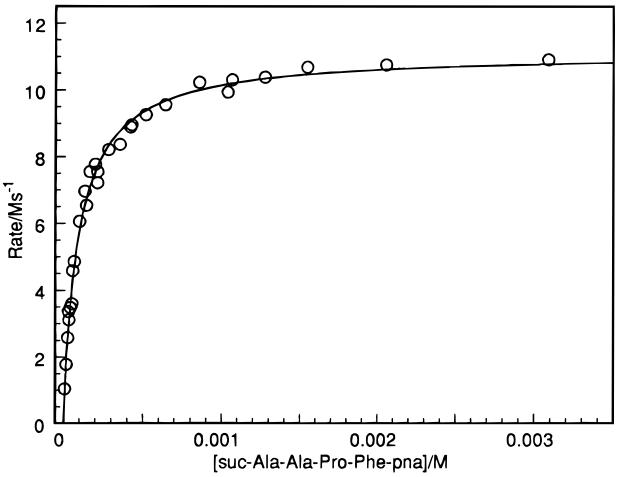

Figure 1. Michaelis−Menten plot for the hydrolysis in D_2O solution of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide catalyzed by wild-type subtilisin.

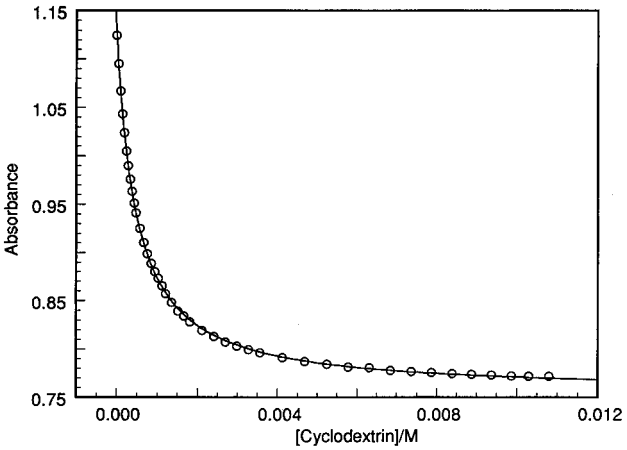

Figure 2. Relationship between absorbance and α-cyclodextrin concentration produced by complex formation with *p*-nitrophenoxide ion in H_2O solution.

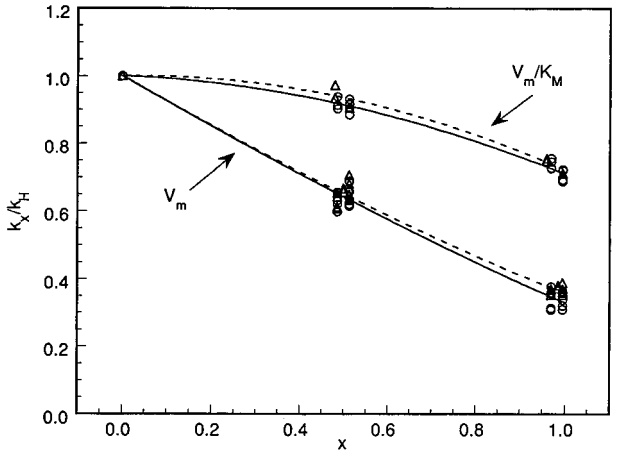

Figure 3. Solvent isotope effects for the hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by subtilisin (○, solid line) and its N155G mutant (△, broken line).

Both kinds of rate measurements (zero- and first-order) were done in TRIS or carbonate buffers at pHs ranging from 8.2 to 10.0; this variation in pH was found to have no systematic influence on the isotope effects. The enzyme stock solutions were made using either H_2O, HDO, or D_2O as the solvent, and this difference was also found to have no influence on the isotope effects.

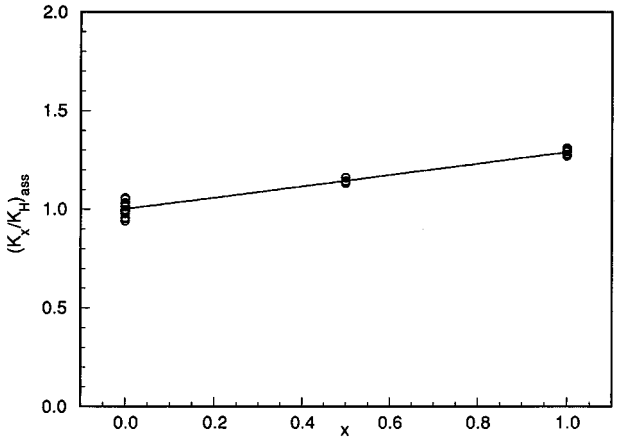

Figure 4. Solvent isotope effects for the association of *p*-nitrophenoxide ion with α-cyclodextrin.

Enzyme concentrations were $10^{-8}-10^{-9}$ *M* for the runs with wild-type enzyme and $10^{-6}-10^{-8}$ M for those with the mutant. We made no effort to fix these concentrations precisely, since our interest was solely in rate ratios determined at the same enzyme concentration, and this was accomplished by using the same enzyme stock solution to measure both constituent rates of a given ratio. Enzyme concentrations were nevertheless known approximately, and use of these to convert the presently evaluated rate constants into k_{cat} and k_{cat}/K_M values gave results consistent with the literature: for the wild-type enzyme, $k_{cat}/s^{-1} = 32$ *versus* 50,[14] 44,[17,20] or 56[11] and $(k_{cat}/K_M)/10^5\ M^{-1}\ s^{-1} = 2.0$ (zero-order) or 2.5 (first-order) *versus* 3.6,[14] 2.2,[20a] 2.5[17,20b] or 3.5[11] and for the mutant, $k_{cat}/s^{-1} = 0.23$ *versus* 0.30[17] and $(k_{cat}/K_M)/10^3\ M^{-1}\ s^{-1} = 1.3$ (zero-order) or 2.6 (first -order) *versus* 1.4.[17]

Cyclodextrin Complex Formation. Association constants, K_{ass}, for complex formation between α-cyclodextrin and *p*-nitrophenoxide ion were determined in H_2O, HDO, and D_2O. Measurements were made in bicarbonate-carbonate buffer solutions at pH = 10.0, midway between the pK_a of the cyclodextrin (p$K_a = 12.33$)[21] and *p*-nitrophenol (p$K_a = 7.15$),[22] in order to ensure that the cyclodextrin was essentially fully unionized and the *p*-nitrophenoxide essentially fully ionized and that the isotope effects were consequently not being influenced by acid−base equilibration of either of these substances. Twenty-six separate determinations of K_{ass} were made in H_2O, eleven in HDO, and fifteen in D_2O, and each determination was based upon some 40 concentration-absorbance data pairs. The results are summarized in Table S3[19] and are displayed in Figure 4.

The average of the determinations made in H_2O is $K_{ass} = (2.264 \pm 0.013) \times 10^3\ M^{-1}$, in good agreement with what appears to be the best value available from the literature, $K_{ass} = (2.178 \pm 0.045) \times 10^3\ M^{-1}$.[23]

Discussion

Oxyanion Hole Interactions. Each of the proton inventories constructed here for hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by subtilisin and its N115G mutant is well represented by a quadratic expression, and least squares fitting gives the following relationships: $(V_m/K_M)_x/(V_m/K_M)_H = 1 - (0.054 \pm 0.019)x - (0.236 \pm 0.021)x^2$ for the wild-type enzyme and $(V_m/K_M)_x/(V_m/K_M)_H = 1-(0.019\pm0.042)x - (0.297 \pm 0.047)x^2$ for N115G, and $(V_m)_x/(V_m)_H = 1 - (0.763 \pm 0.033)x + (0.093 \pm 0.037)x^2$ for the wild-type and $(V_m)_x/(V_m)_H = 1 - (0.757 \pm 0.022)x + (0.115 \pm 0.024)x^2$ for N115G. Comparison of the coefficients of these expressions shows no significant difference between the wild-type and mutant enzymes for either the V_m/K_M or the V_m relationships. The proton inventories based upon these rate constants are therefore the same for both enzymes, within the precision of the present measurements.

The N115G mutation removes the major hydrogen bonding interaction in the oxyanion hole of subtilisin. This mutation reduces the reactivity of the enzyme by two orders of magnitude,[17,24] but the change in hydrogen bond strength to which this rate difference corresponds is apparently not large enough to affect the proton inventory. It is known that only hydrogens involved in very strong hydrogen bonds—the so-called "low-barrier hydrogen bonds" —have fractionation factors sufficiently different from unity to affect proton inventories;[7] evidently the interactions present in the oxyanion hole of subtilisin are not of this nature.

The presently determined absence of an oxyanion-hole influence on proton inventories suggests that the curvature found in the proton inventory for methyl chymotrypsin[10] is not due to oxyanion hole interactions either. Only a single proton transfer can occur in that system, and a linear proton inventory might consequently be expected. However, as was pointed out by one of us some time ago,[25] a linear proton inventory can be converted into a curved relationship by a sufficient number of small changes in fractionation factors in other parts of the system. These changes might be in fractionation factors of exchangeable hydrogens in the enzyme and the substrate or in fractionation factors of water molecules solvating the enzyme and the substrate. In a system as large as an enzymatic reaction, there are of course hundreds of hydrogens of this kind, and it is not unlikely that the fractionation factors of some would be altered by the conformational and other changes that occur as the system moves from initial state to transition state.

A convenient way of handling the aggregate influence of such multiple small changes is to introduce an additional "medium effect" term of the form $(\Phi)^x$ into the basic relationship of eq 1, as shown in eq 5.[15b,26] Such a medium effect does in fact accomodate the curvature of the proton inventory for the methyl chymotrypsin system very well: as Figure 5 shows the fit produced by eq 5 with one transition state

$$\frac{k_x}{k_H} = \frac{\Pi_i(1 - x + x\phi_i^{\ddagger})}{\Pi_j(1 - x + x\phi_j^{IS})}(\Phi)^x \qquad (5)$$

fractionation factor, corresponding to a single proton transfer, plus a medium effect is as good as the one originally proposed[10] with three transition state fractionation factors, corresponding to one proton transfer and two hydrogen bonding interactions. Nonlinear least squares fitting of the medium effect model gives $\phi^{\ddagger} = 0.44 \pm 0.05$ and $\Phi = 0.58 \pm 0.05$. This medium effect is a differential quantity that compares the tightness of bonding in the initial state of the reaction with that of the transition state.

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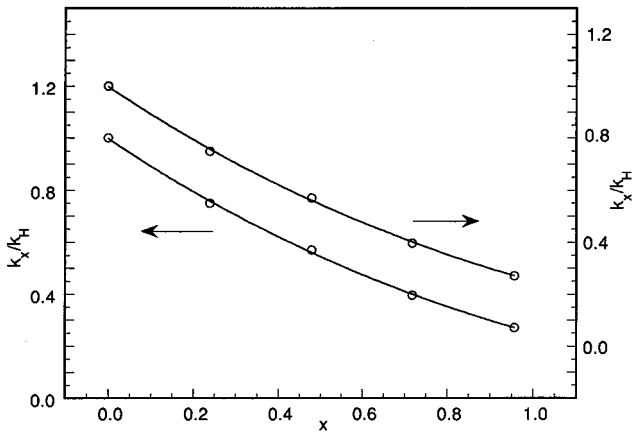

Figure 5. Proton inventories for the hydrolysis reaction of ref 10 catalyzed by methyl chymotrypsin; lower curve: one proton transfer plus two oxyanion hole interactions, upper curve: one proton transfer plus medium effect.

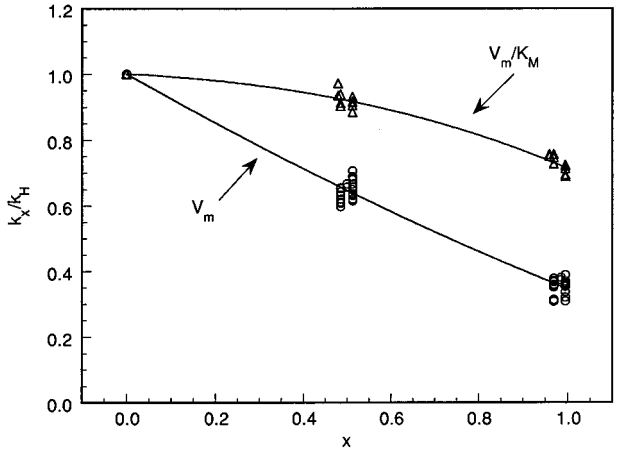

Figure 6. Proton inventories for the hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide using combined data for catalysis by subitlisin and its N155G mutant; V_m: model 3; V_m/K_M: model 6.

The fact that it is less than unity means that the aggregate bonding is loser in the transition state than in the initial state.

Subtilisin Proton Inventories. Hydrolysis reactions catalyzed by subtilisin are believed to follow the two-state enzyme acylation−deacylation mechanism common to serine proteases, with acyl-enzyme, E·S′, formation rate-determining for amide substrates, eq 6.[14] It follows then that K_M is equal to the dissociation constant of the

$$E + S \underset{K_M}{\rightleftharpoons} E\cdot S \xrightarrow[\text{r.d.}]{k_{cat}} E\cdot S' \xrightarrow[\text{fast}]{H_2O} E + P_2 + P_1 \qquad (6)$$

enzyme−substrate complex, k_{cat}/K_M is the rate constant for the reaction starting from free enzyme and substrate as the initial state, and k_{cat} is the rate constant for reaction with enzyme−substrate complex, E·S, as the initial state. This scheme requires that the two rate constants, k_{cat}/K_M and k_{cat}, refer to the same transition state, and that imposes the added restriction that proton inventories based on the analogs of these rate constants used here, V_m/K_M and V_m, must have the same transition state fractionation factors.

A further restriction on analysis of the present data by the proton inventory method is imposed by the fact that, whereas both V_m and V_m/K_M isotope effects are better represented by a quadratic than by a linear function, a cubic relationship gives no further improvement. Fitting of forms of eq 5 with more than two disposable parameters is therefore unwarranted, and that limits to two the number of independent fractionation factors that can be determined by each proton inventory. This reduces considerably the number of proton inventory models that should be examined, for there are only eight variants of eq 5 with no more than two fractionation factors.

Table 1. Models That Provide Acceptable Fits to the Combined Proton Inventory Data for Hydrolysis of Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by Subtilisin and Its N155G Mutant

model	$\phi_1^{\ddagger}$	$\phi_2^{\ddagger}$	ϕ^{IS}	Φ
		V_m:		
1	0.42 ± 0.03	0.82 ± 0.05		
2	0.41 ± 0.02		1.18 ± 0.04	
3	0.41 ± 0.02			0.84 ± 0.03
		V_m/K_M:		
4	0.48 ± 0.01	1.48 ± 0.03		
5	0.27 ± 0.02		0.38 ± 0.03	
6	0.43 ± 0.01			1.65 ± 0.04

Since wild-type subtilisin and its N155G mutant give isotope effects that are not significantly different, data obtained with the two enzymes were combined for fitting of eq 5. Each of the eight possible variants of eq 5 containing no more than two fractionation factors was examined, and three good fits were found for the V_m isotope effects and three also for the V_m/K_M data. The fractionation factors obtained with these models are listed in Table 1.

The requirement that transition state fractionation factors be the same for V_m and V_m/K_M proton inventories eliminates models 1, 4, and 5, leaving a unique solution, model 6, for V_m/K_M, but still two possibilities, models 2 and 3, for V_m. A choice between the latter two can be made, however, by using the fact that they lead to different predictions of proton inventories on $1/K_M$. Division of the expression for model 6 by that for model 2 gives the relationship shown in eq 7 for $(1/K_M)_x/(1/K_M)_H$,[27] whereas division of the expression for model 6 by that for model

$$\frac{\{(V_m/K_M)_x/(V_m/K_M)_H\}_6}{\{(V_m)_x/(V_m)_H\}_3} = \frac{(1/K_M)_x}{(1/K_M)_H} = \frac{(1-x+x\phi^{\ddagger})(\Phi_6)^x}{(1-x+x\phi^{\ddagger})/(1-x+x\phi^{IS})} = (1-x+x\Phi^{FS})(\Phi_6)^x \qquad (7)$$

3 gives the different relationship shown as eq 8. Isotope effects on $1/K_M$ were obtained from the data of Table S1[19] by dividing

$$\frac{\{(V_m/K_M)_x/(V_m/K_M)_H\}_6}{\{(V_m)_x/(V_m)_H\}_3} = \frac{(1/K_M)_x}{(1/K_M)_H} = \frac{(1-x+x\phi^{\ddagger})(\Phi_6)^x}{(1-x+x\phi^{\ddagger})(\Phi_3)^x} = \frac{(\Phi_6)^x}{(\Phi_3)^x} \qquad (8)$$

isotope effects on V_m/K_M by those on V_m, and the results were analyzed using expressions of the form of eqs 7 and 8. Model 2 requires that $\phi^{IS} = 1.18 \pm 0.04$ and $\Phi_6 = 1.65 \pm 0.04$, whereas the fit of $1/K_M$ isotope effects gave $\phi^{FS} = 1.00 \pm 0.02$ and $\Phi = 1.82 \pm 0.03$, and model 3 requires that $\Phi_6/\Phi_3 = 1.97 \pm 0.10$, whereas the fit of $1/K_M$ isotope effects gave $\Phi = 1.82 \pm 0.03$. These comparisons show that model 3 fits the experimental data better than model 2 and is consequently to be preferred. This fit is illustrated in Figure 6 together with that of model 6 for the V_m/K_M isotope effects.

The picture that emerges from these analyses is thus one of a reaction with a single low transition state fractionation factor

modified by substantial medium effects. The low transition state fractionation factor could represent a hydrogen undergoing transfer, whose isotope effect is $k_H/k_D = 1/\phi^{\ddagger} \cong 2.4$, or it could refer to a hydrogen engaged in strong hydrogen bonding. Since at least one hydrogen must be transferred in the catalytic triad mechanism of Scheme 1, the former explanation seems the more likely.[28] The medium effect of $\Phi = 0.84$ on V_m represents a small aggregate loosening of bonds to a number of hydrogens as the system progresses from the enzyme substrate complex to the transition state; these hydrogens could be in the complex itself or in its solvation shell. The larger medium effect of $\Phi = 1.65$ on V_m/K_M, on the other hand, represents a larger aggregate tightening of bonds to a number of hydrogens anywhere in the system in the process starting with free enzyme and substrate.

It is instructive to separate out from the medium effect on V_m/K_M that on the first step of this process, association of the enzyme and substrate to form the enzyme-substrate complex. This may be done by taking the ratio of medium effects on V_m/K_M and V_m, as was done above in deciding between models 2 and 3, or by fitting isotope effects on $1/K_M$ directly, whose result was also summarized above. The weighted average of the two methods gives $\Phi = 1.84 \pm 0.03$. Medium effects may now be assigned to individual parts of the acylation stage of the enzymatic reaction, as shown in eq 9.

$$E + S \underset{}{\overset{\Phi = 1.84}{\rightleftharpoons}} E\cdot S \xrightarrow{\Phi = 0.84} E\cdot S' \qquad (9)$$

The considerable tightening produced by enzyme−complex formation can thus be seen to be offset by a smaller loosening upon progress of this complex to the transition state.

It is likely that solvating water molecules make a significant contribution to the greater than unity character of the medium effect on $1/K_M$, $\Phi = 1.84$. Most substances are less soluble in D_2O than in H_2O,[15b,29] which means that the waters solvating them have $\phi < 1$. If this is true of the substrate and the active site of the enzyme in the present system, then, as the enzyme and substrate associate to form the enzyme−substrate complex, their solvating waters will be transformed into bulk solvent with $\phi = 1.00$. The change from $\phi < 1$ to $\phi = 1.00$ will then make a greater than unity contribution to Φ on the enzyme−substrate association step.

Cyclodextrin Proton Inventory. Cyclodextrins are cyclic glucopyranosides with central cavities—"active sites"—that mimic the catalytic action of some enzymes remarkably closely.[30] They operate, like enzymes, by first forming catalyst−substrate complexes, and in suitable systems association constants for such complex association can be measured without interference from any further reaction. Cyclodextrins are also much smaller than enzymes with far fewer exchangeable hydrogens (only three in each of the six glucose units of α-cyclodextrin), and they would therefore appear to be attractive, simple models that might provide further insight into proton inventories on enzyme-substrate complex formation. The complex between α-cyclodextrin and *p*-nitrophenoxide ion is among the strongest formed, and we consequently chose to investigate this system.

The overall solvent isotope effect on this association reaction proved to be rather weak, $(K_{ass})_D/(K_{ass})_H = 1.29 \pm 0.01$, which puts this system into a region where the difference between various proton inventory models is very small. The data are consequently not very diagnostic, but they nevertheless do conform to a simple medium effect expression well and give the result $\Phi = 1.29 \pm 0.01$. This medium effect is in the same direction, $\Phi > 1$, as that found for complex formation between subtilisin and its hydrolysis substrate and is consequently consistent with the explanation offered above for that effect: as complex formation occurs and solvation is replaced by interactions between cyclodextrin and *p*-nitrophenoxide ion, the more loosely bound solvating water molecules are converted to more tightly bound bulk water. The smaller magnitude of the cyclodextrin medium effect is also consistent with the result for subtilisin in that cyclodextrin and its substrate are smaller than the subtilisin system and have fewer exchangeable hydrogens and solvating water molecules. Thus, although the present solvent isotope effects on cyclodextrin-*p*-nitrophenoxide complex formation do not provide a detailed proton inventory analysis, they nevertheless do support the conclusion reached above that medium effects have a decisive influence on solvent isotope effects in the subtilisin system.

Conclusions

We have found that removing a hydrogen-bonding interaction in the oxyanion hole of subtilisin has no detectable influence on solvent isotope effects for a hydrolysis reaction catalyzed by this enzyme but that curvature in proton inventories for this system stems from medium effects. Medium effects also can account for the curvature of a proton inventory for a reaction catalyzed by methyl chymotrypsin that had previously been attributed to oxyanion hole interactions. This suggests that oxyanion hole interactions are not strong enough to influence proton inventories but that medium effects, which so far have received little attention in proton inventory studies, can play decisive roles.

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Supporting Information Available: Tables of isotope effects (4 pages). See any current masthead page for ordering and Internet access instructions.

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(27) Because the initial state for V_m is the final state for $1/K_M$, the initial state fractionation factor ϕ^{IS} in the expression for model 2 becomes the final state fractionation factor in the expression for $1/K_M$.

(28) A referee has suggestd that the transition state fractionation factor $\phi^{\ddagger} = 0.42$ might be a composite of the fractionation factor for hydron transfer from serine to histidine plus another for a hydron becoming engaged in strong hydrogen bonding between histidine and aspartate. However, such a model with two transition state fractionation factors, $\phi_1^{\ddagger}$ and $\phi_2^{\ddagger}$, produces different values of $\phi_1^{\ddagger}$ and $\phi_2^{\ddagger}$ for V_m proton inventories than for V_m/K_M proton inventories, and it therefore does not meet the criterion that transition state fractionation factors for the two kinds of proton inventory must be the same.

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