J. Am. Chem. Soc. **1990**, *112*, 3122-3125

Exchange of Amide Protons. Effect of Intramolecular Hydrogen Bonding

Charles L. Perrin,*,† Tammy J. Dwyer,† Julius Rebek, Jr.,‡ and Robert J. Duff‡

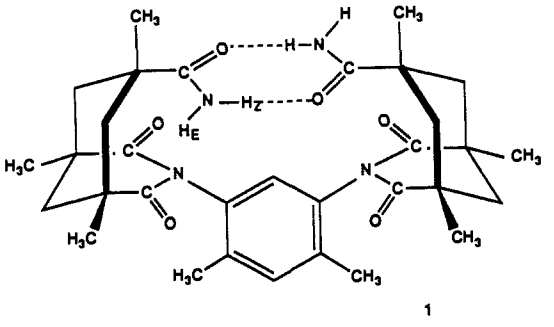
Contribution from the Department of Chemistry D-006, University of California, San Diego, La Jolla, California 92093, and the Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received September 19, 1989*

Abstract: Kinetics of the exchange of the NH protons of a primary amide with intramolecularly hydrogen bonded protons has been studied by NMR saturation transfer methods. The base-catalyzed exchange is retarded ca. 30-fold by the hydrogen bonding. The exchange is considered to proceed by direct abstraction of the proton from the hydrogen bond. The acid-catalyzed exchange proceeds by the N-protonation mechanism, but there is no retardation due to hydrogen bonding. The significance of these results for NH proton exchange in peptides and proteins is discussed.

Proton exchange is a powerful technique for studying structure and dynamics of peptides and proteins.[1] It is found that CONH protons buried in the interior of a protein are slow to exchange. Retardations up to 10^8-fold have been observed. Much of that retardation arises because the interior is inaccessible to solvent. Part of the retardation arises because the protons are internally hydrogen bonded, as in α-helices or β-sheets. The goal of this study is to evaluate the effect of internal hydrogen bonding on NH exchange.

Peptides and proteins themselves are too diverse for such a study. Rates of NH exchange depend too strongly on inductive effects of substituents, electrostatic effects of nearby charges and dipoles, and steric effects due to restricted accessibility to solvent. It is necessary to design a well-defined system, plus a reference system that differs only in lacking the internal hydrogen bond. The solution is to build a framework with "convergent" amide groups that are forced to hydrogen bond with each other.

Such a system is diamide **1**.[2] Molecular models and NMR chemical shifts verify that two NH protons, labeled H_Z, are hydrogen bonded to the opposite carbonyl groups. Moreover, these are primary amide groups, so that there are two other protons, labeled H_E, that are not internally hydrogen bonded but are otherwise chemically equivalent. The slight chemical inequivalence due to diastereotopicity can be corrected for through studies of simple primary amides. Therefore this molecule carries its own reference system. The exchange rates of the two kinds of NH protons can then be compared under identical conditions.



1

Experimental Section

Materials. Diamide **1** was prepared as previously described.[2] It was necessary to find a solvent system that would dissolve the amide and not have interfering NMR peaks. A hydroxylic solvent is necessary for proton exchange, and aqueous dioxane-d_8 was found to be suitable. Dioxane-d_8 and acid or base catalysts were commercially available reagents and were used without further purification.

The diamide (10 mg) was dissolved in dioxane-d_8 and sufficient aqueous acid (HCl + $HClO_4$) or base (1,1,2,2-tetramethylguanidine + tetra-*n*-butylammonium hydroxide) was added to produce a 70% (v/v) aqueous dioxane-d_8 solution that exhibited a measurable exchange rate. Alkaline solutions were deaerated with a stream of N_2. Before and after the NMR measurements the apparent pH was measured with a Corning Model 125 pH meter and Ingold microelectrode capable of fitting into a 5-mm NMR tube. The pH meter was calibrated with 0.001–0.01 M HCl in 70% aqueous dioxane, and the OH^- concentration was calculated from $\log [OH^-] = pH_{obs} - pK_w$, where $pK_w = 17.86$ in this solvent.[3]

NMR Methods. Proton NMR spectra were acquired with a 360-MHz Oxford magnet interfaced to a modified Nicolet 1180 pulse programmer and computer. Probe temperature was 22 °C. The strong OH resonance was suppressed by using a time-shared Redfield 21412 pulse sequence.[4] The carrier frequency was set at the NH signal whose intensity was to be measured. In the aqueous dioxane-d_8 solvent these signals are broadened by the ^{14}N quadrupole, but the line width is only ca. 5 Hz, which is satisfactory. There are singlets at δ 7.2 and 8.6. The downfield peak has been assigned as the internally hydrogen-bonded H_Z.[2]

Nuclear Overhauser enhancements (NOEs) were measured under nonexchange conditions, without added acid or base. Each signal (NH_E, NH_Z, H_S = solvent OH) was saturated and any enhancement of intensity at the other two sites was evaluated from difference spectra. The NOEs $\eta_i(j)$ were calculated according to eq 1, where $I_i(j)$ is the peak height of

$$\eta_i(j) = \frac{I_i(j) - I_i^0}{I_i^0} \tag{1}$$

site i on saturating site j and I_i^0 is its equilibrium peak height on irradiating at an off-resonance position offset from i by the frequency difference $|\nu_i - \nu_j|$. Similarly, saturation-transfer values $t_i(j)$, calculated according to eq 2, were measured under conditions of acid- or base-

$$t_i(j) = \frac{I_i^0 - I_i(j)}{I_i^0} \tag{2}$$

catalyzed exchange. Values of $t_E(Z)$ and $t_Z(E)$ in acid were corrected for NOE by adding the observed $\eta_E(Z)$ and $\eta_Z(E)$. Apparent spin–lattice relaxation rates $R_i(j)$, the reciprocal of the spin–lattice relaxation times, of the NH and OH signals were measured by inversion-recovery while saturating signal j. The 180° inversion pulse was also a 21412 pulse sequence. Intensities were fit to exponential recovery, by using a

† University of California, San Diego.
‡ University of Pittsburgh. Present address: Department of Chemistry, Massachusetts Institue of Technology, Cambridge, MA 02139.

(1) Reviews: (a) Linderstrom-Lang, K. *Spec. Publ. Chem. Soc.* **1955**, *No. 2*, 1. (b) Leach, S. J. *Rev. Pure Appl. Chem.* **1959**, *9*, 33. (c) Hvidt, A.; Nielsen, S. O. *Adv. Protein Chem.* **1966**, *21*, 287. (d) Klotz, I. M. *J. Colloid Interface Sci.* **1968**, *27*, 804. (e) Englander, S. W.; Downer, N. W.; Teitelbaum, H. *Annu. Rev. Biochem.* **1972**, *41*, 903. (f) Otteson, M. *Methods Biochem. Anal.* **1972**, *20*, 135. (g) Craig, L. C.; Cowburn, D.; Bleich, H. *Ibid.* **1975**, *44*, 477. (h) Englander, S. W.; Englander, J. J. *Methods Enzymol.* **1978**, *49*, 24. (i) Woodward, C. K.; Hilton, B. D. *Annu. Rev. Biophys. Bioeng.* **1978**, *8*, 99. (j) Barksdale, A. D.; Rosenberg, A. *Methods Biochem. Anal.* **1982**, *28*, 1. (k) Woodward, C.; Simon, I.; Tuchsen, E. *Mol. Cell. Biochem.* **1982**, *48*, 135. (l) Englander, S. W.; Kallenbach, N. R. *Q. Rev. Biophys.* **1983**, *16*, 521.

(2) Rebek, J., Jr.; Marshall, L.; Wolak, R.; Parris, K.; Killoran, M.; Askew, B.; Nemeth, D.; Islam, N. *J. Am. Chem. Soc.* **1985**, *107*, 7476.

(3) Harned, H. S.; Owen, B. B. *The Physical Chemistry of Electrolytic Solutions*, 3rd ed.; Reinhold Pub. Corp.: New York, 1958; p 756.

(4) Redfield, A. G.; Kunz, S. D.; Ralph, E. K. *J. Magn. Reson.* **1975**, *19*, 114. Wright, J. M.; Feigon, J.; Denny, W.; Leupin, W.; Kearns, D. R. *Ibid.* **1981**, *45*, 514.

0002-7863/90/1512-3122$02.50/0

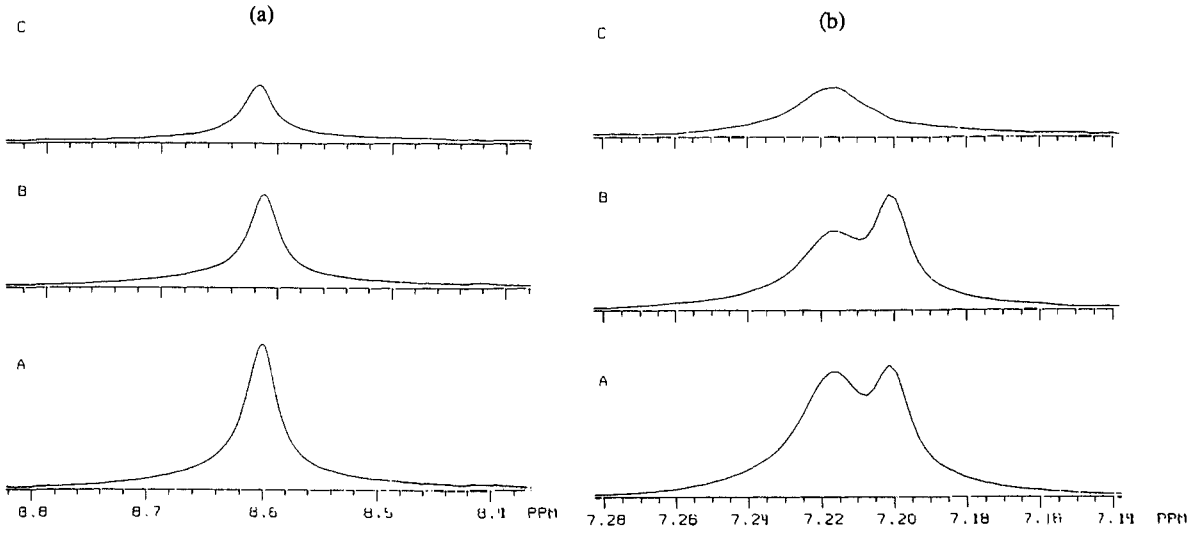


Figure 1. Saturation-transfer experiment for base-catalyzed exchange of diamide **1** (peaks, from left to right, are NH_Z, NH_E, and aromatic CH). Part a shows NH_Z at pH 12.28. Part b shows NH_E at pH 10.43. Figure sections are as follows: A, with off-resonance irradiation; B, with solvent peak (not shown) saturated; C, difference spectrum (A minus B).

Table I. Saturation-Transfer Results for Base-Catalyzed Proton Exchange of Amide **1**

	NH_E	NH_Z
pH	10.82	12.67
$t_{NH}(S)$	0.358 ± 0.003	0.365 ± 0.006
$R_{NH}(S)$, s^{-1}	7.80 ± 0.01	7.86 ± 0.04
k_{HS}, s^{-1}	2.79 ± 0.02	2.87 ± 0.05
$k_{2\cdot HS}$, $M^{-1}s^{-1}$	(3.0 ± 0.2) × 10^7	(4.4 ± 0.2) × 10^5
$\eta_H(H')^a$	0.04 ± 0.01	0.02 ± 0.01

[a] In neutral solution.

weighted linear least-squares method.

Evaluation of Rate Constants. Pseudo-first-order rate constants k_{ij} for site-to-site exchange from site i to site j were evaluated according to eqs 3 and 4.[5] (The additional terms involving $t_S(E)$ and $t_S(Z)$ are negligible, since solvent is in large excess.) The expressions for k_{ES} and k_{EZ} are

$$k_{ZS} = R_Z(S)\,\frac{t_Z(S) - t_Z(E)t_E(S)}{1 - t_Z(E)} \tag{3}$$

$$k_{ZE} = R_Z(S)\,\frac{t_Z(E)}{1 - t_Z(E)} \tag{4}$$

analogous. The upper limit for the rate of stereoisomerization under nonexchange conditions was evaluated according to eq 5. Second-order

$$k_{EZ}^0 < \eta_E(Z)R_E(Z) \text{ or } \eta_Z(E)R_Z(E) \tag{5}$$

rate constants for base-catalyzed exchange were obtained according to eq 6. Errors are standard deviations, calculated according to propagation of errors from replicate determinations of $t_i(j)$ or deviations from the linearized exponential fit.

$$k_{2,ij} = k_{ij}[OH^-] \tag{6}$$

Results

Base-Catalyzed Exchange. It was immediately obvious that the two NH protons exchange at very different rates. In dilute base, H_Z exchange is too slow to measure. In more concentrated base, H_E exchange is so fast that the signal is broadened into the base line. Therefore it was necessary to measure rates in two separate solutions and use the measured pH to compare them. Figure 1a shows saturation-transfer spectra at pH 10.82. Figure 1b shows saturation-transfer spectra at pH 12.67. Table I lists saturation-transfer data and derived rate constants for base-catalyzed exchange.

Acid-Catalyzed Exchange. In contrast to the base-catalyzed exchange, both NH protons undergo acid-catalyzed exchange at nearly the same rate, so that their reactivities could be compared directly in the same solution. Figure 2 shows saturation-transfer spectra in acidic medium. Table II lists saturation-transfer data and derived rate constants for acid-catalyzed exchange.

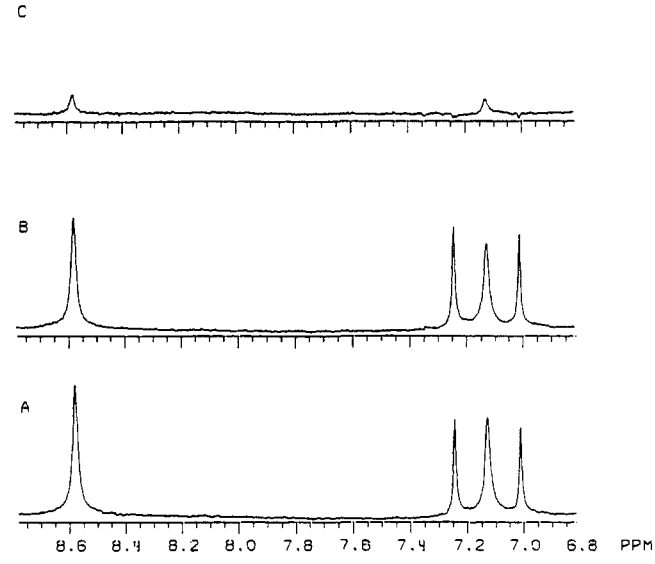


Figure 2. Saturation-transfer experiment for acid-catalyzed exchange of diamide **1** (peaks, from left to right, are NH_Z, aromatic CH, NH_E, and aromatic CH). Figure sections are as follows: A, with off-resonance irradiation; B, with solvent peak (not shown) saturated; C, difference spectrum (A minus B).

Table II. Saturation-Transfer Results for Acid-Catalyzed Proton Exchange of Amide **1**

	NH_E	NH_Z
$t_{NH}(S)$	0.170 ± 0.017	0.149 ± 0.011
$t_{NH}(NH')$	0.082 ± 0.022[a]	0.093 ± 0.014[a]
$R_{NH}(S)$, s^{-1}	8.93 ± 0.29	6.45 ± 0.06
k_{HS}, s^{-1}	1.53 ± 0.17	0.95 ± 0.07
$k_{HH'}$, s^{-1}	0.80 ± 0.22	0.66 ± 0.10

[a] Corrected for NOE.

Discussion

Base-Catalyzed Exchange. The second-order rate constant for exchange of H_E is in adequate agreement with values for H_E of primary amides in asparagine and glutamine side chains.[6]

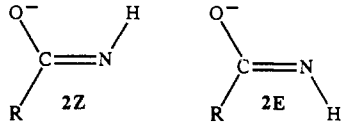
(5) Perrin, C. L.; Johnston, E. R. *J. Magn. Reson.* **1979**, *33*, 619.

(6) Krishna, N. R.; Sarathy, K. P.; Huang, D.-H.; Stephens, R. L.; Glickson, J. D.; Smith, C. W.; Walter, R. *J. Am. Chem. Soc.* **1982**, *104*, 5051.

However, rates are quite sensitive to solvent and substituents,[7] and there are no model primary amides closer to **1**. Therefore we must take the exchange rate of H_E in **1** as our reference, and compare the exchange rate of H_Z with this reference in order to assess the effect of hydrogen bonding.

According to the data in Table I, H_Z undergoes OH^--catalyzed exchange only $^1/_{70}$ as fast as H_Z. These rates could not be compared directly, in the same solution, since they are so disparate. However, second-order rate constants from separate solutions could be compared, since OH^- concentrations are available through the measured pH. Although addition of amide and catalyst may change pK_w from its reported value[3] in 70% aqueous dioxane, such that the OH^- concentration is in error, the relative pH and the ratio of OH^- concentrations in the two solutions are reliable.

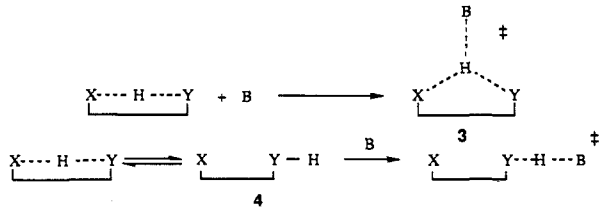
The 70-fold rate retardation is not solely due to hydrogen bonding. Even in simple primary amides H_E undergoes base-catalyzed exchange ca. 2–3 times as fast as H_Z.[6,8] This difference has been attributed to the greater stability of a *Z*-imidate (**2*Z***), relative to the *E* stereoisomer (**2*E***). Such an effect should also be operative on proton exchange of **1**. Then the retardation of H_Z attributable to internal hydrogen bonding is only ca. 30-fold.



Why is the retardation so small? It corresponds to a $\Delta\Delta G^{\ddagger}$ of only 2 kcal/mol, substantially less than 6–7 kcal/mol, which is half the calculated dimerization energy of formamide.[9] Although much of that energy must be expended to break the hydrogen bond and expose H_Z to exchange, nearly as much energy must be expended to expose H_E, which is hydrogen bonded to solvent. The 2 kcal/mol is the empirical difference between these energies, as manifested in the transition states for proton removal.

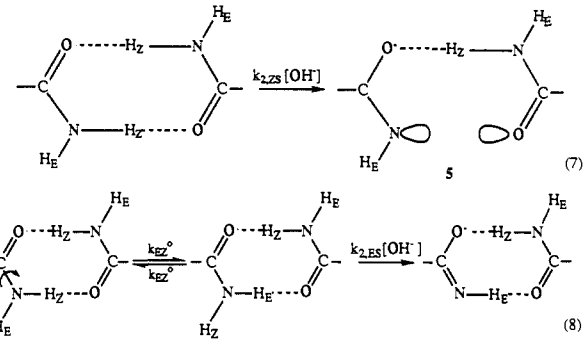
This 2 kcal/mol is not universal. It represents the retardation due to internal hydrogen bonding in this particular amide. Different retardations would be observed for other amides, peptides, or proteins with different forms of hydrogen bonding. However, this value is a reasonable one and probably representative.

Mechanism of Base-Catalyzed Exchange. Two limiting mechanisms have been proposed for exchange of internally hydrogen-bonded protons:[10] (1) Direct abstraction of the proton



from the hydrogen bond, through a transition state with a three-center bond (**3**), or (2) rotation to a conformer in which the proton is not internally hydrogen bonded (**4**), followed by abstraction of the exposed proton. From studies of general-base catalysis[11] it was concluded that the proton is abstracted from open forms of salicylamide and 1,8-bis(dimethylamino)naphthalenes.

In contrast, some (phenylazo)resorcinols may exchange by direct abstraction of an internally hydrogen-bonded proton, but early evidence is equivocal. For amide **1** the alternative mechanisms for exchange of H_Z are direct abstraction (eq 7) or rotation about the C–N bond, which transfers H_Z to the *E* site, from which it can then readily be abstracted (eq 8).



The experimental results exclude the latter possibility. According to eq 8, the overall rate constant k_{ZS} cannot be greater than k_{EZ}^0 the rate constant for uncatalyzed C–N rotation. That rate constant can be evaluated independently, under nonexchange conditions, by saturation-transfer measurements. Experimentally there is no saturation transfer from H_E to H_Z or from H_Z to H_E. Instead there is a small nuclear Overhauser enhancement (NOE) (Table I). Even if the observed NOE is the net result of an NOE and an opposing saturation transfer, it would be very fortuitous if the saturation transfer were larger than the observed NOE. The observed NOE thus represents an upper limit to the saturation transfer. Equation 5 then provides an upper limit to k_{EZ}^0 of 0.1–0.2 s^{-1}, which is far less than the observed 2.87 s^{-1}. Moreover, according to eq 8 and the observed rate constants, the rate of exchange of H_Z would become zero order in OH^-. Since it does not, we reject this mechanism.

We therefore conclude that exchange of H_Z proceeds by direct abstraction of the proton from the hydrogen bond. We cannot exclude reaction through breathing motions of the molecule that break apart the dimer and expose H_Z. Exchange might occur through an open form present in equilibrium to 4–5%. However, such a form is not produced simply by rotation of the proton out of the hydrogen bond, and the distinction between this pathway and direct abstraction is quite subtle. Direct abstraction accounts for an additional reason for the retardation of H_Z exchange, since the intermediate (**5**) suffers repulsions of lone pairs on nitrogen and oxygen.

Acid-Catalyzed Exchange. Two mechanisms have been proposed for acid-catalyzed proton exchange in amides.[12] The imidic acid mechanism (eq 9) is favored for amides with electron-withdrawing substituents. The N-protonation mechanism (eq

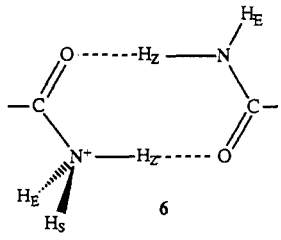
$$RCONH_2 + H^+ \rightleftharpoons RC(OH)=NH_2^+ \rightleftharpoons RC(OH)=NH + H^+ \quad (9)$$

$$RCONH_2 + H^+ \rightleftharpoons RCONH_3^+ \quad (10)$$

10) is more likely for **1**, with its electron-donating alkyl. The mechanisms may be distinguished experimentally because only the N-protonation mechanism allows intramolecular exchange. This is manifested by nonzero k_{EZ} and k_{ZE}, measurable by saturation transfer. The data in Table II show that within experimental error k_{EZ} and k_{ZE} are equal to k_{ZS}, as required for the N-protonation mechanism. Such behavior is inconsistent with the imidic acid mechanism.

Moreover, k_{ZS} is significantly less than k_{ES}. This is evidence that deprotonation of the intermediate $RCONH_3^+$ is competitive with rotation about its C–N single bond.[12] According to MO calculations[13] on $HCONH_3^+$ this intermediate must be created in conformation **6**. Moreover, in order to satisfy the principle

(7) Berger, A.; Loewenstein, A.; Meiboom, S. *J. Am. Chem. Soc.* **1959**, *81*, 62.
(8) Perrin, C. L. *J. Am. Chem. Soc.* **1974**, *96*, 5628. Perrin, C. L.; Johnston, E. R.; Lollo, C. P.; Kobrin, P. A. *J. Am. Chem. Soc.* **1981**, *103*, 4691.
(9) Sagarik, K. P.; Ahlrichs, R. *J. Chem. Phys.* **1987**, *86*, 5117. Dory, M.; Delhalle, J.; Fripiat, J. G.; Andre, J.-M. *Int. J. Quantum Chem., Quantum Biol. Symp.* **1987**, *14*, 84.
(10) Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1964**, *3*, 1. Miles, M. H.; Eyring, E. M.; Epstein, W. W.; Anderson, M. T. *J. Phys. Chem.* **1966**, *70*, 3490.
(11) Hibbert, F. *Acc. Chem. Res.* **1984**, *17*, 115. Hibbert, F. *Adv. Phys. Org. Chem.* **1986**, *22*, 113. Kresge, A. J.; Powell, M. F. *J. Am. Chem. Soc.* **1981**, *103*, 972. Briffett, N. E.; Hibbert, F.; Sellens, R. J. *Ibid.* **1985**, *107*, 6712. But see: Briffett, N. E.; Hibbert, F.; Sellens, R. J. *J. Chem. Soc., Perkin Trans. 2* **1988**, 2123.
(12) Perrin, C. L. *Acc. Chem. Res.* **1989**, *22*, 268.
(13) Perrin, C. L.; Johnston, E. R. *J. Am. Chem. Soc.* **1981**, *103*, 4697.

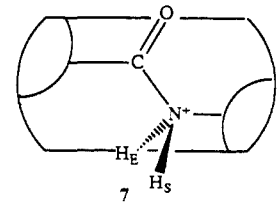


6

of microscopic reversibility, the initial labeling must be as shown (H_S from solvent). Then exchange of H_Z is retarded by the necessity for rotation out of the intramolecular hydrogen bond. That hydrogen bond would appear to be quite strong, imposing a significant barrier to rotation about the C-N^+ bond. Nevertheless there seems to be hardly any barrier to rotation.

Comparison of Acid- and Base-Catalyzed Exchange. Why does internal hydrogen bonding retard base-catalyzed exchange of H_Z ca. 30-fold but acid-catalyzed exchange hardly at all? Indeed, the ratio k_{ES}/k_{ZS} for acid-catalyzed exchange is comparable to that found for ordinary primary amides,[14] so that the hydrogen bonding in **6** does not retard exchange of H_Z. This result is consistent with observations on rotation of ammonium ion within its solvation shell.[15] This rotation is extremely fast, especially in water, despite the necessity for breaking and remaking hydrogen bonds. Nevertheless, it is remarkable that the internal hydrogen bond in **1** is so much more resistant to breaking than that in **6**.

This comparison clarifies an aspect of NH exchange in proteins. Acid-catalyzed exchange had long been thought to occur by the N-protonation mechanism. However, substituent effects in model *N*-methyl amides[16] and considerations of solvent accessibility to nitrogen and oxygen[17] indicate that the imidic acid mechanism is dominant. However, many of these NH are in α-helices, β-sheets, or other environments where the nitrogen is accessible from only one face. Protonation on that face produces an intermediate **7** that can lose only H_S, the proton that came from solvent. Loss



7

of the original NH proton is impossible, since H_E is now embedded in the protein and inaccessible to solvent. In a primary amide, even **1**, the corresponding intermediate **6** can undergo rotation about the C-N single bond and render any NH proton accessible to solvent. However, in a protein the backbone resists such twisting, and **7** cannot lead to proton exchange. In contrast, both the base-catalyzed and imidic acid mechanisms permit removal of the proton without this complication. Thus the N-protonation mechanism is quite unlikely for acid-catalyzed exchange of secondary NH in proteins.

Conclusions. The internal hydrogen bond in diamide **1** retards base-catalyzed exchange of H_Z ca. 30-fold. Exchange is viewed as occurring by direct abstraction of the proton from the hydrogen bond, and this may be the first example in which this one-step mechanism predominates. In contrast, the internal hydrogen bond retards the acid-catalyzed exchange of H_Z not at all. This is a consequence of the nearly free rotation about the C-N single bond of the N-protonated intermediate. However, this mechanism cannot be operative in proteins.

Acknowledgment. This research was supported by NSF Grant No. CHE87-14451.

(14) Hopkinson, A. C.; Csizmadia, I. G. *Can. J. Chem.* **1973**, *51*, 1432.
(15) Perrin, C. L.; Gipe, R. K. *J. Am. Chem. Soc.* **1986**, *108*, 1088. Perrin, C. L.; Gipe, R. K. *Science* **1987**, *238*, 1393.
(16) Perrin, C. L.; Arrhenius, G. M. L. *J. Am. Chem. Soc.* **1982**, *104*, 6693.
(17) Tüchsen, E.; Woodward, C. *J. Mol. Biol.* **1985**, *185*, 421.

Synthesis of Covalently Linked Double-Helical Cross Sections Representative of Purine–Pyrimidine, Purine–Purine, and Pyrimidine–Pyrimidine Duplexes†

Balekudru Devadas and Nelson J. Leonard*,‡

Contribution from the Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, 1209 West California Street, Urbana, Illinois 61801-3731. *Received October 16, 1989*

Abstract: Here described are the syntheses of (1) covalently linked cross sections with molecular architecture similar to Watson–Crick hydrogen-bonded purine–pyrimidine base pairs in RNA, DNA, and RNA/DNA double helices; (2) covalently linked purine–purine cross sections with dimensions such as would be produced in the pairing of A with I or G, generating a bulge in double-helical RNA or DNA; and (3) covalently linked pyrimidine–pyrimidine cross sections with dimensions such as might be produced in the hypothetical pairing of C with U or T, namely, a pinched-in RNA or DNA cross section.

In two preliminary communications,[1,2] we have introduced the concept of covalently linked double-helical cross sections that are representative of purine–pyrimidine, purine–purine, and pyrimidine–pyrimidine duplexes. We described briefly how these complex molecules in the bis(ribonucleoside) and bis(deoxyribonucleoside) series can be synthesized conveniently from the natural ribo- and deoxyribonucleosides in only three steps plus initial O-protection and final O-deprotection. We now provide further rationale and full details of the synthesis and spectroscopic

† Dedicated to the memory of Professor Roger Adams in the centennial year of his birth.
‡ Fogarty Scholar-in-Residence, 1989–1990, National Institutes of Health, U.S. Public Health Service, Bethesda, MD 20892.

(1) Devadas, B.; Leonard, N. J. *J. Am. Chem. Soc.* **1986**, *108*, 5012.
(2) Leonard, N. J.; Devadas, B. *J. Am. Chem. Soc.* **1987**, *109*, 623.

0002-7863/90/1512-3125$02.50/0