Primary Amide Hydrogen Exchange in Model Amino Acids: Asparagine, Glutamine, and Glycine Amides

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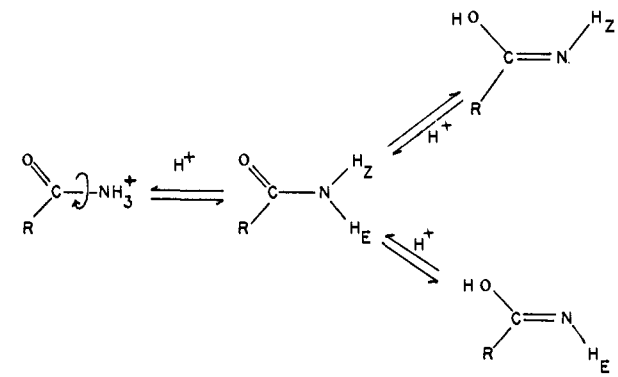
Abstract: We report model compound data for primary amide hydrogen exchange in three protected amino acids: *N*-acetyl-Asn-N_α-methylamide (Asn'), *N*-acetyl-Gln-N_α-methylamide (Gln'), and *N*-acetyl-Gly-NH_2 (Gly'). The solvent exchange rates of the individual *E* and *Z* protons have been measured for each of these amino acids in H_2O by employing double-resonance NMR techniques. The corresponding acid- and base-catalyzed rate constants (M^{-1} s^{-1}) are $k_H^E = 897$, $k_H^Z = 806$, $k_{OH}^E = 9.39 \times 10^7$, and $k_{OH}^Z = 2.2 \times 10^7$ for Asn' at 25 °C; $k_H^E = 1134$, $k_H^Z = 1095$, $k_{OH}^E = 5.52 \times 10^7$, and $k_{OH}^Z = 1.36 \times 10^7$ for Gln' at 22 °C; and $k_H^E = 327$, $k_H^Z = 333$, $k_{OH}^E = 13.48 \times 10^7$, and $k_{OH}^Z = 7.87 \times 10^7$ for Gly' at 25 °C. Our model compound data provide a basis for evaluating the primary amide hydrogen exchange behavior of these amino acids in peptides and proteins. The acid-catalyzed exchange in these model primary amides appears to take place predominantly through N-protonation.

Essential to conformational and dynamic analyses based on hydrogen exchange kinetics is the estimation of the exchange rates of specific labile hydrogens in "completely solvated" environments.[2-6] For proteins and peptides, such estimates have been obtained from studies of model peptides[7] and indoles,[8,9] but only inadequate data are available for the primary amide components of these biomolecules.[10] Here we have employed the NMR double-resonance method[4-6,8,9,11] to measure the individual exchange rates of the *E* and *Z* carboxamide hydrogens of *N*-acetyl-Asn-N_α-methylamide (Asn'), *N*-acetyl-Gln-N_α-methylamide (Gln'), and *N*-acetyl-Gly-amide (Gly'), which serve as models for Asn, Gln, and Gly NH_2 residues.[12] NMR methods have been employed to measure the exchange rates of secondary amides or Trp indole NH hydrogens of peptides[4-6,13-19] and proteins.[20,21]

Scheme I



This paper provides the basis for extending these investigations to the primary amide hydrogens of such biomolecules.

Our study also relates to the mechanism of acid-catalyzed amide hydrogen exchange, a subject of renewed interest and controversy.[22-25] Two mechanisms have been proposed, one involving protonation of the amide nitrogen[22,24,25] and the other, protonation of the carbonyl oxygen[23] according to the Scheme I.

The laboratories of Perrin[24] and Redfield[25] studied several primary amides, and they attempted to explain the observed

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(10) Molday et al.[7] measured the tritium–hydrogen exchange rate of poly-D,L-Asn at 0 °C. The exchange of primary amides was kinetically distinguished from peptide hydrogen exchange, but the individual exchange rates of the carboxamide hydrogens, *E* and *Z*, could not be distinguished by this method. The possibility exists that regions of secondary structure might exist in this polymer. The exchange rates of Gln carboxamide were estimated from the Asn rates but were not measured.

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acid-catalyzed exchange behavior in terms of detailed models of protonation. They both favored the N-protonation mechanism in primary amides in general, even though they differed somewhat in their mechanistic interpretation.[24e,25] These two mechanisms may be distinguished—the imidic acid pathway precludes[24e] an intramolecular E–Z exchange but permits an exchange of these protons with the solvent directly. Further, it has been shown by Perrin through analogy with imidate esters and through molecular orbital calculations that for this mechanism the Z proton would exchange faster.[24e] The N-protonation mechanism, on the other hand, can lead to an intramolecular E–Z exchange through internal rotation[24,25] about the C–N bond due to the formation of transient sp^2–sp^3 species. If the rotational diffusion about the C–N bond is either rapid or comparable to deprotonation, the E and Z protons would be expected to exhibit symmetric behavior with respect to E–Z cross-saturation and solvent exchange.[24,25] Asymmetric behavior can be expected if this deprotonation is more rapid than internal rotation.[25] Our present data also provide additional support to the N-protonation model favored by the laboratories of Perrin[24] and Redfield.[25]

Experimental Section

Materials. Asn′ and Gln′ were prepared from the corresponding benzyloxycarbonyl p-nitrophenyl esters by ammonolysis with methylamine. Following hydrogenolysis over 10% Pd/C catalyst, the acetyl groups were introduced by the method of Cromwell and Stark[26] to give racemic products. Gly′ was purchased from Vega Biochemicals (Tucson, AZ). The samples were studied in H_2O (with 10% D_2O for lock) at amide concentrations of 30 mM (Gln′) and 50 mM (Asn′ and Gly′) and acetate buffer concentrations of 10 mM (Gln′) and 25 mM (for Asn′ and Gly′).

NMR Measurements and Analysis. The NMR measurements were performed in the 2–1–4 long-pulse mode[27,28] on a Nicolet NT-360 spectrometer (for Gln′) and on a Bruker WH-400 spectrometer (for Asn′ and Gly′). A few additional measurements above pH 5 (some with 10 mM phosphate buffer) were also performed on the WH-400 for Gln′. The exchange rates were measured by a combination of three separate experiments:[25,29–31] (i) transfer of solvent saturation, (ii) amide hydrogen saturation–recovery, and (iii) H_E–H_Z cross-saturation experiments. The first two experiments formed the basis for the measurement of secondary amide hydrogen exchange in peptides.[4–6,8,13–19,32] For primary amides, the additional experiment iii is required to isolate the E–Z intramolecular exchange from the solvent exchange. The solvent exchange rates can be extracted from the above three experiments by adapting the Bloch–McConnell formulation[33] to proton exchange between the three sites E, Z, and S (solvent). If I_E, I_Z, and S are the corresponding magnetizations per proton for the E, Z, and S sites, the equation of motion for I_E may be written as

$$-\frac{dI_E}{dt} = (R_E + \rho + k_{ES} + k_r)(I_E - I_E^0) - (k_r - \sigma)(I_Z - I_Z^0) - k_{ES}(S - S^0) \quad (1)$$

where I_E^0, I_Z^0, and S^0 are the thermal equilibrium magnetizations. k_{ES} is the solvent-exchange rate constant for the E proton, while k_r is the rate constant for intramolecular exchange of E and Z protons. ρ and σ terms take into account explicitly the mutual dipolar relaxation of E and Z protons, while R_E is the spin–lattice relaxation rate due to all remaining mechanisms of relaxation. In eq 1 we have neglected intermolecular dipolar relaxation with the solvent protons. These rates are usually negligible[8,32] compared to solvent exchange rates, except perhaps at exchange minima where they may lead to an underestimation of k_{ES} due to an intermolecular nuclear Overhauser effect[34,35] when the solvent is saturated. An important simplification in solving eq 1 for the three experiments considered here is that because of the much larger concentration (110 M) of the water protons compared to the amide concentration (~50 mM), the solvent magnetization S always remains at thermal equilibrium due to its own self-relaxation, unless of course it is directly perturbed, as in experiment i. If $\eta_i(j)$ refers to the fractional intensity change in the magnetization at site i when that at site j is completely saturated, we obtain for the transfer of solvent saturation experiment

$$(R_E + \rho + k_r)\eta_E(S) - (k_r - \sigma)\eta_Z(S) + k_{ES}(\eta_E(S) + 1) = 0 \quad (2)$$

Selective steady-state saturation of the Z proton resonance alone (experiment iii) leads to eq 3. The apparent relaxation rate for the E proton

$$(R_E + \rho + k_r)\eta_E(Z) + (k_r - \sigma) + k_{ES}\eta_E(Z) = 0 \quad (3)$$

in a saturation–recovery experiment in which the Z proton is also continuously kept saturated is given by eq 4. This procedure was used to

$$(R_E + \rho + k_r) + k_{ES} = 1/T_{1app} \quad (4)$$

measure the apparent relaxation rates for Asn′ and Gly′. For Gln′, however, the apparent relaxation rates were measured without keeping the second primary amide proton saturated. In this case, the saturation–recovery curves will be, in general, double exponential due to coupled recovery of the E and Z protons through interchange and dipolar relaxation. We have analyzed the initial part of the recovery curves for Gln′, and the corresponding initial recovery rates[36] are given by

$$(R_E + \rho + k_r) + k_{ES} + (k_r - \sigma)\eta_Z(E) \simeq 1/T_{1app} \quad (4')$$

Equation 4′ will reduce to eq 4 if the last term on the left-hand side is negligible.

The set of simultaneous equations 2, 3, and 4 (or 4′) can then be solved to obtain the solvent-exchange rate constants at any given pH, k_{ES}, from the experimental data. A similar set of equations for analyzing the k_{ZS}

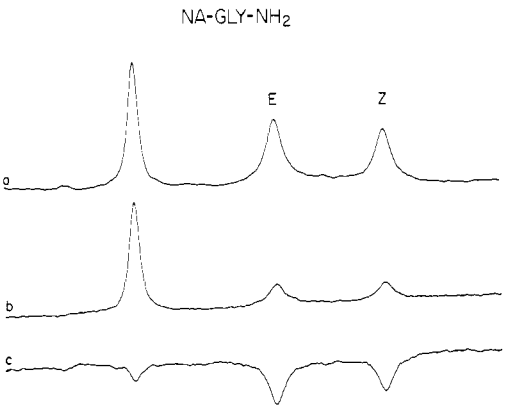

Figure 1. A typical transfer-of-solvent saturation experiment on the E and Z protons of Gly′ in H_2O (pH 2.5): (a) Control spectrum; (b) spectrum obtained after steady-state saturation of the solvent (H_2O); (c) the difference b − a.

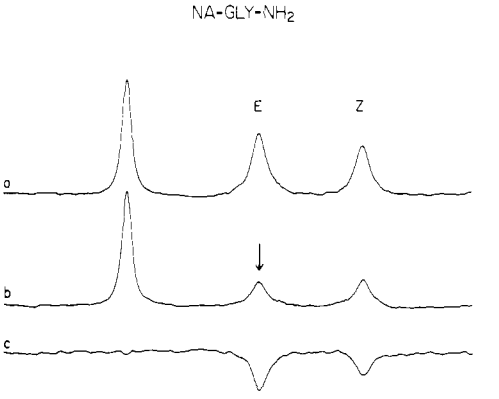

Figure 2. A typical cross-saturation experiment on Gly′ in H_2O (pH 2.5) showing the transfer of saturation to the Z proton through intramolecular exchange when the E proton is continuously saturated: (a) Control spectrum; (b) spectrum of the cross-saturation experiment with irradiation at the E proton; (c) the difference b − a.

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Table I. Carboxamide NH Hydrogen Rate Constants (M^{-1} s^{-1}) and Cross-Saturation Data in Acidic Solutions for the Model Amino Acids at 25 °C

	Gln'[a]		Asn'		Gly'	
	E	*Z*	*E*	*Z*	*E*	*Z*
k_H[b]	1134 ± 303	1095 ± 320	897 ± 156	806 ± 136	327 ± 61	333 ± 50
$k_{OH} \times 10^{-7}$[b]	5.5 ± 0.3	1.4 ± 0.2	9.4 ± 1	2.2 ± 0.2	13.5 ± 2.6	7.9 ± 1.6
$\eta_E(Z)$[c]	0.24 (2.06)		0.46 (2.21)		0.57 (1.84)	
$\eta_Z(E)$[c]		0.28 (2.06)		0.45 (2.21)		0.5 (1.84)

[a] At 22 °C. [b] The reported errors are standard errors. [c] $\eta_i(j)$ refers to the fractional intensity change in the resonance of proton i when the resonance from proton j is saturated completely. Experimentally, these are obtained by dividing the degree of indirect saturation of i by the degree of direct saturation of j. The numbers in parentheses refer to the pH values corresponding to the cross-saturation data reported here.

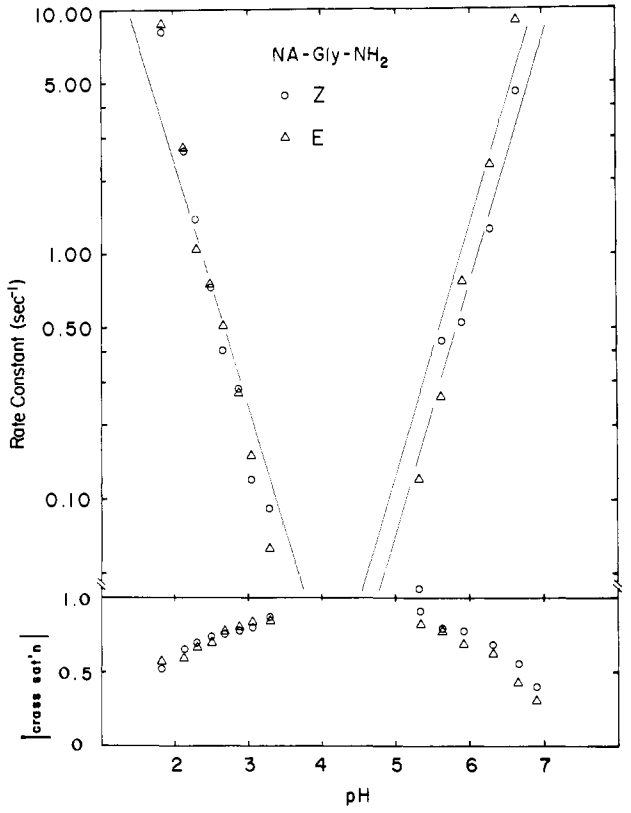

Figure 3. Solvent-exchange rate constants and cross-saturation data for the *E* and *Z* protons of Gly' as a function of pH.

may be obtained by interchanging the indices *E* and *Z* in the above equations.

Results and Discussion

Figure 1 shows a typical transfer of solvent saturation experiment on Gly'. Figure 2 shows a typical cross-saturation experiment on the same compound in which steady-state saturation of the *E* proton leads to a decrease in the intensity of *Z* proton due to intramolecular exchange. The experimentally determined solvent-exchange rates (k_{ES} and k_{ZS}) and the cross-saturation data for Gly' are shown in Figure 3. Similar results were obtained on the primary amides Asn' and Gln'. For Gln', there was some degree of scatter in the cross-saturation data above pH 5, but the scatter was not significant enough to affect the solvent-exchange rate constants. The cross-saturation values in the acid-catalyzed range and the solvent-exchange rate constants (k_H and k_{OH}), determined from a least-squares fit of the experimental data in the acid- and base-catalysis ranges separately to straight lines, are given in Table I.

The base-catalyzed rate constants for the *E* proton are consistently larger than those for the *Z* proton in all three amides studied. Similar behavior has been observed earlier by Perrin et al.[24a,b,37] and Redfield and Waelder[25] in other amides. The faster exchange of the *E* proton is compatible with what one would predict on the basis of lone-pair repulsions, viz., on deprotonation: the species with a lone *Z* proton is more stable.[24a,b,37]

The acid-catalyzed rate constants are equal, within experimental error, for the *E* and *Z* protons of each primary amide. Furthermore, the *E* and *Z* protons exhibited essentially symmetric cross-saturation behavior (Table I). These observations are completely compatible with an N-protonation model in which at least a moderate amount of internal rotation about the C–N bond[25] takes place before deprotonation. If the rate of internal rotation is much faster than that for deprotonation, then a maximum of 50% for the degree of cross-saturation is expected.[24e,25] This circumstance appears to be realized for the glycine amide, for which the degree of cross-saturation approaches this limit. Asparagine amide, with about ~45% for the degree of cross-saturation, is also fairly close to this limit. In the case of glutamine amide, the degree of cross-saturation is smaller (~25%) but still symmetric. This behavior can be expected if the rate of deprotonation is comparable to that of internal rotation of the sp^3 nitrogen about the C–N bond.[25] The observed exchange behavior is not compatible with the imidic acid pathway for which, as explained earlier, negligible *E*–*Z* cross-saturation and faster solvent-exchange rates for the *Z* proton are predicted.[24e]

Finally, and most importantly, the acid- and base-catalyzed rate constants given in Table I for the three primary amides serve as model compound data and provide a basis for evaluating the exchange behavior of similar amides in peptides and proteins. Currently we are undertaking a detailed investigation of primary amide hydrogen exchange in oxytocin and its analogues with a view to refine our earlier measurements,[4] which were only preliminary because of possible uncertainties due to complex resonance overlap, limited number of data points, and unincorporation of *E*–*Z* interchange in the analysis.

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