Mechanisms of NH Proton Exchange in Amides and Proteins: Solvent Effects and Solvent Accessibility

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Abstract: Site-to-site rate constants have been measured for acid-catalyzed proton exchange of *N*-methylformamide and four primary amides in a series of solvents of varying polarity. The method involves NMR saturation-transfer measurements, sometimes combined with line-shape analysis. According to the rate ratio k_{ZE}/k_{ZS}, the mix of exchange mechanisms in primary amides does not change significantly with a moderate decrease in solvent polarity. For secondary amides there is a substantial changeover from the N-protonation mechanism to the imidic acid mechanism. This changeover is also seen for acetamide when the solvent is changed to 90% aqueous tetrahydrofuran. The solvent effect on mechanism is rationalized in terms of p*K*s of model compounds. The implications of these results for proton exchange in proteins are discussed. In particular, it is concluded that the NH protons of peptide backbones, both solvent exposed and buried, exchange >99% via the imidic acid. The accessibility requirements of the imidic acid mechanism then support the local-unfolding model for exchange of buried protons.

Proton exchange in amides has long been of wide interest,[1] especially since proton-exchange kinetics of amides, peptides, and proteins can provide information about the structure of peptides and proteins in solution and about the accessibility of slowly exchanging protons buried in the interior of proteins.[2] The exchange reaction is observed to be both base and acid catalyzed. There is no problem with the mechanism of the base-catalyzed reaction. It occurs simply by removing the NH proton and replacing it by another one from solvent (eq 1). Two mechanisms

$$\mathrm{RCONHR' + OH^- \rightleftharpoons RC(\!-\!O^-)\!=\!NR' + H_2O} \quad (1)$$

have been proposed for the reaction. One, long accepted, is the N-protonation mechanism (eq 2), which is related to the base-

$$\mathrm{RCONHR' + H^+ \rightleftharpoons RCONH_2{}^+R'} \quad (2)$$

catalyzed reaction in that it only reverses the order of proton addition and removal. The other is the imidic acid mechanism (eq 3), which is more circuitous but more attractive on chemical grounds.

$$\mathrm{RCONHR' + H^+ \rightleftharpoons RC(OH)\!=\!NHR'^+ \rightleftharpoons RC(OH)\!=\!NR' + H^+} \quad (3)$$

Since this exchange reaction continues to be used to probe peptide and protein structure,[3] protein dynamics,[4] and protein interactions,[5] it is important[6] to answer the mechanistic question. Various attempts have been made to do so,[7] including our own. We have concluded[8,9] that exchange in "ordinary" primary amides, $RCONH_2$, occurs predominantly via N-protonation (eq 2), but with the novel features[8b,c] that the intermediate $RCONH_3{}^+$ is so strong an acid that its lifetime is too short—ca. 10^{-11} s—to permit rotational equilibration about its C–N single bond. Subsequently, we have used saturation-transfer techniques to obtain evidence[9] that primary amides with electron-withdrawing substituents exchange at least partly via the imidic acid (eq 3). A similar changeover in mechanism is seen with *N*-methylamides[10] and with some formamides,[11] and the changeover can be understood in terms of transition-state structures. On the basis of such studies we have concluded[10,11] that the NH protons of peptide or protein backbones exchange predominatly by the imidic acid mechanism (eq 3). In contrast, Krishna et al.[7e] have observed that the primary amide side chains of glutamyl and asparagyl residues of proteins exchange via N-protonation, in agreement with our studies on simple primary amides.

All these studies have been in aqueous solution or other solvents of high polarity, such as ethylene glycol. The interior of a protein is often a less polar environment, and the mechanism may change, especially in view of a balancing so close that different mechanisms are operative for NH and NH_2 groups of a protein. We therefore have undertaken a study of the effect of solvent polarity on the mechanisms of acid-catalyzed exchange for both primary and secondary amides, and we here report that solvent effects do not upset the previous conclusions.

To distinguish these mechanisms unambiguously, it is necessary to compare rates of intermolecular proton exchange with rates of either intramolecular exchange in primary amides or E/Z isomerization in secondary amides. For primary amides (**1**) it

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Table I. Saturation-Transfer Data for Acid-Catalyzed Exchange of Primary Amides[a]

amide	$t_E(Z)$	$t_Z(E)$	$t_S(E)$	$t_S(Z)$	$t_E(S)$	$t_Z(S)$	$M_E(S)$, s^{-1}	$M_Z(S)$, s^{-1}
acetamide	0.502 ± 0.014	0.494 ± 0.015	0.264 ± 0.006	0.243 ± 0.004	0.703 ± 0.014	0.648 ± 0.013	4.44 ± 0.05	5.14 ± 0.14
acrylamide	0.624 ± 0.007	0.690 ± 0.005	0.463 ± 0.003	0.403 ± 0.004	0.680 ± 0.007	0.656 ± 0.003	6.84 ± 0.31	6.06 ± 0.22
cyanoacetamide	0.318 ± 0.010	0.294 ± 0.018	0.031 ± 0.007	0.024 ± 0.006	0.264 ± 0.010	0.252 ± 0.013	2.84 ± 0.02	2.61 ± 0.05
malonamide[b]	0.131 ± 0.026	0.161 ± 0.016	0.011 ± 0.015	0.004 ± 0.017	0.219 ± 0.005	0.179 ± 0.015	3.42 ± 0.05	3.27 ± 0.06

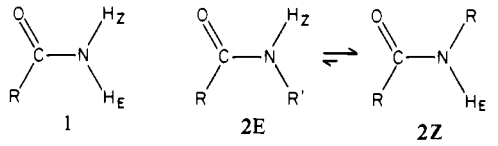
[a] Unless otherwise specified, the solvent was 1:1 (v/v) cyclohexanol–dioxane. [b] 1:1 (v/v) ethylene glycol–dioxane.

Table II. Site-to-Site Rate Constants, in s^{-1}, for Acid-Catalyzed Proton Exchange of Amides in Less Polar Solvents

amide	solvent[a]	k_{EZ}	k_{ZE}	k_{ZS}	k_{ES}
acetamide	A	2.2 ± 0.3	2.7 ± 0.3	3.1 ± 0.2	3.3 ± 0.2
acetamide-^{15}N	B	2 ± 1	2 ± 1	24 ± 1	41 ± 2
acrylamide	A	3.6 ± 0.5	5.3 ± 0.5	3.8 ± 0.2	4.8 ± 0.2
cyanoacetamide	A	0.45 ± 0.09	0.24 ± 0.11	0.64 ± 0.06	0.77 ± 0.05
malonamide	C	0.3 ± 0.1[b]	0.4 ± 0.1[b]	0.56 ± 0.06[b]	0.77 ± 0.04[b]
N-methylformamide	B		0.1	6.7 ± 0.8	3.3 ± 0.6
	D		0.4 ± 0.04	9.3 ± 1.2	5.5 ± 0.8

[a] A = 1:1 cyclohexanol–dioxane, B = 90% aqueous THF, C = 1:1 ethylene glycol–dioxane, D = cyclohexanol. [b] Per NH.

can be shown[8c,9b] that the N-protonation mechanism requires $k_{ZE} = k_{ZS}$, where k_{ij} is the rate constant for proton exchange from site i to site j (i, j = *E* or *Z* or Solvent). In contrast, there is no intramolecular exchange in the imidic acid mechanism, so $k_{ZE} = 0$. For secondary amides (**2E** ⇌ **2Z**) the kinetics become more complicated,[11] but the mechanisms can still be distinguished by comparing k_{ZS} (of the *E* amide) with k_{ZE} (for isomerization of the *E* isomer to the *Z*).



1 2E 2Z

There are some severe constraints on choosing suitable systems. Secondary amides, RCONHR′, are restricted to formamides (R = H), since with larger R there is too little of the isomer **2E**. Moreover, only those amides where the two mechanisms are closely balanced in polar solvents are suitable to test for a change of mechanism. Consequently, potential substrates are restricted to primary amides with weakly electron-withdrawing substituents and to simple *N*-alkylformamides. The site-to-site rate constants can be measured by NMR saturation-transfer techniques,[12] perhaps combined with line-shape analysis.[11] Ordinarily the NMR signals of NH protons of amides are excessively broadened by quadrupolar relaxation, so it is most convenient to use viscous solvents, such as ethylene glycol or cyclohexanol, to sharpen them. In order to cover a wide range of solvent polarity, 90% aqueous tetrahydrofuran (THF) and mixtures including dioxane have also been utilized. Quantitative measures of solvent polarity are not necessary, and it suffices to conclude from such measures[13] that solvent polarity decreases in the order water > ethylene glycol ≫ 1:1 glycol–dioxane ~ cyclohexanol > 1:1 cyclohexanol–dioxane > 90% aqueous THF.

Experimental Section

Materials. Amides, reagent-grade solvents, acids, and buffer components were commercial samples from Eastman Kodak, Mallinckrodt, Aldrich, or Matheson Coleman and Bell, used without further purification. Acetamide-^{15}N was obtained from Stohler Isotope Chemicals. Cyclohexanol was redistilled from CaO. Solvent mixtures were prepared by volume/volume dilution, with a weighed proportion of amide to produce a solution ca. 1 M in amide. The acidity of the solution was adjusted by adding small amounts of base or acid so as to produce either nonexchange conditions or acid-catalyzed exchange at a rate readily measureable by NMR techniques.

Instrumentation. FT-NMR spectra were obtained with a Varian HR220 spectrometer adapted for FT or with a Nicolet 1180E computer interfaced to an Oxford 360-MHz magnet. Probe temperature was 22–23 °C. The downfield NH or NMe was assigned as *E*.[14] Saturation-transfer experiments utilized strong irradition at a specified frequency during the delay part of the pulse sequence. To improve precision, each spectrum represented 4 to 20 acquisitions. An initial acquisition was always discarded, to ensure establishment of saturation. Spillover of the strong saturating irradiation, from one NH peak to the other, was accounted for by comparing the intensity of an NH peak to its intensity acquired with (off-resonance) irradiation at the same frequency difference from that NH peak but in the opposite direction. Apparent spin–lattice relaxation rate constants were determined by the 180°–*τ*–90° inversion–recovery method, under conditions of selective saturation. Since the saturating irradiation can create *xy* magnetization, this was rejected with a 90° observation pulse. Owing to the high sensitivity of a 90° pulse and the high concentration of solvent, it was also necessary to attenuate the signal.

Kinetic Methods. Rate constants for *N*-methylformamide were obtained by a combination of line-shape analysis, for intermolecular proton exchange, and saturation transfer, for *E*/*Z* isomerization, as described previously.[11] Site-to-site rate constants for primary amides were obtained by a series of saturation-transfer measurements (eq 4 and 5, and equivalently for k_{ZS} and k_{ZE}.[12d,14] The experimentally determined quantities

$$k_{ES} = M_E(S)\,\frac{t_E(S) - t_E(Z)t_Z(S)}{1 - t_E(Z) + t_S(Z)[t_E(S) - t_Z(S)]} \tag{4}$$

$$k_{EZ} = M_E(S)\frac{t_E(Z) - t_E(S)t_S(Z)}{1 - t_E(Z) + t_S(Z)[t_E(S) - t_Z(S)]} \tag{5}$$

were six values of saturation transfer, the fractional loss of intensity at site i, I_i, on saturating site j (eq 6), $M_i(S) = 1/T_{1i}^{app}$, measured under

$$t_i(j) = \frac{I_i^0 - I_i(j)}{I_i^0} \tag{6}$$

conditions of selective saturation of solvent and evaluated by weighted linear least squares from the time dependence of H_E or H_Z intensity following a 180° pulse. For acetamide-^{15}N in aqueous THF signal overload prevented measurement of $t_E(Z)$ or $t_Z(E)$, but measurement of $t_S(E,Z)$ and $M_S(E,Z)$ under conditions of saturating both H_E and H_Z provided a value for the sum $k_{ES} + k_{ZS}$, and the sums $k_{ES} + k_{EZ}$ and $k_{ZS} + k_{ZE}$ could be obtained from line broadening. Intensities were simply peak heights, rather than integrals. Additional line broadening, ca. 2 Hz, was introduced before Fourier transformation in order to reduce the variability in peak heights due to digital resolution and changes in homogeneity. All saturation-transfer values are averages of at least three separate determinations, and the standard deviations in derived quantities were calculated by propagation of errors. Further details are avialable.[15]

Results

Saturation-transfer data for primary amides in some less polar solvent mixtures are tabulated in Table I. Pseudo-first-order rate

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Table III. Solvent Effect on Rate Ratio k_{ZE}/k_{ZS} in Acid-Catalyzed Proton Exchange of Amides

amide	ethylene glycol	1:1 cyclohexanol-dioxane	other
acetamide	0.91 ± 0.17[a]	0.79 ± 0.14	0.1 ± 0.05[b]
acrylamide	0.80 ± 0.21[a]	1.17 ± 0.11	
cyanoacetamide	0.17 ± 0.04[a]	0.54 ± 0.23	
malonamide	0.40 ± 0.05[a]		0.61 ± 0.21[c]
N-methylformamide	0.2,[d] 0.2[d,e]	0.043 ± 0.007[f]	<0.01[b]

[a] Reference 9b. [b] 90% aqueous THF. [c] 1:1 ethylene glycol–dioxane. [d] Reference 11. [e] Water. [f] Cyclohexanol.

constants for these amides, as well as for *N*-methylformamide, are collected in Table II. Values for k_{EZ} and k_{ZE} are corrected for uncatalyzed rotation about the C–N bond, determined independently. In all cases, the absolute rate constants are not of significance, since they were set to a convenient range by adjusting the acidity. It is the relative site-to-site rate constants in a given solvent mixture that are of interest. In particular, the ratio k_{ZE}/k_{ZS} is diagnostic for mechanism, and values of this ratio are compiled in Table III. For primary amides this ratio was computed as $^1/_2(k_{ZE} + k_{EZ})/k_{ZS}$, so as to utilize the additional determination.

The data in Table I show the high precision attainable, especially in intensity measurements. As a result, relative rates are quite reliable, more so than absolute rate constants determined from the time dependence of magnetization, which are known to be subject to error.[16] The data in Table II provide another measure of reliability. By detailed balance k_{EZ} must equal k_{ZE} for primary amides. These values, determined independently, are not always equal, within experimental error, but the discrepancy is not so large as to force rejection of the error estimates. Similarly, the value of k_{ZE}/k_{ZS} for acrylamide in cyclohexanol–dioxane is greater than 1, which is impossible by any mechanism, but the discrepancy suggests that our error estimates ought only to be doubled.

Discussion

Solvent Effect on Mechanism. The values of k_{ZE}/k_{ZS} in Table III are diagnostic for possible changes of mechanism with solvent. For primary amides there is no significant change in k_{ZE}/k_{ZS} on changing from ethylene glycol to 1:1 glycol–dioxane or 1:1 cyclohexanol–dioxane. There seems to be no major change of mechanism due to this moderate reduction of solvent polarity. Amides such as cyanoacetamide or malonamide, with weakly electron-withdrawing substituents, still exchange by a combination of N-protonation and imidic acid mechanisms. Ordinary amides such as acetamide or acrylamide still exchange predominantly by the N-protonation mechanism. By analogy, the NH protons of the amide side chains of glutamyl and asparagyl residues of proteins also exchange predominantly by the N-protonation mechanism, even in moderately nonpolar media, just as they do in aqueous solution.[7e]

It should also be noted in Table II that k_{ES} for acrylamide is significantly greater than k_{ZS}, just as in ethylene glycol.[9b] This is the evidence that the N-protonated intermediate, $RCONH_3^+$, is so strong an acid that its lifetime is too short to permit rotational equilibration about its C–N single bond.[8b,c] Of course, it is unlikely that solvent polarity would affect this phenomenon.

In contrast, k_{ZE}/k_{ZS} for *N*-methylformamide decreases significantly on reducing the solvent polarity from water or ethylene glycol through cyclohexanol to 90% aqueous THF. This conclusion is quite apparent from qualitative inspection of NMR spectra. In 90% THF, at higher acidities than those designed to measure the rate constants, the two *N*-methyl doublets coalesce to separate singlets without yet coalescing with each other. Also, the formyl CH doublets of *N*-*tert*-butylformamide show this same behavior in both cyclohexanol and 90% THF, although it was not possible to analyze the spectra for rate constants. It is mathematically possible to fit such data to the N-protonation mechanism,[11] but that would require unreasonable constraints on the conformational equilibria in $HCONH_2^+R'$ and on the competition between its deprotonation and rotation about its C–N bond. Certainly the simplest interpretation of the sharp reduction in k_{ZE}/k_{ZS} is that the imidic acid mechanism becomes increasingly important as solvent polarity decreases, until it is the exclusive mechanism in 90% THF. This interpretation is supported by the behavior of acetamide, which also shows a decreased k_{ZE}/k_{ZS} in 90% THF. It is impossible to fit so small a ratio to the N-protonation mechanism, and therefore the imidic acid mechanism has again become dominant in this nonpolar medium, even for this primary amide.

In summary, there is a changeover toward the imidic acid mechanism as solvent polarity decreases. This changeover is more pronounced for secondary amides than for primary. The implication for NH protons of protein backbones is quite clear. Those protons are in secondary amides, RCONHR′, with electron-withdrawing substituents in both R and R′. Even in aqueous solution these undergo acid-catalyzed exchange predominantly (>99%) by the imidic acid mechanism.[10,11] In less polar media, as in the interior of proteins, this dominance becomes still stronger, and the N-protonation mechanism becomes entirely insignificant.

These solvent effects may be rationalized by reference to solvent effects on p*K*s of model compounds. According to eq 8 of ref 9b, the ratio of exchange rates v_I and v_N, via imidic acid and N-protonation pathways is given by eq 7, where k_i and k_d are rate

$$\frac{v_I}{v_N} = \frac{k_i}{k_d}\frac{K_a^N K_a^I}{K_a^O} \qquad (7)$$

constants for diffusion-controlled proton transfers and K_a^N, K_a^I, and K_a^O are acidity constants of $RCONH_2^+R'$, $RC(OH)=NHR'^+$, and $RC(O\mathit{H})=NHR'^+$, respectively. The ratio K_a^I/K_a^O is simply the equilibrium constant for tautomerization of amide to imidic acid, both of which are uncharged, so that this ratio ought to be nearly independent of solvent polarity. Likewise, k_i and k_d are determined by diffusion rates and ought not depend on solvent polarity. Thus the ratio v_I/v_N is approximately proportional to K_a^N. Such an acidity constant is not directly measurable, because $RCONH_2^+R'$ is not stable. However, it does resemble an anilinium ion, $PhNH_2^+R'$, and we may use acidities of anilinium ions to model the solvent dependence of K_a^N or v_I/v_N. The pK_as of $PhNH_3^+$ and $PhNH_2^+Me$ decrease steadily from 4.60 and 4.80, respectively, in water to 3.53 and 3.29, respectively, in 82% aqueous dioxane.[17] Not only does the pK_a decrease as solvent polarity decreases but also the solvent effect is larger for the secondary amine than for the primary. Similar behavior is seen in aqueous mixtures of acetone, methoxyethanol, or dimethoxyethane, and also for methylamine and dimethylamine in aqueous ethanol.[18] By analogy then, K_a^N increases with decreasing solvent polarity, and so does v_I/v_N (eq 7). Thus the imidic acid mechanism, especially for secondary amides, RCONHR′, becomes more important in less polar media.

Solvent Accessibility to the Interior of Proteins. These results support the interpretation that the mechanism for accessibility of solvent to buried protons is via a local unfolding, rather than a solvent penetration that requires only small-amplitude protein motions. This has been a subject of vigorous controversy, and evidence has been presented for both models, but it has not yet been possible to distinguish between them.[2h–j,4c,19] In the past, details of the exchange mechanism did not enter into the question of accessibility, inasmuch as the accepted N-protonation mechanism and the base-catalyzed reaction have very similar accessibility requirements. Figure 1 illustrates these requirements, including the internal hydrogen bonds between the amide fragment

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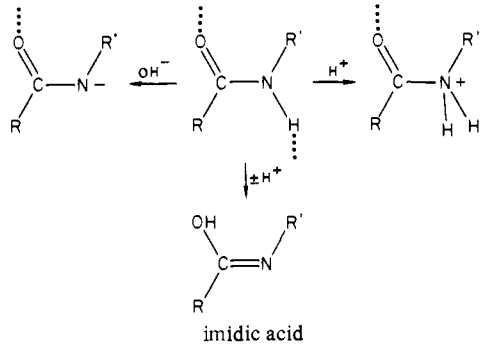

Figure 1. Comparison of accessibility requirements of base-catalyzed, N-protonation, and imidic acid mechanisms for NH exchange in the interior of proteins. The dotted lines represent internal hydrogen bonds that may need to be broken to permit the exchange.

and other regions of the protein. In exchanging an NH proton, by whatever mechanism, its hydrogen bond must be broken and the NH exposed to solvent. If exposure of NH alone is sufficient, the hydrogen bond to oxygen remains intact and small-amplitude motion might suffice.

The accessibility requirements for the imidic acid mechanism are more demanding. Not only must the NH be exposed to solvent but so must the oxygen which is to accept a proton from solvent. As Figure 1 shows, the imidic acid has the wrong donor–acceptor properties to form the hydrogen bonds that the amide formed within the protein. Both the nitrogen and the oxygen are dislodged from their original environment. Although small-amplitude motion might suffice to permit solvent to penetrate to the NH alone, the requisite exposure of the entire amide fragment would seem to demand more extensive motion, such as a partial unfolding.

Might the mechanism revert to N-protonation for those protons buried in the interior of proteins? Then the solvent would need access only to the NH, and the solvent-penetration model could still apply. If so, though, the strong inherent preference for the imidic acid mechanism would retard the acid-catalyzed reaction, relative to model compounds,[20] which do exchange via the imidic acid mechanism.[10,11] No such additional retardation would be operative on the base-catalyzed reaction. As a result, pH_{min}, the pH at which the exchange rate is minimum, would decrease substantially. This is opposite to what is observed at low temperatures for the slowly exchanging protons of bovine pancreative trypsin inhibitor,[21] where pH_{min} is ca. 1 unit greater than pH_{min} for model compounds. Although other proteins might show an increased pH_{min} for buried protons, which would be suggestive of the N-protonation mechanism, we conclude that the imidic acid mechanism is operative even for buried protons.

Might the base-catalyzed reaction occur via solvent penetration but the acid-catalyzed reaction via local unfolding, as required by the greater accessibility demands of the imidic acid mechanism? Both reactions would be retarded, relative to model compounds, but the acid-catalyzed reaction would be subject to a greater retardation, since it would require more extensive protein motion. Again, though, there would result a decrease in pH_{min}, contrary to one observation.[21] We therefore conclude that both reactions involve a partial unfolding, whereby the entire amide group can be exposed to solvent, even though such accessibility is not necessary for the base-catalyzed reaction.

The imidic acid mechanism also rationalizes this increase of pH_{min}. This increase has generally been attributed to a reduced solvent polarity in the protein interior, although variants have also been proposed.[19b,21b,22] Such a solvent effect is well established experimentally for model amides in dioxane–water mixtures.[23] Leichtling and Klotz[23] have derived eq 8, where K_w is the auto-

$$pH_{min} = \tfrac{1}{2}pK_w + \tfrac{1}{2}\log\,(k_{H^+}/k_{OH^-}) \qquad (8)$$

protolysis constant of water and k_{H^+} and k_{OH^-} are second-order rate constants for acid- and base-catalyzed exchange. The increase of pH_{min} in less polar solvents is then attributed to the increase in pK_w as solvent polarity decreases. This interpretation is valid for changes of solvent, but it cannot apply to the interior of a protein. Even though that environment may be less polar than bulk water, equilibrium is established throughout the solution, so that K_w and the activities a_{H^+} and a_{OH^-} cannot differ from their values in bulk water. The increase of pH_{min} must therefore be attributed to an increase of k_{H^+}/k_{OH^-}. While the N-protonation mechanism was accepted, such an increase was unreasonable since the solvation requirements of the N-protonation mechanism are quite similar to those of the base-catalyzed reaction. Figure 1 shows the symmetry between positive and negative charges in these two mechanisms. However, the operation of the imidic acid mechanism destroys the symmetry. The base-catalyzed reaction is retarded in the less polar interior of a protein because of the difficulty of solvating the transition state's negative charge there. In contrast, substituent effects[10] show that the transition state for the imidic acid mechansim resembles the imidic acid. A proton is not localized on the nitrogen, as in the N-protonation mechanism, but has been transferred to the solvent. In consequence, this transition state bears little positive charge, so there is less need for solvation, and therefore less retardation. Thus, for buried protons, k_{OH^-} is decreased to a greater extent than k_{H^+} is, and according to eq 8, pH_{min} increases, as observed. The increase is small, though, because the polarity decrease is moderated by the local unfolding.

Conclusions

According to the solvent dependence of k_{ZE}/k_{ZS} for model amdies, we conclude that the imidic acid mechansim for acid-catalyzed proton exchange becomes more important as solvent polarity decreases. This effect is more pronounced for secondary amides, and it can be rationalized in terms of pK_as of model compounds. We further conclude that the previously accepted N-protonation mechanism is insignificant for the NH protons of protein backbones, not only in aqueous solution but also in a less polar environment such as might be encountered in the interior of a protein. In view of the accessibility demands of the imidic acid mechanism, these results are taken to support the local-unfolding model for solvent accessibility to protein interiors. Moreover, the pH dependence of the rates of exchange of buried protons is interpreted as a consequence of the transition-state structure for the imidic acid mechanism.

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Registry No. Acetamide, 60-35-5; acetamide-^{15}N, 1449-72-5; acrylamide, 79-06-1; cyanoacetamide, 107-91-5; malonamide, 108-13-4; *N*-methylformamide, 123-39-7.

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