Hydrogen Bonding in Proteins As Studied by Amide Hydrogen D/H Fractionation Factors: Application to Staphylococcal Nuclease†

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ABSTRACT: The D/H fractionation factor (φ) is the extent to which a hydrogen at a particular site becomes enriched in ^2H over ^1H relative to the solvent. A growing body of experimental evidence suggests that there is a correlation between the value of the fractionation factor and hydrogen-bond strength, with a lower φ value reflecting a stronger hydrogen bond. Fractionation factors of 60% of the individual backbone amide hydrogens in the staphylococcal nuclease V8 variant (H124L) have been measured for the enzyme in the presence and absence of bound ligands (the activating ion Ca^{2+} and the inhibitor thymidine 3′,5′-bisphosphate). The method used employed two-dimensional ^1H–^{15}N nuclear magnetic resonance analysis of uniformly ^{15}N-labeled protein in mixed H_2O/D_2O solvents. Fractionation factors of individual residues were found to range from 0.3 (T120) to 1.5 (L38). The φ value of 0.3 for the NH of T120, which is the lowest fractionation factor reported for any system yet studied, suggests that the hydrogen bond between T120 H^N and D77 $O^{\delta 1}$ is unusually strong. The results of previous site-directed mutagenesis experiments [Hinck, A. P. (1993) Ph.D. Thesis, University of Wisconsin—Madison, Madison, WI] support the notion that formation of this hydrogen bond is important to maintain the stability and conformation of the native state. The φ value averaged over all residues was ~0.85 for both the unligated and ligated enzymes. Residues in α-helices displayed a slightly lower average φ value (0.79), whereas residues with solvent-exposed amide hydrogens exhibited a slightly higher average figure (0.98). These results suggest that the hydrogen-bond network present in protein α-helices may make these intramolecular amide–amide hydrogen bonds stronger than those formed between amide groups and H_2O. However, this conclusion must be tempered by the fact that several solvent-exposed amide hydrogens had low φ values. No correlation was found between the φ values and the N–O distances of amide hydrogen-bonded residues, as determined from the X-ray structure of unligated staphylococcal nuclease [Hynes, T. R., & Fox, R. O. (1991) *Proteins: Struct., Funct., Genet. 10,* 92–105].

One of the central questions in protein chemistry is to what extent the formation of intrachain hydrogen bonds contributes to the stability of the folded state. Self-assembly processes such as oligomerization, ligand binding, and protein folding all involve the formation of extensive networks of protein–protein (or protein–ligand) hydrogen bonds, particularly of the intramolecular amide–amide type. This topic remains controversial a half-century after Mirsky and Pauling (1936) first suggested that intramolecular hydrogen bonding may drive protein folding. The principal reason for the longevity of this debate has been the difficulty in assessing the energetics of solute–solute hydrogen bonds relative to those of solute–solvent hydrogen bonds, as evidenced by the abundance of contradictory reports on the strength of amide–amide hydrogen bonds in aqueous solution.[1]

The isotopic fractionation factors of labile hydrogens in proteins may hold an important clue to the resolution of this key issue. The equilibrium constant φ of isotope exchange[2] at a site in a molecule, for example, a backbone amide group within a protein that exchanges its proton with a solvent deuteron,

$$\mathrm{NH} + \mathrm{D\ (bulk)} \overset{\varphi}{\rightleftarrows} \mathrm{ND} + \mathrm{H\ (bulk)} \qquad (1)$$

is known as the fractionation factor. A φ value of unity reflects an equal distribution of protons and deuterons between amide groups and bulk solvent: a value greater than unity indicates a preference for D over H on the amide nitrogen, and a value less than unity indicates a preference for H over D. The position of the equilibrium is thought to depend primarily on the relative zero-point vibrational energies of the N–H and N–D bonds [for reviews, see Hibbert and Emsley (1990), Schowen and Schowen (1982), Melander and Saunders (1980), Gold (1969), Hvidt and Nielsen (1966), and Kresge (1964)]. Although deuterium fractionation factors for most low molecular weight alcohols, phenols, carboxylates, hydroxyls, imidazoles, amines, and amides are close to unity when measured in aqueous solution[3] (Jarret & Saunders, 1985, 1986; Cleland, 1980), deviations of 50% and more have been

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[1] This issue has been reviewed recently (Creighton, 1991; Dill, 1990).

[2] Abbreviations: HSMQC, heteronuclear single- and multiple-quantum correlation; φ, fractionation factor; NMR, nuclear magnetic resonance; nuclease H124L, staphylococcal nuclease V8 variant; nuclease wt, staphylococcal nuclease Foggi variant; pdTp, thymidine 3′,5′-bisphosphate; in this article, H is used to represent ^1H and D to represent ^2H.

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observed in protic as well as aprotic solutions (Jarret & Saunders, 1985, 1986; Kreevoy & Liang, 1980; Kreevoy et al., 1977; Lang & Mason, 1960; Kwart et al., 1954; Small & Wolfenden, 1936).

This result is of considerable interest in the context of determining the presence and strength of hydrogen bonds. A substantial body of experimental evidence suggests that strong hydrogen bonds tend to accumulate protium relative to its content in a mixed H_2O/D_2O solution, while weak hydrogen bonds accumulate deuterium. This effect presumably originates from the antagonistic consequences of loosening the in-line hydrogen-stretching mode and restricting the off-line bending motions that result from hydrogen-bond formation. The fractionation factor can be either raised or lowered from unity, depending on which mode is more affected (Schowen & Schowen, 1982). For example, D-bonds are stronger than H-bonds in the weakly bound solute–solvent complexes of water–dioxane (Bell & Wolfenden, 1935), water–methanol (Benjamin & Benson, 1963), and tetrahydrofuran–fluoroform (Creswell & Allred, 1962). Consistent with these findings, the fractionation factors of most alcohols and amines (in aqueous solution) have been found to lie between 1.0 and 1.2, with a few as high as 1.4 (Jarret & Saunders, 1985; Kooner et al., 1980; Phutela et al., 1979; Gold, 1968; Hobden et al., 1939).

By contrast, compounds that form strong hydrogen bonds generally exhibit low fractionation factors. The most extreme examples are homoconjugate complexes such as F_2H^- in water ($\varphi = 0.6$), dimers of 4-nitrophenolate (0.31), trifluoroacetate (0.42), 3,5-dinitrobenzoate (0.30), 3,5-dinitrophenolate (0.36), and pentachlorophenolate (0.40) in acetonitrile (Kreevoy & Liang, 1980) and the methoxide anion dimer ion in methanol and 25% methanol/75% dimethyl sulfoxide (0.74 and 0.38, respectively; Baltzer & Bergman, 1982). Hydrogen-bond competition with the solvent presumably explains why fractionation factors of acids in water are not as low as those of the diacid complexes in aprotic solvents; measured φ values of acids in aqueous solution are only slightly smaller than unity (Jarret & Saunders, 1985; Kreevoy et al., 1977; Gold & Lowe, 1968). One exception is the monovalent anion of maleic acid, which has a fractionation factor of $\sim$0.8 in water owing to the formation of a strong internal hydrogen bond (Jarret & Saunders, 1985; Kreevoy & Liang, 1980; McDougall & Long, 1962; Dahlgren & Long, 1960).

Gas-phase fractionation factors tend to reflect the trends observed in solution. For the isotopic substitution reaction of D_2O in bulk solution with the hydronium ion, φ was found to be 0.69 for H_3O^+ in the liquid phase (Kresge & Allred, 1963; Gold, 1963; Heinzinger & Weston, 1964) and $\sim$0.75 for H_3O^+ in the gas phase (Graul et al., 1990; Larson & McMahon, 1986, 1988). Larson and McMahon (1988) suggest that the observed lowering of the φ value in going from the gas to the liquid phase is due to the hydrogen-bond network present in solution. The fractionation factors of the shared hydrogen in homoconjugate complexes are low in both the vapor and liquid phases; examples include the monoanionic dimers of methoxide and ethoxide, which have gas-phase φ values of 0.33 and 0.46, respectively (Weil & Dixon, 1985). In addition, Graul et al. (1990) found that gas-phase $(H_2O)_nH^+$ clusters, when mixed with D_2O vapor, preferentially accumulate deuterium at the terminal positions and exclude it from the bridging positions (the φ value of the shared hydrogen in $(H_2O)_2H^+$ is 0.55).

[3] Sulfhydryl groups prove to be the exception, with φ values around 0.5 (Schowen, 1972; Small, 1937).

These findings are consistent with the notion that protium is preferred over deuterium in strong hydrogen bonds.

With regard to fractionation factors in proteins, few data are available. Kreevoy and coworkers made an important contribution with the discovery that the hydrogen shared between two groups of similar pK_a, as in the homoconjugate complexes described above, exhibits an abnormally low φ value (<0.5; Kreevoy & Liang, 1980; Kreevoy et al., 1977). This exceptionally strong hydrogen bond is known as a single-well or low-barrier H-bond, owing to the shape the potential well adopts due to the short heavy-atom interatomic distance. Such effects have recently been found in proteins; for example, the hydrogen shared between E168 and E211 in enolase and that between E217 and N1 of adenosine in adenosine deaminase each have φ values of $\sim$0.4 (Cleland, 1992; Weiss et al., 1987a,b). The role that low-barrier hydrogen bonds potentially play in enzymatic catalysis has been discussed (Cleland, 1992). However, fractionation data in proteins have thus far been limited to a relatively low number of sites. We recently outlined a method for obtaining both site-specific and comprehensive fractionation factor data for proteins in solution (Loh & Markley, 1993). This technique, which employs ^{15}N-resolved proton nuclear magnetic resonance (NMR) spectroscopy of ^{15}N-labeled proteins, allows the determination of φ values for all of the backbone NH hydrogens that are resolved in two-dimensional NMR spectra.

In the present article, we report the deuterium fractionation factors of the backbone amide hydrogens of the enzyme staphylococcal nuclease H124L (nuclease H124L), both in its unliganded state and in its ternary complex with Ca^{2+} and the inhibitor thymidine 3′,5′-bisphosphate (pdTp). The goal of this study is to determine whether this information can be used to characterize the strength and importance of individual hydrogen bonds in staphylococcal nuclease. Staphylococcal nuclease is an excellent model system for this analysis. It is a small (MW 17 000), monomeric enzyme lacking disulfide linkages and containing a typical distribution of $\alpha+\beta$ secondary structure. It has been used as a prototype for structure–function and protein-folding studies for many years. Most importantly, the backbone ^{15}N and ^1H NMR resonances have been assigned for both the unligated and ligated enzymes (Wang et al., 1990a,b, 1992; Torchia et al., 1989), and high-resolution X-ray crystal structures are available for both protein species. In addition, amide hydrogen-exchange kinetics has been characterized extensively for the protein in the presence and absence of ligands (Ca^{2+} and pdTp) (Loh et al., 1993; Wang et al., 1990a; Torchia et al., 1989; Schecter et al., 1968).

MATERIALS AND METHODS

^{15}N-labeled protein was prepared as outlined by Wang et al. (1990b). The method for measuring fractionation factors was described previously (Loh & Markley, 1993). Briefly, seven protein samples were prepared that differed only in the ratio of H_2O to D_2O in the solvent (0.14, 0.28, 0.43, 0.57, 0.71, 0.86, and 1.0 mol fraction H_2O). Particular care was taken to insure that protein concentrations were uniform and that solvent composition was precise (Loh & Markley, 1993). To establish that an exchange had gone essentially to completion, we dissolved a nuclease sample in 100% D_2O under the conditions of the experiment (45 °C, pH 5.5) and observed the ^1H signals as a function of time as they decayed to values beyond detection. This took approximately 1 day for unligated nuclease H124L and 10 days for the nuclease H124L·Ca^{2+}·pdTp ternary complex. Samples studied by NMR were allowed to equilibrate for times at least as long as these.

Each sample used for NMR analysis consisted of 3.0 mM protein in 50 mM succinate-d_4 at pH 5.5. Samples of the nuclease H124L·Ca^{2+}·pdTp ternary complex contained an additional 9.0 mM pdTp (Pharmacia LKB Biotechnology) and 18 mM ultrapure $CaCl_2$ (EM Science). Spectroscopy was performed at 45 °C on Bruker AM500 and AM600 spectrometers. Two-dimensional ^1H–^{15}N spectra were collected by using the HSMQC pulse sequence of Zuiderweg (1990). Solvent suppression was by means of a 2.0-s DANTE presaturation pulse train (Morris & Freeman, 1978), and ^{15}N decoupling was achieved by a GARP pulse sequence applied during acquisition (Shaka et al., 1983). Since it was anticipated that the replacement of a substantial number of labile protons with deuterons would eliminate cross-relaxation pathways and cause an increase in amide proton T_1 relaxation times, a relatively long recycle time (2.3 s) was employed in the NMR experiments. The average T_1 for the backbone amide protons of nuclease H124L in 10% D_2O was determined by inversion–recovery experiments to be ~0.5 s (data not shown).

HSMQC spectra consisted of 512 t_1 increments (32 transients each) of 2048 complex data points. Prior to Fourier transformation, the t_1 and t_2 dimensions were apodized by shifted sine-bell and Gaussian functions, respectively, and zero-filled to yield final digital resolutions of 2 Hz/point on each axis. Cross-peak volumes were measured by using the volume integration utility of the FELIX software package (release 2.05; Hare Research, Inc.) and were normalized to that of an external ^{15}N-acetylglycine reference, which was contained in a capillary (present in the 5-mm NMR tube) that was transferred from sample to sample. Fractionation factors were obtained by linear least-squares analysis of

$$1/y = C[\varphi(1-x)/(x) + 1] \quad (2)$$

where y is the peak volume, x is the mole fraction of H_2O, and C is a normalization parameter. Each $1/y$ point was weighted by y[4]. The φ values reported are the average of those obtained in duplicate experiments. On the basis of the confidence limits of the least-squares fits and the reproducibility of the data, the uncertainty in φ values is estimated to be ~10%.

RESULTS

Over 80% of the 143 non-proline backbone ^{15}N and ^1H resonances of unligated and ligated nuclease H124L have been assigned previously (Wang et al., 1990a,b, 1992). Of these, approximately 80 NH's were sufficiently well resolved in two-dimensional spectra to permit analysis of their isotope fractionation ratios. Figure 1 contains portions of the ^1H–^{15}N data sets obtained for seven samples differing in their mole fraction of H_2O. The region of the spectra that contains the NH resonance of T120 is shown to illustrate the observed differences in φ values. Of the peaks displayed in Figure 1, the volume of T120 (in natural isotopic abundance H_2O solution) is smaller than those of all residues except R35, D77, Y93, D95, and M98. It is approximately one-half the volume of the V111 cross peak under these conditions. Yet in the spectrum of the 14% H_2O sample, the T120 cross peak is the only signal still present at the contour level plotted. This is not due to incomplete exchange; the half-life of the T120 NH was observed to be less than 0.5 h in D_2O at 37 °C and pH 5.5 (Loh et al., 1993). Rather, this result emphasizes the strong preference for H over D at this position.

Although fractionation factors can be obtained, in principle, from a single spectrum of known intermediate solvent composition, for example, 50% D_2O, it is more accurate to estimate φ from a series of samples containing different H_2O/D_2O ratios. This is illustrated in Figure 2 for two of the cross peaks shown in Figure 1. The implicit assumption is that φ at a single site is independent of the difference in occupancy (H or D) at the other sites over the rage of the solvent composition. The plot of the normalized peak volume as a function of the mole fraction of H_2O in the solvent displays an upward or downward bowing of the straight line of unit slope (φ = 1.0) as a particular site becomes enriched in H or D, respectively (Figure 2A). When plotted in the form given by eq 2, the effect of different φ values is on the slope of the linearized function (Figure 2B).

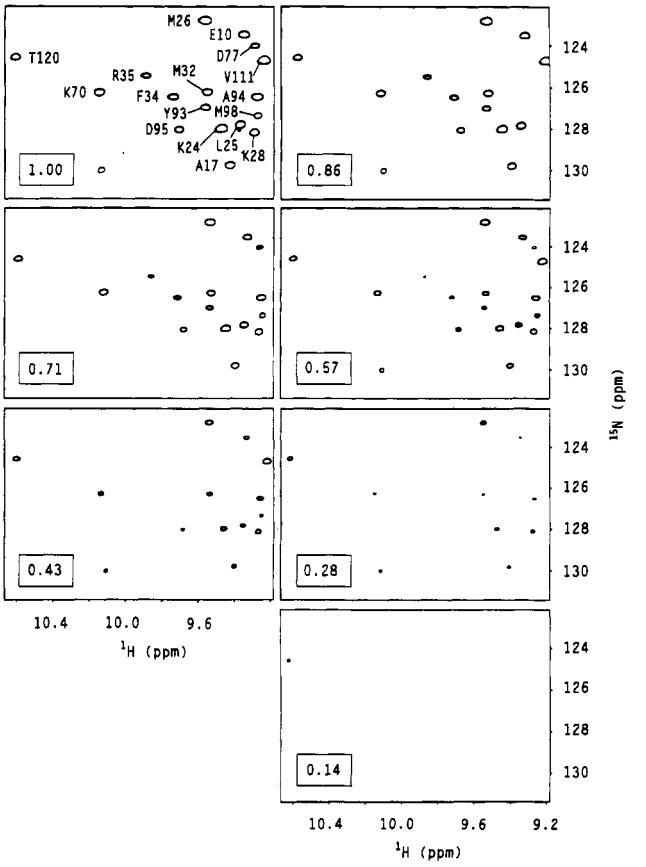

FIGURE 1: Portion of the 500-MHz ^1H–^{15}N HSMQC spectra of the nuclease H124L·Ca^{2+}·pdTp ternary complex acquired for samples at equilibrium in a solvent containing seven different mole fractions of H_2O/D_2O (as indicated in insets to each spectrum).

The fractionation factors of the backbone amide hydrogens in ligated and unligated nuclease H124L are displayed by residue number in Figure 3 and in tabular form in the supplementary material for this article (Table S-I, supplementary material). The distributions of φ values for both proteins species are roughly bell-shaped and centered around 0.8 (Figure 4). In order to draw qualitative comparisons between fractionation factors and hydrogen-bonding patterns, residues were grouped by secondary structure and by solvent exposure of their amide hydrogens, as determined from the X-ray crystal structures of unligated (Hynes & Fox, 1991) and ligated nuclease wt (Loll & Lattman, 1989); results are

[4] Although examination of the X-ray crystal structures revealed no solvent-exposed amide hydrogen (with the exception of E43) to be involved in an intramolecular hydrogen bond, several of these hydrogens (most notably V39) exhibited significant protection in hydrogen-exchange experiments (Loh et al., 1993). These residues were not considered in the statistical analysis since slow-exchange kinetics is not consistent with a solvent-exposed hydrogen being bonded only to the solvent.

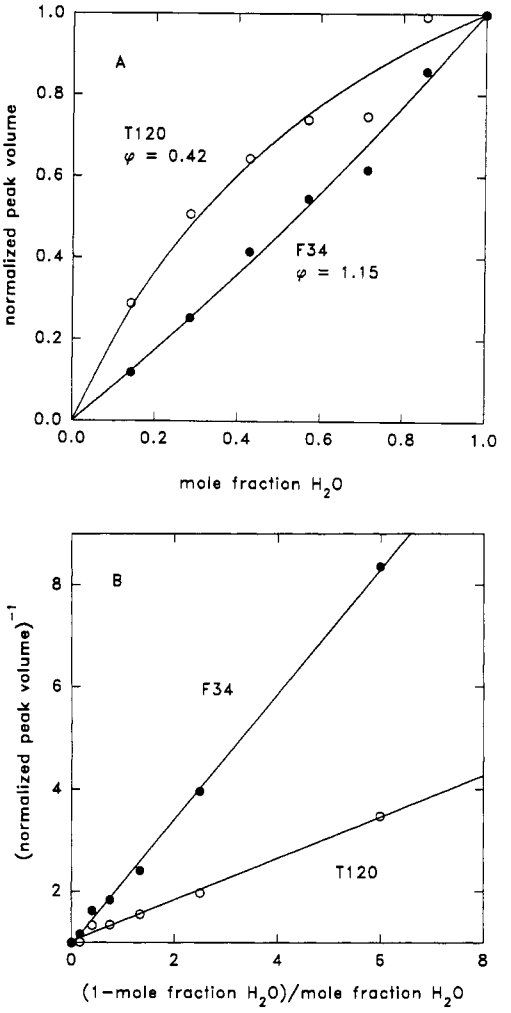

FIGURE 2: (A) Plots of the relative NH peak volumes of T120 and F34 (see Figure 1) as a function of the mole fraction of H_2O in the solvent. (B) The same data plotted in the form given by eq 2.

summarized in Table 1. Only those solvent-exposed NH's that were observed to exchange rapidly with the solvent (Loh et al., 1993) were included in the latter category. Rapid exchange was taken as an indication of hydrogen bonding to the bulk solvent and not to other protein groups.[4] In addition, the solvent-exposed NH's of T82 and T33 were not included for the reason that threonine residues exhibit φ values that are consistently well below those of other residues (Table S–I, supplementary material). The reason for this discrepancy is not known. One possibility is that the $O^{\gamma 1}$ of the side chain is able to accept a hydrogen bond from the amide group. We do not regard this as likely, however, since inspection of wire models shows the geometry of these bonds to be unfavorable. In addition, this mechanism predicts that serine residue should show the same anomalous behavior, whereas in fact they do not.

Previous studies of low molecular weight model compounds have established a correlation between low fractionation factors and short heavy-atom interatomic distances (Kreevoy & Liang, 1980). Although none of the amide hydrogens can participate in low-barrier hydrogen bonds when free in solution (this requires two groups of similar pK_a), it is conceivable that geometric constraints within the protein might act to shorten the heavy-atom distance to a value approaching the low-barrier limit of ~2.55 Å. A plot of fractionation factor as a function of N–O distance for the amide–amide hydrogen bonds present in unligated nuclease wt (Hynes & Fox, 1991) is shown in Figure 5.

The residues that consistently displayed the lowest and highest fractionation factors (Figure 3) in unligated and ligated nuclease were T120 (0.28, unligated; 0.39, ligated) and L38 (1.23, unligated; 1.47, ligated). The amide hydrogen of L38 is solvent-exposed in the X-ray crystal structures of both the unligated and ligated proteins and is not involved in a hydrogen bond to a protein acceptor group. In contrast, T120 participates in a number of hydrogen bonds that are believed to be important to the conformation and stability of nuclease. The environment surrounding T120 is depicted in Figure 6.

DISCUSSION

Distribution of Fractionation Factors. Staphylococcal nuclease exhibits a large range in fractionation factors. Values as low as 0.3 and as high as 1.5 were observed (Figures 3 and 4), with the average value being ~0.85 for both the ligated and unligated species (Table 1). This result was not anticipated since, in previous model compound studies, fractionation factors below ~0.8 were only observed in strong hydrogen bonds involving a proton shared between two groups of similar pK_a. Moreover, φ values in water are closer to unity than values obtained for similar compounds in acetonitrile or dimethyl sulfoxide (Kreevoy & Liang, 1980). However, the environment around the majority of NH's in the present study cannot be described as aqueous, since the interior of a protein is less similar to an aqueous solution that it is to an organic solution. One might expect the fractionation factors of solvent-exposed NH's to more closely reflect the (near-unity) φ values obtained for amide groups in water, especially since none of them (with the exception of the E43 NH) were observed in the crystal structures to be intramolecularly hydrogen-bonded. The average fractionation factors of these residues (0.98 compared to the overall average of 0.85; Table 1) would seem to indicate that this is so, although the large standard deviations associated with these values caution against assigning too much importance to the difference. Individual solvent-exposed amides exhibited large departures from the average value: examples include Q123 (in α-helix 3) and Y113–K116 (involved in a type VIa turn), both of which have low φ values (Figure 3).

Among the categories listed in Table 1, residues in α-helices exhibited the lowest average fractionation factor (0.79), as well as the lowest standard deviation (0.10). The average φ value for these residues is 20% lower than that of the solvent-exposed NH's, although the difference still falls within the sum of the standard deviations. It is tempting to use this result to speculate on the origin of the stability of the α-helix in aqueous solution. It has recently been shown that short, alanine-containing peptides form stable helices in H_2O and that this process is enthalpy-driven (Marqusee et al., 1989; Marqusee & Baldwin, 1987). One possible source of this driving force is a favorable enthalpy change upon the formation of amide H-bonds in H_2O, as suggested by Schellman (1955). The experimental observation that the helix is destabilized by solvents that form strong hydrogen bonds (and stabilized by solvents that form weak hydrogen bonds) lends support to this view (Nelson & Kallenbach, 1986; Nemethy et al., 1981). Our results indicate that the NH sites of all three α-helices in staphylococcal nuclease become significantly enriched in protium relative to its content in the solvent. This suggests a difference in the energetics of amide–amide hydrogen bonds present in α-helices and those of the amide–H_2O bonds present in solvent-exposed residues. Given the experimental prece-

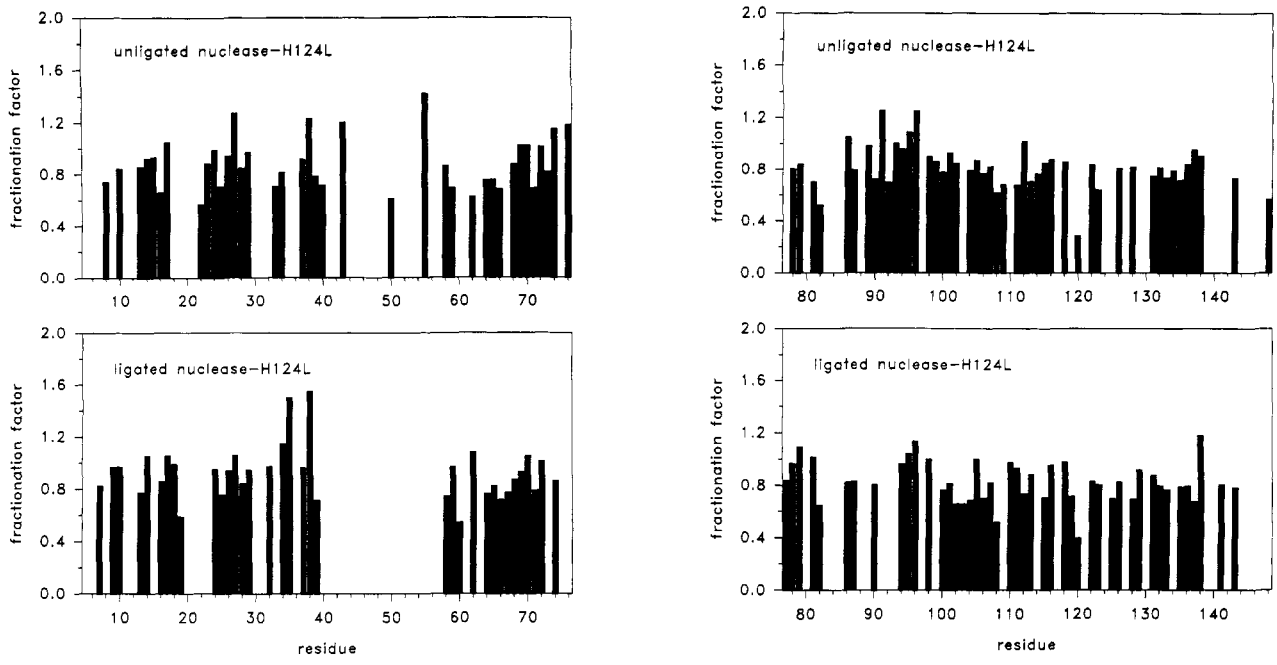

FIGURE 3: Fractionation factors for the backbone amide hydrogens of unligated (top) and ligated (bottom) nuclease H124L displayed by residue number. Errors are estimated to be ~10%.

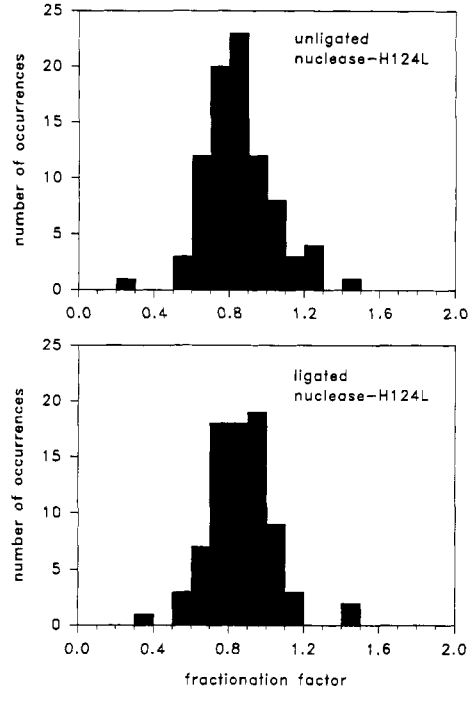

FIGURE 4: Histograms showing the distribution of backbone amide hydrogen fractionation factors for unligated (top) and ligated (bottom) nuclease H124L.

Table 1: Distribution of Fractionation Factors in Staphylococcal Nuclease

residues	average φ	standard deviation	number of residues
all (unligated nuclease)	0.84	0.19	83
all (ligated nuclease)	0.86	0.17	79
α-helix[a]	0.79	0.10	51
β-sheet[a]	0.91	0.15	36
turn[a]	0.88	0.25	29
solvent-exposed[a,b]	0.98	0.18	25

[a] For both ligated and unligated nuclease. [b] Includes only NH's known to exchange rapidly with the solvent (Loh et al., 1993) and excludes threonine residues (see text).

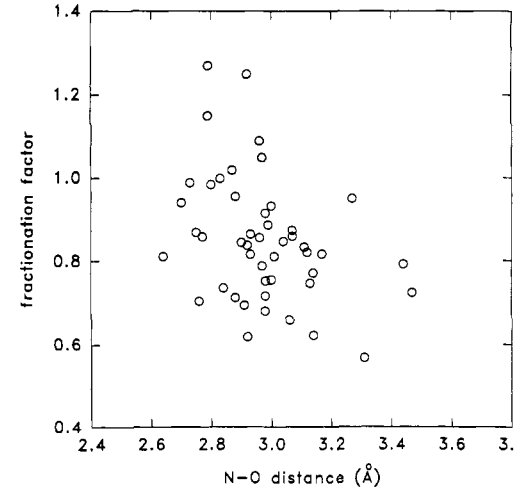

FIGURE 5: Relationship between the N–O hydrogen-bond distances in unligated nuclease wt and fractionation factors of unligated nuclease H124L. Interatomic distances were taken from the X-ray crystal structure of Hynes and Fox (1991).

dence for low fractionation factors, this result is consistent with intrachain amide hydrogen bonds in α-helices being stronger than those formed between the peptide and the H_2O in the random coil and those formed between H_2O and H_2O in the bulk solution.

The average φ values for β-sheet and reverse turn structures were found to lie between the extremes set by the solvent-exposed and α-helical residues (Table 1). No trends are obvious from these data. We do note, however, that turns exhibited a particularly broad distribution of fractionation factors. The highest φ value (1.42 for G55 in unligated nuclease) as well as the lowest (0.34 for T120, averaged over all measurements) were observed in type II and type I turns, respectively.

The plot of fractionation factors as a function of N–O distance for the amide–amide hydrogen bonds observed in the crystal structure of unligated nuclease wt shows no obvious

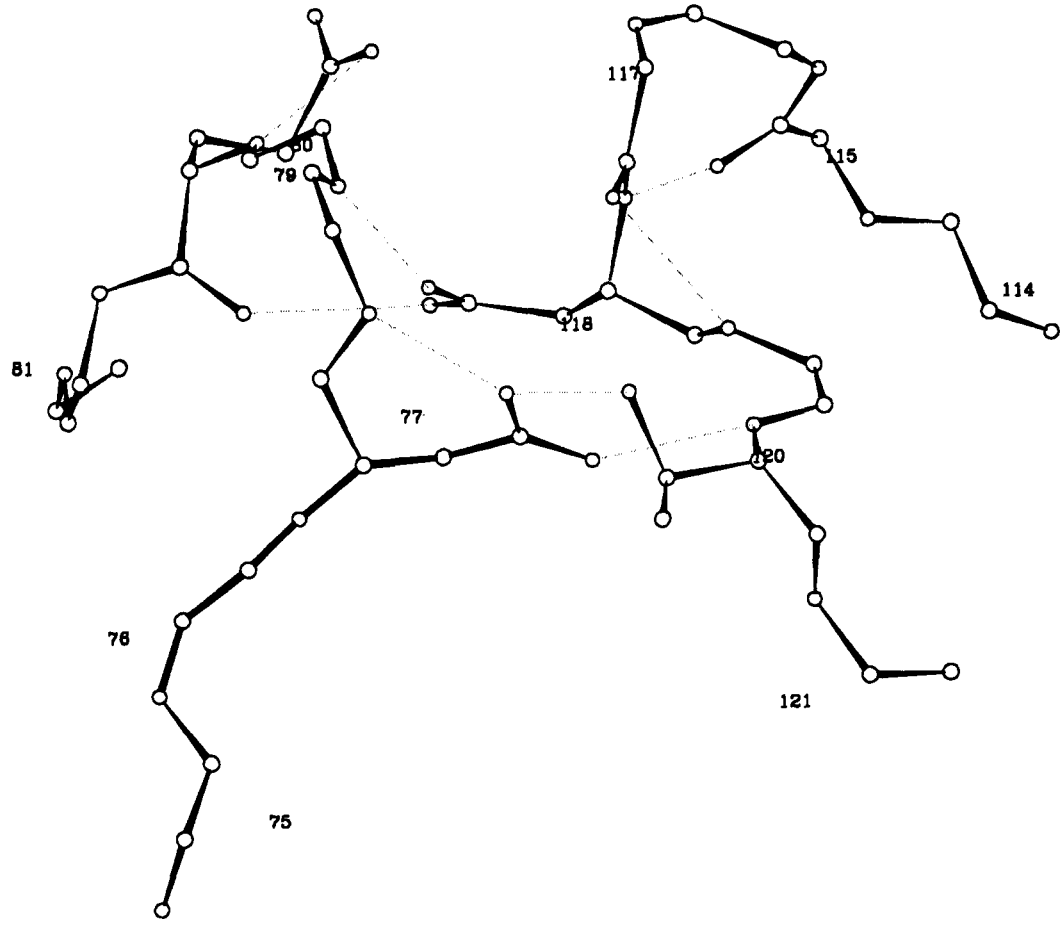

FIGURE 6: Backbone trace of a portion of the X-ray crystal structure of unligated nuclease wt (Hynes & Fox, 1991) showing the environment around T120. The left- and right-side loops consist of residues 75–81 and 114–121, respectively. Hydrogen bonds are indicated by dashed lines. Side chains and carbonyl oxygen atoms involved in hydrogen-bonding interactions have been included to illustrate the contacts between the two loops.

correlation (Figure 5). The NH hydrogens displaying the lowest φ values display typical N–O hydrogen-bond lengths (e.g., T120, 2.89 Å; L108, 2.92 Å; T22, 3.31 Å). There are several possible causes for this lack of correlation. First, the difference in heavy-atom distance between "normal" and low-barrier hydrogen bonds can be less than 0.3 Å (Kreevoy & Liang, 1980). The changes in bond length expected to correlate with the φ values may be below the limit of detection of the X-ray method in this case. Constraints imposed during the crystallographic refinement process (such as peptide group planarity, bond length, and bond angle constraints) may serve to obscure what is expected to be an already delicate relationship between bond length and the fractionation factor (see below). An additional consideration is that the fractionation factor may indeed be correlated with N–O distance, but that the hydrogen-bond strengths and N–O distances are different in the solution and crystalline states. The remaining possibility is that other mechanisms besides bond length are responsible for the observed lowering of φ values. When interpreting fractionation factors in terms of low-barrier hydrogen bonds (i.e., in the framework of in-line oscillations of the hydrogen between the two heavy atoms), other bending modes tend to be overlooked. Theories on the relative importance of off-line motions with regard to fractionation factors have not been developed by either theoretical or experimental means. Schowen and Schowen (1982) argue that hydrogen bonding and other interactions can either raise or lower the fractionation factor, depending on which vibrational mode is most affected. The extent to which the off-line frequencies may be mediated by the many noncovalent interactions present in the interior of a protein remains unknown. Therefore, the lack of a simple relationship between bond lengths from X-ray data and φ values may not be unexpected.

Comparison of Equilibrium and Kinetic Isotope Exchange Data. In native proteins, amide hydrogens that are highly protected from exchange with the solvent are found overwhelmingly in regions of secondary structure (α-helices and β-sheets) that are stabilized by extensive hydrogen bonding (Loh et al., 1993; Radford et al., 1992). According to the unfolding model, exchange of these hydrogens is dependent on breakage of the intramolecular H-bonds (Englander & Kallenbach, 1984; Hvidt & Nielsen, 1966; Linderstrøm-Lang, 1955). One may expect *a priori* that residues with the strongest H-bonds (and lowest fractionation factors) might exhibit the slowest exchange kinetics. The amide groups identified as being highly protected in nuclease H124L (Loh et al., 1993) did not display φ values significantly different from those of residues showing little or no protection. This is the expected result if the opening reaction (local and global) permitting hydrogens to exchange with the solvent is controlled by cooperative breakage of many hydrogen bonds rather than one or two. Without knowledge of the structural features of an unfolding unit, it is difficult to assess the effect of individual hydrogen-bond strengths on exchange rates. The present results, however, predict that the exchange out rates of deuteriated nuclease H124L should be greater than those of the correspondingly protiated enzyme.

The T120–D77 Hydrogen Bond. The results of many experimental and theoretical studies indicate that low frac-

tionation factors correspond to very strong hydrogen bonds. It is therefore of interest to examine in greater detail the residues possessing the lowest observed φ values. The only residue type in nuclease that exhibits a clear trend in fractionation factors is threonine, with an average value of 0.63. This is especially puzzling since T82 and T33 are both solvent-exposed and yet possess φ values of 0.70 or lower. It is difficult to explain these values by a systematic error in the measurements. Incomplete exchange is ruled out by the observation that T120 and T82, the two most anomalous residues, readily exchange their NH's with the solvent (Loh et al., 1993). Other effects such as saturation, rapid exchange, and T_1 perturbation would have the effect of *raising* φ. The NH of T120 reported the lowest fractionation factor of all of the residues in staphylococcal nuclease. These values (0.28 and 0.39 in the absence and presence of bound ligands, respectively) are among the lowest experimental value yet reported for any system. Recent theoretical and experimental results have provided insights into a possible basis for the extremely low fractionation factor of T120 and into the importance of the T120 H^N–D77 $O^{\delta 1}$ hydrogen bond. *Ab initio* calculations of a dimer of two amino acid derivatives, *cis-N*-(2-carboxyethyl)formamide and 3-ammoniopropanamide, yielded φ values of approximately 0.6 for the two amide hydrogens involved in *intra*molecular H-bonds to the charged carboxy and amino groups (Edison, 1993; A. S. Edison, F. Weinhold, and J. L. Markley, manuscript in preparation). Interestingly, these values returned to near unity when the *inter*molecular hydrogen bond was broken. The aforementioned intramolecular H-bonds were found to more than double in strength as a result of dimerization through formation of the intermolecular amide–amide H-bond. This result, which draws a comparison to the case of the charged T120 H^N–D77 $O^{\delta 1}$ hydrogen bond found in nuclease H124L, suggests that a charged H-bond can experience a dramatic decrease in φ—together with a significant increase in strength—as a result of *cooperative* hydrogen-bond formation facilitated by an amide–amide interaction. Cooperativity is enhanced by the presence of positive and negative charges at the ends of the amide groups, which serve to stabilize the alternate amide resonance form.

Site-directed mutagenesis experiments support the notion that the T120 H^N–D77 $O^{\delta 1}$ hydrogen bond is of prime importance to the structure and stability of the native state of nuclease H124L. T120 is found in a loop that is in close proximity to another loop centered around D77. An extensive hydrogen-bond network links residues 118–121 and 75–80 on opposing loops (Figure 6). T120 donates its H^N and $H^{\gamma 1}$ to $O^{\delta 1}$ and $O^{\delta 2}$ of D77, respectively. Removal of the carboxyl group of D77 by the D77 → A substitution produces two conspicuous effects: (1) the position of the cis ⇌ trans equilibrium about the K116–P117 peptide bond shifts from 95% cis to ≥98% trans; (2) the stability of the enzyme toward denaturation by guanidine hydrochloride decreases by 3.1 kcal mol^{-1} (approximately 50% of the total free energy of stabilization; Hinck, 1993). Replacement of T120 with alanine presumably eliminates the side chain–side chain hydrogen bond, leaving the 120 H^N–77 $O^{\delta 1}$ bond intact. The effects of this mutation on the cis ⇌ trans equilibrium and on stability are much less pronounced. These results illustrate the importance of the hydrogen bond formed between the NH of T120 and the side chain of D77.

Effects of Ligand Binding. The residues that exhibited significant increases in φ values (>20%) as a result of ligand binding are F34, L38, S59, T62, G79, R81, V111, A112, and N138. Those residues whose fractionation factors decreased significantly on complex formation are Y27, V74, and L137. Large differences were seen in the crystal structures of complexed and uncomplexed nuclease wt in the segment from A112 to Y115 (Hynes & Fox, 1991). Differences in $^1H^N$ and ^{15}N chemical shifts upon complexation were found to be largest around residues 19, 34, 38, 110, and 115 (Wang et al., 1992). In addition, we note that R81 is close to the nucleotide-binding pocket consisting of residues K84, Y85, and R87. The amide groups of G79 and R81 are connected through mutual hydrogen bonds to the side chain of N118, providing a means for propagating changes at the nucleotide-binding site to G79. Thus, one-half of the residues that show significant changes in φ on binding ligands also exhibit changes when comparing X-ray or NMR data for the uncomplexed and complexed enzymes.

It is more difficult to reconcile the observed differences in the remaining residues for which φ values were found to change appreciably upon complexation (Y27, S59, T62, V74, L137 and N138). S59 and T62 are linked by a hydrogen bond between their amide groups; L137 and N138 are linked sequentially. These connectivities may provide an as yet unknown mechanism by which changes in the φ values of one residue might be transmitted to its partner. The fact that differences in φ were noted where differences in chemical shifts were not suggests that fractionation factors may be a probe for subtle changes in protein structure, hydration, or other effects.

The basis for the observed changes in the fractionation factors as a result of inhibitor binding remains unknown, as do the origins of the extreme φ values displayed by residues such as T120, L108, T22, G55, and L38. The present results do, however, suggest an important role for the T120–D77 hydrogen bond in maintaining the stability and conformation of the native state of the protein. Overall, we can conclude that staphylococcal nuclease becomes enriched in protium relative to its content in the mixed H_2O/D_2O solvent. On average, protium is accumulated to the greatest extent in α-helices and to the least extent in sites that are exposed to the solvent. Whether or not this isotope preference indicates that intrachain amide hydrogen bonds are stronger than those formed between amide groups and water is a question we are attempting to answer with studies of model compounds.

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SUPPLEMENTARY MATERIAL AVAILABLE

Table listing amide hydrogen D/H fractionation factors of staphylococcal nuclease H124L (5 pages). Ordering information is given on any current masthead page.

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