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 $\sim$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]

Acknowledgments. We are much indebted to Dr. M. W. Hunkapiller and Professor J. H. Richards for a gift of α-lytic protease and for helpful advice.

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

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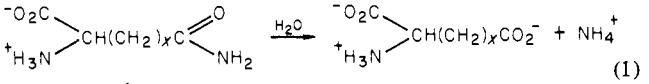
Abstract: Asparaginases, catalyzing the hydrolysis of asparagine, and glutaminase, catalyzing the hydrolysis of glutamine, exhibit modest solvent isotope effects which seem to arise from transition-state reorganization at two (or perhaps more) protonic sites. Thus, asparaginases from *Escherichia coli*, *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



1 (1)

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 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.

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

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

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

(1) This research was supported by the National Institutes of Health. Further information may be found in the doctoral theses of D. M. Quinn (1977) and K. S. Venkatasubban (1974) at the University of Kansas. M. Kise was on leave from Nihon Shinyaku Co., Kyoto.
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Table I. Proton Inventories of the Catalytic Action of Amidohydrolases on the Hydrolysis of Natural and Unnatural Substrates

enzyme, substrate, and reaction conditions[a]	atom fraction of deuterium n [V_n/V_1, partial solvent isotope effect]
1. L-asparaginase (*Escherichia coli*), 0.83 unit mL^{-1}; L-asparagine, 5×10^{-3} M; pH 7.12 and equivalent (0.10 M Tris); 37.00 ± 0.02 °C; $V_1 = 4.51 \times 10^{-6}$ M s^{-1}	0.000 [2.886, 2.867, 2.800]; 0.099 [2.619, 2.519, 2.619]; 0.198 [2.371, 2.442, 2.349]; 0.298 [2.124, 2.138]; 0.395 [1.924, 1.879, 1.902]; 0.495 [1.697, 1.750, 1.713]; 0.592 [1.565, 1.554, 1.521]; 0.699 [1.376, 1.398]; 0.795 [1.239, 1.268]; 0.896 [1.021, 1.036, 1.080]; 0.995 [0.991, 1.002, 1.006]
2. (a) L-asparaginase (*Erwinia carotovora*), 0.67 unit mL^{-1}; L-asparagine, 5×10^{-3} M; pH 7.12 and equivalent (0.10 M Tris); 37.00 ± 0.02 °C; $V_1 = 4.35 \times 10^{-6}$ M s^{-1}	0.000 [2.631, 2.619, 2.617, 2.635]; 0.099 [2.500, 2.397, 2.428]; 0.196 [2.144, 2.274]; 0.302 [2.040, 2.036]; 0.491 [1.695, 1.737]; 0.705 [1.469, 1.450]; 0.789 [1.197, 1.258, 1.177]; 0.894 [1.158, 1.105, 1.128]; 0.996 [0.993, 1.020, 1.020, 0.966]
(b) L-asparaginase (*Erwinia carotovora*), 1.8 mg mL^{-1}; L-asparagine, 7.41×10^{-3} M; pH 7.20 and equivalent (0.10 M Tris); 37.00 ± 0.02 °C; $V_1 = 1.97 \times 10^{-6}$ M s^{-1}	0.000 [2.615, 2.627, 2.636]; 0.244 [2.069, 2.103]; 0.488 [1.724, 1.707, 1.785]; 0.732 [1.279, 1.280, 1.235]; 0.975 [1.043, 0.955, 1.089]
3. L-asparaginase (*Proteus vulgaris*), 6 mg mL^{-1}; L-asparagine, 7.32×10^{-3} M; pH 7.10 and equivalent (0.05 M Tris); 37.00 ± 0.03 °C; $V_1 = 1.98 \times 10^{-6}$ M s^{-1}	0.012 [3.336, 3.268]; 0.108 [2.719, 2.854]; 0.205 [2.698, 2.657]; 0.299 [2.347, 2.326]; 0.395 [2.191, 2.202]; 0.497 [1.882, 1.944]; 0.591 [1.664, 1.674]; 0.690 [1.513, 1.491]; 0.784 [1.324, 1.321]; 0.881 [1.192, 1.196]; 0.978 [1.019, 0.995]
4. L-glutaminase (*Escherichia coli*), 0.1 mg mL^{-1}; L-glutamine, 2×10^{-2} M; pH 5.50 and equivalent (0.1 M acetate buffer); 37.0 ± 0.2 °C; $V_1 = 3.39 \times 10^{-6}$ M s^{-1}	0.000 [1.734, 1.818, 1.834]; 0.195 [1.553, 1.698, 1.566]; 0.389 [1.458, 1.345, 1.380]; 0.495 [1.382, 1.371, 1.304]; 0.596 [1.257, 1.271, 1.224]; 0.795 [1.121, 1.135, 1.152]; 0.995 [1.007, 0.987, 1.006]
5. L-asparaginase (*Erwinia carotovora*), 7.2 mg mL^{-1}; L-glutamine, 1.98×10^{-2} M; pH 7.13 and equivalent (0.10 M Tris); 37.00 ± 0.02 °C; $V_1 = 9.8 \times 10^{-7}$ M s^{-1}	0.000 [1.853, 1.958, 1.931]; 0.098 [1.744, 1.863, 1.818]; 0.244 [1.749, 1.737]; 0.390 [1.683, 1.651, 1.623]; 0.488 [1.589, 1.510, 1.467]; 0.586 [1.472, 1.433, 1.421]; 0.732 [1.289, 1.264]; 0.781 [1.199, 1.232, 1.264]; 0.980 [1.060, 1.005, 1.045]
6. L-asparaginase (*Erwinia carotovora*), 6.8 mg mL^{-1}; succinamate ion, 3.9×10^{-2} M; pH 7.34 and equivalent (0.05 M Tris); 37.00 ± 0.02 °C; $V_1 = 1.05 \times 10^{-6}$ M s^{-1}	0.000 [2.354, 2.295, 2.285, 2.263, 2.172, 2.223]; 0.125 [2.044, 1.987]; 0.238 [1.796, 1.913, 1.946, 1.878]; 0.366 [1.661, 1.589, 1.727]; 0.484 [1.598, 1.573, 1.550]; 0.610 [1.444, 1.438, 1.403]; 0.731 [1.282, 1.303, 1.372]; 0.850 [1.110, 1.109]; 0.971 [1.025, 1.013, 0.972, 1.047]
7. L-asparaginase (*Eschericha coli*), 0.48 mg mL^{-1}; L-glutamine, 1.9×10^{-2} M; pH 7.09 and equivalent (0.10 Tris); 37.00 ± 0.02 °C; $V_1 = 9.6 \times 10^{-7}$ M s^{-1}	0.000 [1.672, 1.655]; 0.103 [1.634, 1.650]; 0.279 [1.497, 1.506]; 0.375 [1.444, 1.410]; 0.467 [1.398, 1.337]; 0.562 [1.307, 1.281]; 0.751 [1.164, 1.194]; 0.843 [1.098, 1.107]; 0.939 [1.026, 1.057]

[a] The K_m values are about 10–40 μM for asparagine with asparaginases, 0.5 μM for glutamine with glutaminase, 1–6 mM for glutamine with asparaginases, and 15 mM for succinamate ion with *Erwinia* asparaginase. The enzymes were thus 95–99% saturated except in case 6, where the extent of saturation was about 70%. Velocities are therefore determined in the former cases by V_{max} to about 95–99% and by V_{max}/K_m to about 5–1% in H_2O, and in the latter case by V_{max} to about 70% and by V_{max}/K_m to about 30% in H_2O. If isotope effects on V_{max} and V_{max}/K_m are equal, then this weighting will be the same in D_2O and the transition-state information derived will be similarly weighted with respect to V_{max} and V_{max}/K_m transition states. If isotope effects on the two terms are unequal, then the balance of rate limitation will shift toward the term of smaller (normal) isotope effect in D_2O. The proton-inventory curves are altered in the direction of "bulging up"[7] in such a case (sense of curvature opposite to that for multiproton catalysis). The effect should be most serious for case 6 (70% saturation) but, as shown in Table II, this curve "bulges down". It is possible that some reduction from the true curvature for V_{max} has occurred from this source in case 6.

system. When the substrate structure is truncated so that only catalytic-site interactions, and no remote-site interactions, are possible, the function of the charge-relay system becomes unreliable. For some such substrates, the rate is linear in atom fraction deuterium (one-proton catalysis, as in model systems); for others, it is quadratic. Finally, minimal substrates like *p*-nitrophenyl acetate show simply one-proton catalysis.[9]

In this paper, we report an examination by the same method of seven cases of bacterial amidohydrolase action. The L-asparaginases of *Escherichia coli*, *Erwinia carotovora*, and *Proteus vulgaris* were studied with L-asparagine and the L-glutaminase of *Escherichia coli* with L-glutamine, all cases of natural substrates. In addition, the *Erwinia* L-asparaginase was studied with the unnatural substrates L-glutamine and succinamate ion, and the *Escherichia* L-asparaginase was examined with L-glutamine.

Results

Table I gives proton-inventory[7] data for seven examples of amidohydrolase catalysis. The results are presented as V_n/V_1 (velocity in binary mixture of protium and deuterium oxides of atom fraction deuterium n, V_n, divided by the velocity in deuterium oxide, V_1, thus, partial solvent isotope effects) with the value of V_1 given in the leftmost column so that the original data can be calculated. All rates were measured spectrophotometrically, except for those of reaction 4 (*E. coli* glutaminase with glutamine) which were determined with an ammonium-selective electrode. In the case of reactions 2a and 2b, measurements on the same system were made 23 months apart by two different workers; the strong similarity illustrates the good reproducibility of these rates.

The general precision of the measurements is sufficient to be useful, although not so good as that achieved in similar work with serine proteases.[9] We will cite the *ranges* (i.e., differences between the mean and the largest and smallest values determined, as a fraction about the mean value) of the rate determinations at a constant value of n; this measure of dispersion in the data will of course be larger than the standard deviation or other customary measures. The largest range for any set of rates at constant n was ±5% (for reaction 4 of Table I, $n = 0.195$). For the entire set of seven reactions of Table I, the average range of rates at constant n was ±1.7%. The best data are for reaction 3 (*Proteus* asparaginase with asparagine; average range at constant n, ±0.9%) and the worst are for reactions 4 (*Escherichia* glutaminase with glutamine, ±2.7%) and 6 (*Erwinia* asparaginase with succinamate ion, ±2.7%).

A good method for surveying the significance of proton-inventory experiments at a superficial level is based on the demonstration by Albery[10] that (for simple situations) the maximum dispersion of the function $V_n(n)$ from linearity is at the midpoint

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of the binary isotopic solvent mixture, $n = 0.5$. The general expression[7] for $V_n(n)$ is given by eq 3, where the ϕ_{Ti} and ϕ_{Rj} are

$$V_n(n) = V\prod_i^{\nu_T}(1 - n + n\phi_{Ti})/\prod_j^{\nu_R}(1 - n + n\phi_{Rj}) \quad (3)$$

deuterium isotopic fractionation factors for exchangeable hydrogenic sites i and j in the transition state and reactant state, respectively, and ν_T and ν_R are the numbers of such sites in the two states with nonunit fractionation factors. First we assume provisionally (see below for further discussion) that all $\phi_{Rj} = 1$ (no isotope discrimination relative to water in the reactant state), which sets the denominator of eq 3 to unity. Then we consider the three simplest cases: $\nu_T = 1$ ("one-proton catalysis"), $\nu_T = 2$ ("two-proton catalysis"), and ν_T very large ("solvation changes"; i.e., changes in the status of solvating water molecules or in the structural hydrogens of the protein, each site generating a small effect, but multiplying to give a large net effect). These generate eq 4, 5, and 6, respectively.[7,9,10] Equation 6 arises because when

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

$V_n(n)$ linear; one-proton catalysis

$$V_n(n) = V_0(1 - n + n\phi_{T1})(1 - n + n\phi_{T2}) \quad (5)$$

$V_n(n)$ quadratic; two-proton catalysis

$$V_n(n) = V_0 \exp[\nu_T(\phi_T - 1)n] \quad (6)$$

$V_n(n)$ exponential; solvation changes

a large number of sites each generates a small isotope effect, $V_n(n)$ approaches an exponential form. Of course, three-proton, four-proton, and other models could also be considered, but these are often difficult to distinguish from one another with data of ordinary precision.

Returning now to Albery's viewpoint mentioned above, we want to compare observed midpoint partial solvent isotope effects (i.e., $V_m/V_1 = V_n/V_1$ at $n_m = n \approx 0.5$) with predictions for various models from eq 4–6. For two-proton catalysis, we take the special case of $\phi_{T1} = \phi_{T2}$. Since $V_n = V_1$ for $n = 1$, we have eq 7–9 for prediction of V_m/V_1. Table II compares the observations with these predictions.

one-proton catalysis

$$\frac{V_m}{V_1} = (1 - n_m)\frac{V_0}{V_1} + n_m \quad (7)$$

two-proton catalysis

$$\frac{V_m}{V_1} = \left[(1 - n_m)\left(\frac{V_0}{V_1}\right)^{1/2} + n_m\right]^2 \quad (8)$$

solvation changes

$$\frac{V_m}{V_1} = \left[\frac{V_0}{V_1}\right]^{(1-n_m)} \quad (9)$$

The first four cases of Table II are for catalysis of the hydrolysis of natural substrates by amidohydrolases. In all these cases, V_m/V_1 falls between the predictions for two-proton catalysis and for generalized solvation changes. The result should therefore be taken as indicating *multiproton catalysis*, i.e., involvement of reorganization at two or more specific protonic sites in catalysis, and possibly some generalized reorganization of solvent or protein structural sites. With *Escherichia* asparaginase (asparagine substrate) and *Escherichia* glutaminase (glutamine substrate), it is possible that the entire solvent isotope effect arises from solvent or protein reorganization, although with the other two asparaginases, from *Erwinia* and *Proteus*, it would appear that two or several specific sites are involved.

Cases 5–7 of Table II correspond to catalytic reactions with unnatural substrates. In the two cases in which glutamine hydrolysis is catalyzed by asparaginases from *Erwinia* and *Proteus*, the data are most nearly consistent with one-proton catalysis. However, as the table shows, even the one-proton value of V_m/V_1

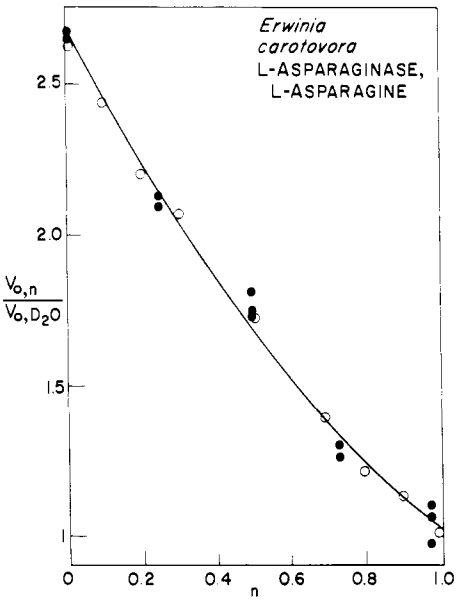


Figure 1. Partial solvent isotope effect as a function of atom fraction of deuterium n for the hydrolysis of asparagine catalyzed by *Erwinia* asparaginase (data from Table I). The open circles represent measurements made in 1973 (by K.S.V.); the filled circles are for data from 1975 (D.M.Q.).

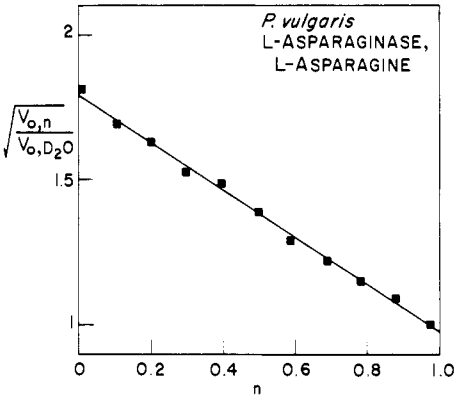


Figure 2. The square root of the partial solvent isotope effect is linear in atom fraction of deuterium n for *Proteus* asparaginase catalyzed hydrolysis of asparagine (data from Table I). This shows that V_n/V_1 is described by $(V_n/V_1) = (1.8)^2(1 - n + n/1.8)^2$.

lies *below* the observations for both of these cases, significantly so for the *Erwinia* enzyme. This betrays the "bowed upward" character indicative of an inverse-isotope-effect contribution or of a change in the rate-determining step.[7] The mechanistic implications of this feature are considered below. When succinamate ion is the substrate for *Erwinia* asparaginase, however, two-proton catalysis is again observed. It may be significant that glutamine is "longer" by one methylene group than the natural substrate asparagine, while succinamate ion is the same "length" as asparagine but merely lacks the positive ammonium function at the α-amino acid head.

Typical graphical representations of the data are given in Figures 1–4. Figure 1 shows the curved, multiproton plot for *Erwinia* asparaginase with asparagine. Figure 2 presents similar data for the *Proteus* enzyme, here plotted as $(V_n/V_1)^{1/2}$, showing by the linearity of the result that the data are consistent with two-proton catalysis with each proton contributing an isotope effect of about 1.8 (left-hand intercept). A corresponding demonstration of two-proton catalysis, with $k_H/k_D \approx 1.5$ for each proton, appears in Figure 3 for *Erwinia* asparaginase with succinamate ion. Figure 4 shows how a linear proton-inventory plot for the *Erwinia* asparaginase–glutamine system can be generated by one possible model for the inverse-isotope-effect contribution, namely a transition-state fractionation factor of 1.25. The linearity of the plots

Table II. Comparison of Observed Midpoint Partial Solvent Isotope Effects with Predictions for Various Mechanistic Models

enzyme and substrate[a] (av range of data, ppt); overall effect, V_0/V_1	obsd V_m/V_1 (n_m)	predicted V_m/V_1 (deviation from obsd, ppt)		
		one-proton catalysis	two-proton catalysis	solvation changes
1. *Escherichia* asparaginase, asparagine (±14); 2.93	1.720 (0.495)	1.935 (+125)	1.816 (+56)	1.697 (−13)
2. *Erwinia* asparaginase, asparagine (±17); 2.62	1.716 (0.491)	1.828 (+65)	1.731 (+9)	1.635 (−47)
3. *Proteus* asparaginase, asparagine (±9); 3.31	1.913 (0.497)	2.158 (+128)	1.990 (+40)	1.824 (−47)
4. *Escherichia* glutaminase, glutamine (±27); 1.80	1.352 (0.495)	1.401 (+36)	1.373 (+16)	1.343 (−7)
5. *Erwinia* asparaginase, glutamine (±23); 1.90	1.522 (0.488)	1.468 (−35)	1.431 (−60)	1.394 (−84)
6. *Erwinia* asparaginase, succinamic acid (±27); 2.27	1.574 (0.484)	1.652 (+50)	1.589 (+10)	1.525 (−31)
7. *Escherichia* asparaginase, glutamine (±10); 1.69	1.368 (0.467)	1.354 (−10)	1.332 (−26)	1.312 (−41)

[a] The numbering scheme corresponds to that of Table I.

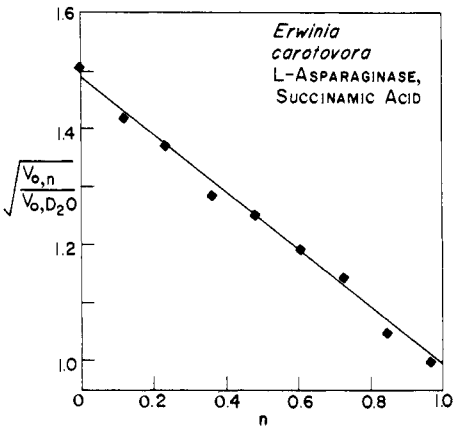


Figure 3. A demonstration, similar to Figure 2, of two-proton catalysis in succinamate ion hydrolysis catalyzed by *Erwinia* asparaginase (data from Table I). The linearity of the plot shows that the proton inventory is described by $(V_n/V_1) = (1.5)^2(1 - n + n/1.5)^2$.

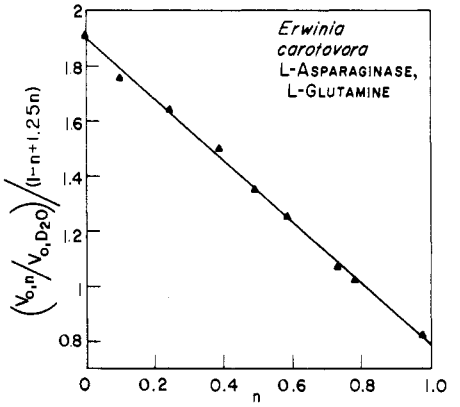


Figure 4. One model which describes the proton-inventory data (Table I) for glutamine hydrolysis catalyzed by *Erwinia* asparaginase. The function which generates the linear fit is $(V_n/V_1) = (1.9)(1 - n + 1.25n)(1 - n + n/2.4)$.

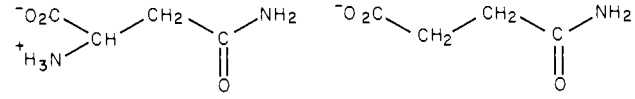
of $(V_n/V_1)^{1/2}$ for the cases shown demonstrate that, although the midpoint partial solvent isotope effect may not exactly correspond to the two-proton expectation, an adequate fit of the entire data set is possible on such a model.

Discussion

When any of the amidohydrolases studied here were presented with the substrate for which their catalytic power has been developed in the course of biological evolution—asparagine for asparaginases; glutamine for the glutaminase—then a proton inventory consistent with *multiproton catalysis* was observed. All of these examples (cases 1–4 of Tables I and II) produced data which could be fit to within the limits of experimental error by the two-proton function of eq 5, with $\phi_{T1} = \phi_{T2}$ (equal isotope effects arising from the two transition-state protonic sites). The best fit values of ϕ_T^{-1} (the isotope effect k_H/k_D for each one of the two transition-state sites) were as follows: 1.75 ± 0.16 (*Escherichia* asparaginase), 1.62 ± 0.08 (*Erwinia* asparaginase), 1.82 ± 0.03 (*Proteus* asparaginase), 1.32 ± 0.07 (*Escherichia* glutaminase). Although these fits are satisfactory, they are not unique. Other models could be fit to the data, incorporating, for example, a third protonic site with correspondingly smaller isotope effects, or, as another example, some contribution of solvent or protein-structural sites in the direction of either a normal or inverse isotope effect. Thus, while the general conclusion of multiproton catalysis seems secure, a deduction of two-proton catalysis is speculative. If it is correct, the contribution of each site seems to be around $k_H/k_D \approx 1.3$–1.8.

Isotope effects in the range of 1.3–1.8 per proton are consistent with contributions of protonic sites participating in acid–base catalysis according to the "solvation catalysis" model, as studied in nonenzymatic systems,[7] although their magnitude is definitely at the lower end of the range expected. Simultaneous participation of two protonic sites in catalysis of the hydrolysis of near-natural substrates has been observed for the serine proteases.[8,9] The observations reported here are consistent with the presence in amidohydrolases of a "charge-relay system", a chain of hydrogen bonds forming a potential multiproton catalytic entity, such as that observed crystallographically for serine proteases.[6] Its efficacious function with the evolutionarily selected structure as substrate is consistent here, as with the serine proteases,[9] with the concept that a transition-state set of specific attractive interactions between substrate and enzyme groups produces a compression of the distances across the component hydrogen bonds of the chain. This is then expected, on theoretical grounds, to favor coupled reorganization at the various protonic sites.[11,12]

When the natural substrate structure is truncated, as in the omission of the ammonio group of asparagine to make succinamate ion, many of the important transition-state interactions leading to compression of hydrogen-bond chains and coupled reorganization of protonic sites should still be present. As structures **2** (asparagine) and **3** (succinamate) emphasize, succinamate should



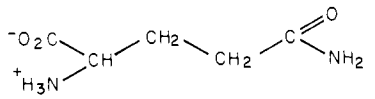
2 3

(11) Elrod, J. P.; Gandour, R. D.; Hogg, J. L.; Kise, M.; Maggiora, G. M.; Schowen, R. L.; Venkatasubban, K. S. *Symp. Faraday Soc.* **1975**, *10*, 145–153.

(12) Gandour, R. D.; Maggiora, G. M.; Schowen, R. L. *J. Am. Chem. Soc.* **1974**, *96*, 6967–6979.

readily fit into an evolutionarily determined "pocket" for asparagine in the enzymatic transition state. The absence of the ammonio group should lead, however, to loss of some of the compressive force that the substrate can exert on the enzyme framework. As can be seen by a comparison of cases 2 and 6 of Tables I and II, only a small change in protonic reorganization is occasioned by truncation of the substrate structure. Asparagine as substrate produces apparent two-proton catalysis with each site contributing an isotope effect of 1.62 ± 0.08. Succinamate produces apparent two-proton catalysis, with the best-fit isotope effect per protonic site being 1.51 ± 0.02.

On the other hand, if an asparaginase is presented with a substrate such as glutamine (**4**) which is geometrically ill-con-



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ditioned for a fit to the transition-state "pocket" for asparagine, a disruption of the catalytic entity should be anticipated. Indeed, this is observed. In case 7 of the tables, *Escherichia* asparaginase exhibits apparent one-proton catalysis with glutamine, the isotope effect of 1.69 ± 0.03 being similar in magnitude to the contribution from one of the two sites active with asparagine as substrate (1.75 ± 0.16). In case 5, *Erwinia* asparaginase with glutamine presents a more complex situation. The plot of $V_n(n)$ is now "bowed upward".[7]

Two simple models which describe such a circumstance are represented by eq 10 and 11. According to eq 10, an inverse-

$$V_n(n)/V_1 = (1.90 \pm 0.06)(1 - n + n/[2.39 \pm 0.07]) \times (1 - n + n[1.25 \pm 0.02]) \quad (10)$$

$$V_n(n)/V_1 = (1.90 \pm 0.06) \times (1 - n + n/[2.39 \pm 0.07])/(1 - n + n[0.80 \pm 0.01]) \quad (11)$$

isotope-effect contribution is imagined from a transition-state site with a fractionation factor of 1.25. This is exactly what is expected for an OH group of a tetrahedral carbonyl adduct.[7] At the same time, another protonic site produces a normal effect of 2.39: this is just what should arise from one-proton acid–base catalysis as observed in nonenzymatic reactions.[7] These data are thus consistent with the idea that the ill-adapted substrate glutamine leads to exposure of a transition state in which proton donation to, or proton abstraction from, a tetrahedral adduct with an intact OH group is occurring, with the enzymatic catalytic group acting as a simple one-proton reagent as in nonenzymatic reactions. Equation 11 is equally consistent with the data. Here a reactant state site with a fractionation factor of 0.80 is envisioned, for which some evidence exists in serine-protease chemistry.[9] Again, the transition-state acid–base catalysis arises from a single site. The two models cannot be distinguished at these levels of precision. Figure 4 shows, in effect, the agreement of both with the data. Furthermore, the contribution of 1.25 or 0.80 could readily be subdivided into smaller contributions, which could be ascribed to reorganization at solvent or protein-structural sites. Plots of this kind are also consistent[7] with shifts in the rate-determining step, a circumstance which could also be reconciled quantitatively with the data at this point.

Conclusions

Amidohydrolases appear to be equipped by molecular evolution with a multiproton catalytic entity (possibly a charge-relay chain), the full function of which is called up by their natural substrate structures (asparagine for asparaginases, glutamine for glutaminases) or by related structures capable of an accurate fit to the enzymatic transition-state structure (succinamate with asparaginase). When the capacity for an intimate transition-state fit is removed (glutamine with asparaginases), the function of the enzymatic acid-base catalytic entity deteriorates; the mechanistic character becomes variable and resembles the one-proton catalysis seen in nonenzymatic model systems.

Experimental Section

Materials. Protium oxide was distilled and deionized (Barnstead mixed-bed ion-exchange column, Ultrapure, Sybron Corp.). Deuterium oxide (Stohler, "99.7%" or "99.8%") was fractionally distilled through a short Vigreux column and analyzed for deuterium content by NMR with acetonitrile as internal standard. Typical atom fractions of deuterium found were 0.9971 ± 0.0002 and 0.9977 ± 0.0003. Sometimes deuterium oxide was employed without distillation with no difference in properties ever observable. Mixtures of isotopic waters were prepared gravimetrically. Tris buffer components (Sigma) were desiccated. L-Asparagine monohydrate and L-glutamine (both from Sigma) were recrystallized and dried over phosphorus pentoxide. Succinamic acid (Pfaltz and Bauer) was recrystallized once from water and once from acetone and dried in vacuo over phosphorus pentoxide.

Enzymes were either L-asparaginase (EC 3.5.1.1) or L-glutaminase (EC 3.5.1.2). *Escherichia* glutaminase was obtained from Sigma. *Escherichia* asparaginase was from Sigma or Biochemical Laboratories. *Erwinia* asparaginase was obtained as a solid pellet from Dr. H. E. Wade. *Proteus* asparaginase was isolated from *Proteus vulgaris* and purified by established procedures.[13] Ammonium sulfate was removed by repeated dialysis at 5 °C against distilled, deionized water.

Kinetics. Some measurements were made by using an ammonium-sensitive electrode (Beckman 39137) vs. calomel electrode to follow the rate of ammonium ion production with a pH meter. The output signal was digitized and stored in a Hewlett-Packard 2100A computer. Calibration experiments showed that the Nernst equation was obeyed in all mixtures of protium and deuterium oxides between 10^{-4} and 10^{-1} M ammonium ion. A constant charge of 10^{-3} M ammonium ion was employed to ensure linearity of response, and good zero-order kinetics were obtained during the initial 10% of the reaction. In a typical experiment, 10–15 mL of reaction solution (except for enzyme) was thermally equilibrated for 15 min, and electrodes were then inserted for a 10-min equilibration time. Reaction was initiated by micropipet injection of 50 or 100 μL of enzyme stock solution.

Most measurements were made spectrophotometrically, with the photomultiplier output of a Cary 16 spectrophotometer being digitized and the resulting values converted to absorbance in a Hewlett-Packard 2100A computer in the manner previously described.[14] In a typical experiment, a cuvette containing 1 or 2 mL of reaction solution (except for enzyme) was thermally equilibrated, with temperature being digitally monitored by a glass-covered thermistor. Reaction was initiated by injection of 25 μL of enzyme stock solution. Absorbance was monitored at 233 (asparagine), 229 or 232 (glutamine), or 231.5 (succinamate ion) nm. Rates were calculated by linear least-squares fits of the zero-order data.

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