Structure and Dynamics of Cytochrome c in Nonaqueous Solvents by 2D NH-Exchange NMR Spectroscopy

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Abstract: Recent biocatalysis studies have shown that many enzymes exhibit catalytic activity and enhanced thermal stability in essentially anhydrous nonpolar solvents. At present we do not understand the important role that water plays in protein structure stabilization and in enzyme mechanisms in nonaqueous environments. In this study, the NH-exchange rates of oxidized horse cytochrome *c* suspended in tetrahydrofuran (THF) containing 1% D_2O (vol/vol) at 37 °C, pH 8.9, have been determined indirectly by 2D NH-exchange NMR spectroscopy. In such a solvent system, we have allowed just enough water molecules to form approximately a monolayer surrounding the protein. A hydrophobic tripeptide *N*-acetyl-$(Ala)_3$-OCH_3 was used to calculate the intrinsic amide-exchange rate directly for a random-coil peptide in THF/1% D_2O. The relative solvent protection factors for amide exchange of cytochrome *c* in THF/1% D_2O have been determined for 35 amide protons and compared with the results obtained at the same pH in aqueous solution. Almost all of these protected protons are located in the three major α-helical regions or involved in crystallographically defined intramolecular hydrogen bonding. In both aqueous solution and THF/1% D_2O at pH 8.9, the NH-exchange profile for cytochrome *c* is similar to that in the native state (pD 7.0: Jeng, et al., *Biochemistry* **1990**, *29*, 10433–10437). The protection factors against exchange were greatest for the NH protons of Phe10 and those in the central part of the C-terminal helix, Ile95, Tyr97, and Leu98. The protection factors from the majority of observable NH protons for cytochrome *c* in purely aqueous solution, pH 8.9, vary between 10^4 and 10^8. These are about <50-fold smaller than those in the native state at pD 7.0 but are 10^3–10^4 larger than those in the molten globule state. The protection factors in THF/1% D_2O at pH 8.9 vary between 10^2 and 10^5, which are in turn 10^2–10^3-fold smaller than those observed at pH 8.9 in purely aqueous solution. These results suggest that while cytochrome *c* remains folded and compact at pH 8.9 in both purely aqueous solution and THF/1% D_2O, the flexibility of the protein is clearly enhanced, especially in the organic medium. However, some differences were also observed between the two solvent systems. Most noticeably part of the C-terminal helix (Leu94, Ala96, and Lys99) has the lowest NH-exchange rates in D_2O, whereas in THF/1% D_2O these exchange rates increased dramatically. This may reflect differences in tertiary interactions in the two solvents. The N-terminal and C-terminal helices fold over each other in the native structure. The decrease in protection factor for those residues of the C-terminal helix facing the N-terminal helix suggests that this helix interaction is destabilized in the hydrated organic solvent, exposing to the surface residues Leu94, Ala96, and Lys99. These hydrogen-exchange results on cytochrome *c* suggest that water serves to "lubricate" the local dynamics of the protein in THF/1% D_2O and that the slightly hydrated organic solvent enhances the dynamic flexibility of all regions of the native structure.

Introduction

What is the role of water in the stabilization of protein structure and the catalytic activity of enzymes? It is generally believed that water stabilizes the native conformation of proteins by driving the hydrophobic regions into the interior as well as specifically stabilizing various ionic and polar interactions at the surface. While nonaqueous environments completely or partially denature selected portions of proteins, surprisingly many enzymes have catalytic activity in essentially anhydrous nonpolar solvent.[2-5] However, enzyme activity can be greatly altered in nonaqueous media,[4-6] and to understand these changes we must understand the structural changes of proteins in these unusual environments.

With the development of two-dimensional NMR methods for the specific assignment of many of the ^1H NMR signals of small proteins,[7,8] it is now possible to determine three-dimensional solution structures for these proteins. Until quite recently the NMR study of non-native protein structures was not possible. An amide proton-exchange methodology[9-15] makes it possible to study the structure of proteins in native, partially and transiently unfolded states of proteins. Although nearly all studies have been limited to the study of protein structures in soluble aqueous solutions, the methodology can be extended to other unusual environments as well.[16,17]

In this study, we extend the NMR NH-exchange methodology to allow us to probe the role of water in defining the structure and dynamics of proteins in nonaqueous solutions. Essentially we take advantage of the differential rate of amide proton exchange with D_2O in proteins dependent upon the secondary and tertiary structure of the protein (as well as the exposure of

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the amide protons to solvent).[12-15,18] If the protein is partially unfolded or in an altered conformation when it is in a nonaqueous solution (even a suspension in a nearly anhydrous solvent), the rate of NH exchange will be perturbed for individually identified residues in the protein. By quenching the NH exchange and stabilizing the protein in solution where no further exchange will take place, a 2D TOCSY NMR spectrum will reveal which protons have been exchanged (this assumes that the proton spectrum of the protein has been previously assigned). The important point is that we do not have to find particular NMR conditions where high-quality 2D NMR spectra must be taken in non-native environments to observe the various resolvable NH signals. The protein can be studied under conditions where high-resolution resolvable signals would otherwise not be observed—this would be true for a protein in suspensions of mixed water/nonaqueous solutions or even essentially anhydrous solvents. We can also study transient changes under these conditions and not be concerned about the long acquisition times normally required for 2D NMR spectra. Similar methods have most recently been successfully used to study the rate of NH exchange of proteins in the crystalline[17] and denatured states.[19]

Horse cytochrome *c*, an important heme-containing protein functioning principally as an electron carrier in the mitochondrial electron-transport chain, has been chosen as the model protein in this study because of its reasonable size, the globular structure, and the availability of both its X-ray crystal[20] and solution NMR[21] structures. A solvent system of tetrahydrofuran (THF) (a water-miscible nonpolar solvent) containing 1% D_2O (vol/vol) was used in the study.

Experimental Procedures

Materials. Horse heart cytochrome *c*, *N*-acetyl-$(Ala)_3$-methyl ester ($NAc-(Ala)_3-OCH_3$), Gly-Gly-His, and deuterated tetrahydrofuran were purchased from Sigma Chemical Co. The anhydrous tetrahydrofuran (THF) was purchased from Aldrich Chemical Co.

Horse heart cytochrome *c* was fully oxidized with a slight excess of $K_3Fe(CN)_6$ and dialyzed using a home-made hollow cellular fiber. The protein solution (4 mM) was adjusted to pH 8.9 and then lyophilized to dryness. The dried protein (~32.2 mg) was incubated with 3 mL of anhydrous THF containing 1% D_2O buffer (50 mM NaPi, 0.1 M NaCl, pD 8.1—pD represents the pH-electrode reading in D_2O). This mixture was magnetically stirred at 37 °C for varying times to maintain a uniform suspension throughout the THF. In a separate pH measurement, adding such small amounts of pure D_2O buffer did not alter the pH of the protein solution. The solvent was removed by suction filtering, and cytochrome *c* was frozen at liquid nitrogen temperatures and lyophilized. To obtain the first time point ($t = 0$) for unexchanged protein, the same amount of dried protein was incubated with 3 mL of anhydrous THF containing 1% H_2O buffer (50 mM NaPi, 0.1 M NaCl, pH 7.7) at 37 °C for 24 h with magnetic stirring and then treated in a similar manner.

For the NH exchange in slightly alkaline purely aqueous solution, dry protein (~32.2 mg, obtained from lyophilization at pH 8.9) was incubated with 0.65 mL of D_2O containing 30 μL of D_2O buffer (50 mM NaPi, 0.1 M NaCl, pD 8.1) at 24 °C for varying amounts of time with magnetic stirring of the homogeneous solution. The final pD of the cytochrome *c* solution in D_2O was 9.3. The exchange was quenched by freezing the sample in liquid nitrogen and subsequent removal of D_2O by lyophilization. For the NMR study the lyophilized protein was dissolved in 0.65 mL of D_2O buffer (50 mM NaPi, 0.1 M NaCl, pD 2.1), and the final NMR sample contained ~4 mM protein at pD 5.1.

The NH exchange of $NAc-(Ala)_3-OCH_3$ was performed by dissolving 1.0 mg of dry peptide in 0.65 mL of deuterated THF containing 1% D_2O (pH 7.6). The homogeneous solution was transferred to a 5-mm NMR tube, 1D ^1H NMR spectra were collected immediately at 20 °C, and subsequent NMR spectra were obtained at 5-min intervals to monitor the intensity decrease for the NH resonances.

The NH exchange of Gly-Gly-His was performed by dissolving 1.0 mg of dry peptide (obtained from lyophilization of the peptide in 3.5 mM NaPi buffer, pH 7.3) in 0.60 mL of THF containing 1% D_2O at 24 °C for various times with magnetic stirring of the heterogeneous suspension. The solvent was removed by suction filtration, and the peptide was frozen at liquid nitrogen temperature and lyophilized. The lyophilized peptide was dissolved in 0.65 mL of DMSO-d_6, and the 1D ^1H NMR spectra were collected. To obtain the first time point ($t = 0$) for unexchanged peptide, the same amount of peptide was incubated with 0.60 mL of THF containing 1% H_2O at 24 °C for 3 h with magnetic stirring.

The water content of the cytochrome *c* and THF was determined by ^1H NMR spectroscopy. The dry protein (10 mg, obtained from lyophilization at pH 8.9) or THF (4% volume) was added to 0.65 mL of D_2O containing 0.75% 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The solution was transferred to a 5-mm NMR tube, and 1D ^1H NMR spectra were collected with a long delay time (30 s) between acquisitions to ensure complete relaxation. The H_2O resonance was integrated relative to the internal DSS protons. The H_2O integral in the protein solution represents the total H_2O retained by the protein and all exchangable NH and side-chain protons from the protein (179 per cytochrome *c* molecule) because most of these protons completely exchange with D_2O within 20 min at pH 8.9. For calibration, various amounts of H_2O (up to 2.0% by volume) were added to the D_2O containing 0.75% DSS and the 1D ^1H NMR spectra collected. The H_2O signal was integrated to obtain a calibration curve of H_2O content vs H_2O integral value using the same DSS reference peak.

All 1D and 2D ^1H NMR spectra were obtained on a Varian VXR-500 NMR spectrometer. The pure absorption phase 2D TOCSY[22,23] NMR spectra were acquired with 512 complex points in the t_1 dimension and 2048 points in the t_2 dimension using a mixing time of 30 ms and a sweep width of ~8000 Hz. Water suppression was obtained by irradiation of the HOD signal. Thirty-two scans were acquired per t_1 value with a delay time of 0.8 s between scans, and total acquisition time was about 5.5 h per sample. Zero-filling (×2) was applied in the t_1 dimension. The data were processed with an 85° shifted sine-bell function. The final digital resolution was 4 Hz/point in both dimensions.

The intensity of individual NH–$C_\alpha H$ crosspeaks in the 2D TOCSY spectra was integrated relative to the aromatic H3–H6 crosspeak of Tyr97. The error in peak volume measurement was estimated to be about ±15%. For the NH exchange of $NAc-(Ala)_3-OCH_3$ and Gly-Gly-His, the 1D NH peaks were integrated relative to the $C_\alpha H$ protons of the Ala residues and the H2 and H4 protons of the His residue, respectively. The exchange rates were then determined using a nonlinear least squares fit (MathCAD, MathSoft, Inc., Cambridge, MA) of the exponential decay of NH resonance or NH–$C_\alpha H$ crosspeak as a function of H–D-exchange time.

In D_2O, the average value for residual NH signal after complete exchange for most residues is ~3%, whereas in THF containing 1% D_2O the residual NH signal for most of the NH protons after complete exchange is 25–55% (average ~36%; of course there is much less D_2O in the hydrated THF). For the random-coil peptide $NAc-(Ala)_3-OCH_3$, the three NH protons exchange up to ~91% in THF containing 1% D_2O. By taking into account all of the exchangeable protons, these differences can be attributed to the presence of ~79 internal H_2O's per cytochrome *c* (10% by weight) in the dried lyophilized powder. This is not unreasonable, since many protein crystals can contain up to 90% water and lyophilization does not completely strip this water from protein in the solid state.[24] In the NH-exchange media of THF/D_2O (99/1, vol/vol), the molar ratio between added D_2O and cytochrome *c* is ~640 or on a per residue basis 6:1. At the completion of NH exchange, the NH–$C_\alpha H$ crosspeak intensities in terms of proton occupancy represent the total amount of H_2O molecules in the solvent and protein and all exchangeable NH and side-chain protons from protein. Therefore, the total additional number of H_2O molecules present in the exchange medium of THF/1% D_2O was 265 per cytochrome *c* molecule. These H_2O molecules orginated from the protein, THF solvent, and moisture absorbed while the sample was exposed to the atmosphere. On the basis of the effective surface area of a bulk water molecule and the protein surface, we estimate that there are enough water molecules to form a monolayer surrounding the cytochrome *c* molecule.

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Results and Discussion

Experimental Design. In order to examine the structure and dynamics of proteins in hydrated organic media, the NH-exchange methodology[12-14,17] was modified to determine the amide hydrogen-exchange rates of ferricytochrome *c* in THF/1% D_2O. In this study 2D NMR methods were used to identify amide protons of individual amino acid residues. The rate of hydrogen exchange depends on the pH (being both acid and base catalyzed), the accessibility of the proton to solvent, the stabilization by hydrogen bonding, the secondary as well as tertiary structure, and the chemical environment surrounding the NH.[12,25,26] The NH-exchange rates of different residues in proteins can vary by as much as 10^8.[1,12]

The measure of relative NH-exchange rates is expressed as a protection factor, $P = k_{in}/k_{obs}$, where k_{in} is the intrinsic rate of exchange for a random-coil protein and k_{obs} is the observed rate of exchange under comparable conditions.[27] These are determined for as many specifically assigned NH protons in the protein as experimentally possible. The solvent exposed intrinsic exchange rate, k_{in}, is measured from simple random-coil peptides in the solvent under study following established procedures.[18] For the intrinsic exchange rate in THF/1% D_2O (k_{in}^{THF}) no corrections for residue type have been made (see below). In this study, we have defined several additional ratios and protection factors. Thus an R_1 factor is defined as k_{D_2O}/k_{THF}, where k_{D_2O} and k_{THF} are the NH-exchange rates of the protein in D_2O and THF/1% D_2O, respectively. Protection factors P_1 and P_2 are defined as k_{in}^{THF}/k_{THF} and $k_{in}^{D_2O}/k_{D_2O}$, respectively. For each amide proton, the intrinsic exchange rate in purely aqueous D_2O, $k_{in}^{D_2O}$, was calculated from data obtained on random-coil peptides according to the pH, temperature, and sequence dependence of NH exchange as described previously.[18,28] The exchange rates were normalized at the same temperature of the protein exchange using the activation energy of 18 kcal/mol for base catalysis of NH exchange.[28]

Because the intrinsic rate of NH exchange is dependent upon solvent conditions, it is important to measure the k_{in}^{THF} for exchange of a random-coil peptide in the mixed nonaqueous solvents. Therefore, a hydrophobic tripeptide, *N*-acetyl-$(Ala)_3$-OCH_3, was dissolved in deuterated THF containing 1% D_2O and used to measure the intrinsic exchange rate directly for a random-coil peptide in THF/1% D_2O, thus allowing the calculation of the protection factors for cytochrome *c* in THF. The exchange rates for the three NH protons of the tripeptide are 0.089, 0.069, and 0.074 min^{-1} at pH 7.6 and 20 °C, giving an average value of 0.077 min^{-1}. The similarity of the exchange rates for the above three amide protons confirms that the tripeptide has little structure in THF. The exchange rates for the two NH protons of Gly-Gly-His in a heterogeneous suspension in THF/1% D_2O (pH 7.3, 24 °C) measured indirectly are 1.6 and 2.7 h^{-1}, respectively. The normalized average value of k_{ex} at pH 7.6 and 20 °C is 0.049 min^{-1}, which is close to the above exchange rate of NAc-$(Ala)_3$-OCH_3 (0.077 min^{-1}). In the calculation of the protection factors (P_1) in THF/1% D_2O, the intrinsic NH-exchange rate determined directly from NAc-$(Ala)_3$-OCH_3 was employed.

In order to obtain the necessary resolution for identifying and measuring NH exchange of individual NH protons, exchange was followed by the time-dependent changes to the 2D spectra. 2D Total correlation spectroscopy (TOCSY) NMR spectra in aqueous solution show crosspeaks between the $C_\alpha H$ and NH proton chemical shifts due to the three bond scalar spin–spin-coupling interactions between these protons (further coherence transfer to other scalar coupled protons is small at the short 30-ms mixing time used) and provided signal to noise ratios comparable to those of the normally used COSY experiment. The intensities vary from one crosspeak to another as a result of differences in scalar coupling constants and transverse relaxation times. The TOCSY experiment gives an in-phase multiplet structure of the crosspeaks, and even crosspeaks between weakly coupled spins can be observed. When D_2O is added to this solution, these crosspeaks gradually disappear due to deuterium exchange of the amide proton. Thus measurement of the volume integral for individual crosspeaks in the TOCSY spectrum at various time intervals and simple nonlinear least squares treatment of the intensity vs time generate the rate constant for exchange, as illustrated in Figures 1 and 2.

Conditions were used such that exchange is slow relative to the time it takes to obtain good-quality 2D TOCSY spectra. Exchange is generally slowest at pH about 3. However, for cytochrome *c*, the minimum NH exchange occurs at pH about 5, probably due to its large excess of basic residues and unusually high isoelectric point (pI > 10). At this pH the rate of exchange is slow enough that a number of 2D amide crosspeaks are observed for residues that are stabilized by the secondary and tertiary protein structure (or alternatively protected from the solvent). In a protein such as cytochrome *c*, many amide crosspeaks are stable to exchange for hours to days or longer at the minimum exchange pH. It is these relatively slowly exchanging signals that can be used to monitor the rate of hydrogen exchange. Almost all of the observed slowly exchanging NH protons are located in buried α-helical regions or involved in the crystallographically defined intramolecular hydrogen bonding. When viewing CPK molecular models of cytochrome *c* (MIDAS Plus software[29]), only small portions of the amide protons of Lys8, Gln12, Lys13, Ile75, Ile85, Glu90, and Arg 91 can be seen. The rest are completely buried.

Hydrogen exchange requires dynamic accessibility of the exchanging solvent with the amide proton. There are currently two different limiting models to explain the variation in NH-exchange rates in proteins.[12-14,27,30] The penetration model assumes that the NH-exchange rate depends on the rate of diffusion of hydroxide ion (at pH > 3, the only effective catalyst in these exchange reactions is hydroxide) to the interior, solvent protected part of the protein. The local unfolding model proposes that exchange occurs via local unfolding of secondary/tertiary structure which allows the NH to contact solvent, leading to rapid exchange followed by refolding. Amide exchange in stable proteins has been analyzed in terms of either model,[27] and significant disagreement still exists as to which one is correct. Certainly, internal hydrogen bonding in secondary structures appears to significantly reduce the rate of NH exchange.[31]

Denaturation of proteins is often a problem for mixed water/organic solvents. It was found that cytochrome *c* exists as a two-phase particle suspension in THF or acetonitrile (either solvent miscible with H_2O) containing 1% H_2O, whereas in cyclohexane or hexane (nonmiscible with H_2O) the protein precipitated out or bound to the glass wall in a three-phase suspension. After the organic solvent was removed and cytochrome *c* was redissolved in aqueous solution, there was no evidence of irreversible denaturation of the protein as examined by 2D TOCSY, NOESY, and CD spectroscopy (data not shown). Almost all of the protein was recovered after exposure to the organic solvent.

Experimentally it was found to be difficult to develop reproducible protocols for measuring exchange under these extreme conditions. The protein must be lyophilized to near dryness before suspending in the hydrated organic solvents. The pH was determined by the pH of the protein solution prior to lyophilization. Zaks and Klibanov[3] have shown that enzymes (or proteins) remain in the same ionization states when transferred from aqueous solutions to anhydrous organic solvents. A pH of

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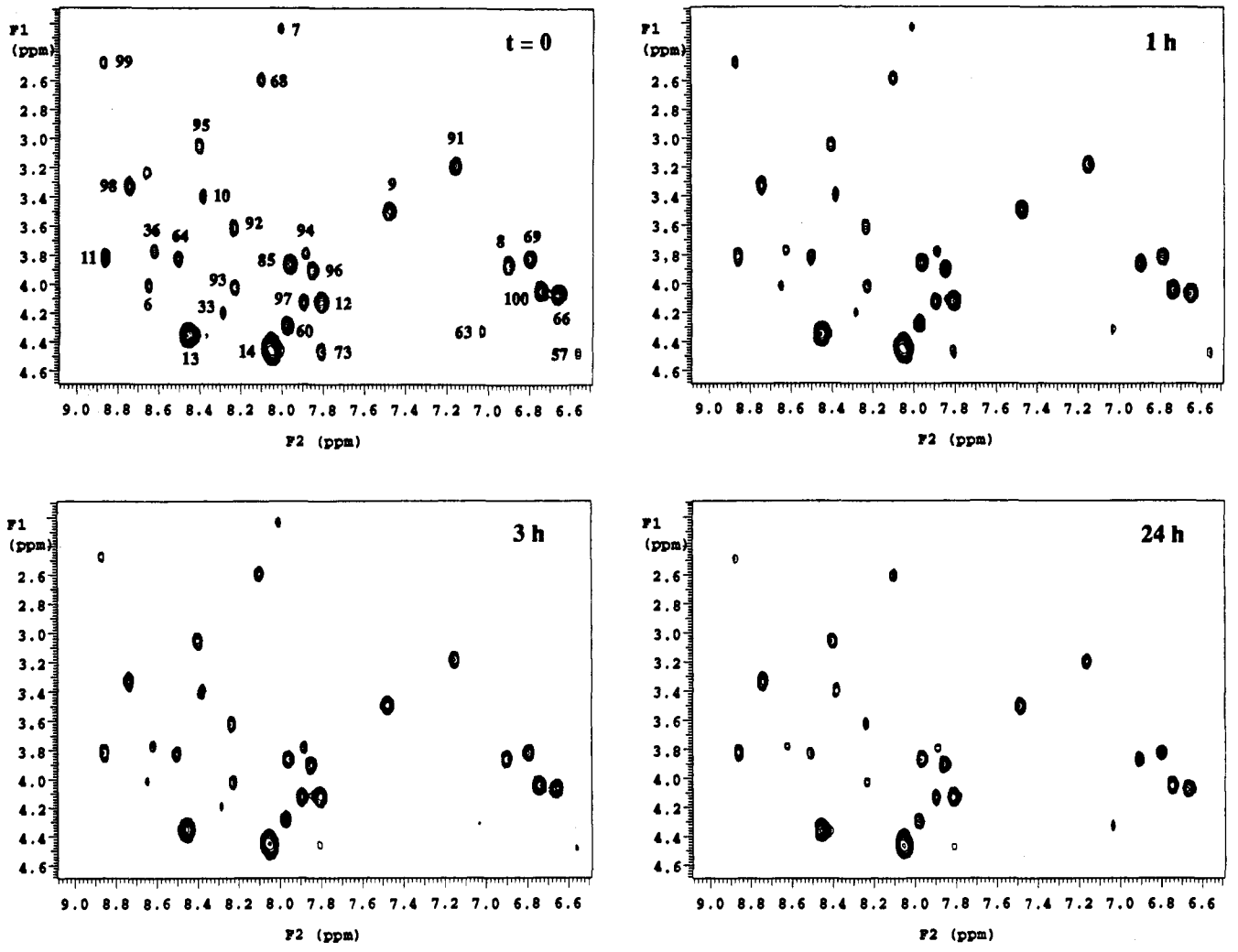

Figure 1. Partial NH–C_αH crosspeaks of 2D TOCSY spectra of horse ferricytochrome *c* in D_2O buffer (pD 5.1), after incubating with THF containing 1% D_2O for various times indicated.

8.9 and a solvent system of THF/1% D_2O were chosen because the rates of NH exchange for cytochrome *c* were much slower when pH < 8.5 or when the D_2O content in THF is below 1% at 20–37 °C. By removing the solvent via filtration and then freezing the protein at liquid N_2 temperature and lyophilizing, residual amide proton intensities could be monitored under slow-exchange conditions (low pH). By following this protocol, reproducible 2D integrations and exchange rates were obtained.

Protection Factors in Purely Aqueous Solution. In order to compare the effect of the THF/1% D_2O suspension on hydrogen exchange, rates were measured at comparable pH in purely aqueous solution. Jeng et al.[1] and Wand et al.[32] have previously measured the protection factors at pD 7.0 (protection factor P_3, Table I) for more than one third of the amide protons (44). The rate of NH exchange in a neutral or alkaline aqueous solution for ferricytochrome *c* can be quenched by lowering the pH to the acid side. Under these conditions this set of relatively slowly exchanging protons can be detected via 2D NMR spectroscopy (Figure 1) and the rate of exchange measured (Table I, k_{D_2O}). From these rates and the intrinsic exchange rates in D_2O, $k_{in}^{D_2O}$,[18,28] the protection factors can be calculated (Table I, $P_2 = k_{in}^{D_2O}/k_{D_2O}$; aqueous, pD 9.3). The P_2 protection factors of the observable NH protons for cytochrome *c* varied from ca. 10^4 to 10^8. These are <50-fold smaller than or close in magnitude to those in the native state at pD 7.0 (P_3). The exceptions are Lys8, Thr19, Ile57, and Ile85, for which the protection factors at pD 9.3 are 6–21-fold larger than those at pD 7.0. (The 2D NH–C_αH crosspeak for Ile57 is very weak; thus, its integration and protection factor may be subject to larger errors). The amide protons of Lys7, Gln12 (part of the N-terminal helix), Phe36, Ile75, Ile85 (part of a loop region), Lys60, Leu64, Glu66, Glu69, Asn70 (part of the 60s helix), Arg91, Glu92, Asp93, and Lys100 (part of the C-terminal helix) exchanged very rapidly in D_2O (Table I, k_{D_2O}) and had correspondingly low protection factors (P_2).

In solution cytochrome *c* undergoes several pH-dependent conformational transitions, at pH 9.4 and 13.[33] By the first transition, cytochrome *c* loses reducibility. The transition is believed to be associated with a loss of Met80 as the sixth ligand, with a new strong-field ligand (either Lys72 or Lys79) coordinating to the heme iron. At pD 9.3 (pH 8.9) cytochrome *c* is thus partially in this non-native state although the similarity between the P_2 and P_3 protection factors indicates that cytochrome *c* in alkaline solution retains much of its native, compact structure. It is, however, certainly more flexible, as indicated by the lower protection factors. Unfortunately exchange of native cytochrome *c* at neutral pH in THF/1% D_2O was so slow as to preclude accurate measurement of protection factors under these conditions.

Protection Factors in THF/1% D_2O. The alkaline NH-exchange rates in THF containing 1% D_2O (k_{THF}, Table I) are about 100–1000-fold slower than those in D_2O (R_1; corrected for temperature) for most of the residues. The overall slower rate of NH exchange of cytochrome *c* in THF/1% H_2O is largely a

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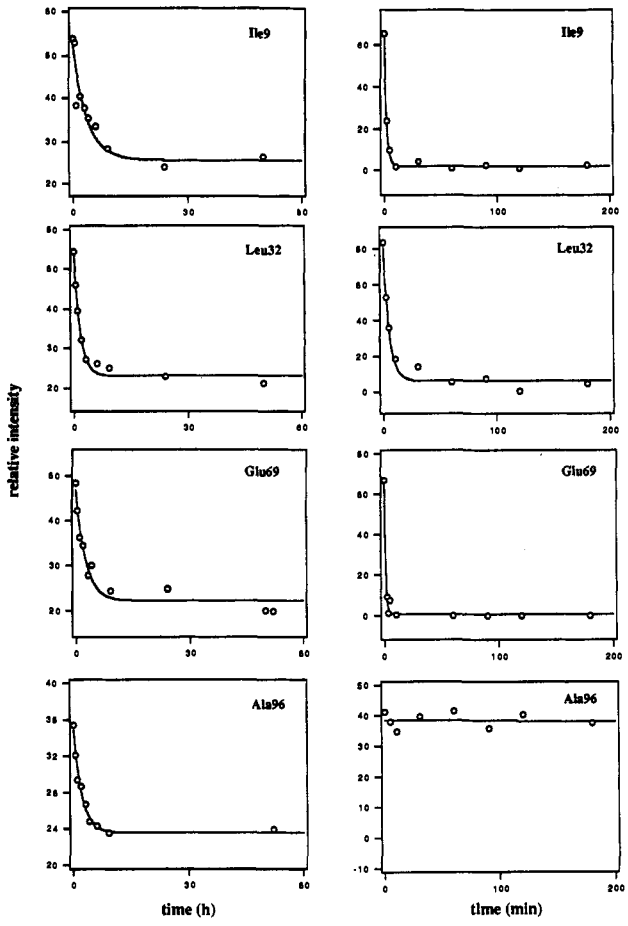

Figure 2. Plots of integral volumes of individual NH–C_αH crosspeaks in 2D TOCSY spectra vs exchange time for selected residues: panels on the left, THF/1% D_2O, pH 8.9; panels on the right, D_2O, pD 9.3. Calibration of integral volume is to the aromatic H3–H6 crosspeak of the Tyr97 residue. The solid lines represent a nonlinear least square fit of their exponential decay as a function of H–D-exchange time. Note the difference in time scale for the plots in THF and D_2O.

simple solvation effect, as judged by the intrinsic rate of exchange for a random-coil peptide in the organic solvent. Thus for the NAc-Ala-Ala-Ala-OMe tripeptide, $k_{in}^{D_2O}/k_{in}^{THF} = 2.1 \times 10^4$. Much of this difference simply represents the decreased molarity of water (and hydroxide) in the 1% aqueous solution/THF. Note that the intrinsic rates of amide exchange for both tripeptides are essentially the same whether in a homogeneous THF/1% D_2O solution or heterogeneous suspension in the same solvent. Thus solvent access to the exchangeable protons of the suspended protein is also probably not a significant contributing factor to the variations in the protection factors.

While the rates decrease by 100–1000-fold, the protection factors (P_1) in THF/1% D_2O are also generally 100–1000-fold smaller than those in D_2O (P_2, Table I). The ratios and protection factors are not, of course, all independent variables. Thus

$$R_1\frac{P_2}{P_1} = (2.1 \times 10^4)f$$

where f is the residue-based correction factor for the intrinsic rate of hydrogen exchange of a peptide and varies over a factor of 10 for different tripeptide sequences. We do not observe a 2.1×10^4–2.1×10^5 range for R_1 (only a range of 100–1000) because the ratio of protection factors (P_2/P_1) varies as well by ca. 100–1000. The larger the R_1 ratio, the more closely the protection factor in THF/1% D_2O resembles that in D_2O.

The smaller protection factors in the organic solvent could reflect a loosening of secondary and tertiary structure. Does this mean that the protein is denatured or partially unfolded in THF/1% D_2O? This is unlikely because the overall pattern of protection (Figure 3) follows that of the protein in aqueous solution (both P_2 and native P_3). Residues 95, 97, and 98 are strongly protected in both solvents.

In contrast, Jeng et al.[1,34] have recently shown that at acid pH and high ionic strength the three major helices of oxidized horse cytochrome c remain folded but most of the amide protons involved in nonhelical H-bonds are only marginally protected. Under these conditions the protein is believed to assume a "molten globule" type state. The protection factors from the majority of observable NH protons for cytochrome c in the organic solvent, pH 8.9, are 10^2–10^3 larger than those in the molten globule state. The protection factors from nonhelical protons are about 200-fold smaller than those for helical protons in this molten globule state.

The P_1 and P_2 protection factors against exchange were greatest for NH protons of Phe10 and those located in the central segment of the C-terminal helix—Ile95, Tyr97, and Leu98. It should be noted that these residues are in close proximity to the heme group and are nearly completely buried in the hydrophobic core of the protein (Figure 4). In THF/1% D_2O, the P_1 protection factors for protons in the three α-helices were also relatively large, further suggesting cytochrome c remains compact and resembles the native state. The similarity between the variations in the P_1, P_2, and P_3 protection factors (Table I, Figure 3) indicates that the protein maintains its essential conformation in both THF and basic aqueous solution.

However, some substantial differences in relative exchange rates were observed. For example, as shown in Table I and Figures 2 and 3, parts of the C-terminal helix (Leu94, Ala96, and Lys99) have the lowest NH-exchange rates in D_2O, whereas in THF/1% D_2O these exchange rates increase dramatically. Note that for Ala96 the change is $>10^4$, resulting in the only negative log R_1 (Figure 3). This must reflect differences in tertiary interactions between the two helices in the different solvents. The N-terminal and C-terminal helices fold over each other in the native structure (Figure 4). Residues Leu94, Ala96, and Lys99 of the C-terminal helix are located at the interface with the N-terminal helix and are thus buried from the surface. The decrease in P_1 protection factors for these residues suggests that this helix interaction is disrupted in the hydrated organic solvent. This allows at least transient exposure of residues Leu94, Ala96, and Lys99 to the surface. Note, however, that residues on the opposite face of the C-terminal helix (95, 97, and 98) will still remain buried within the hydrophobic core and thus retain large P_1 protection factors in the organic solvent.

While a majority of the amide protons have R_1 ratios (k_{D_2O}/k_{THF}) ranging from 100 to 1000, residues Phe10 and Thr63 and part of the C-terminal helix, Leu94, Ala96, Leu98, and Lys99, have the smallest R_1 ratios ($R_1 < 55$). It is interesting to note that all these amide protons except Thr63 are located in the same interior region, as illustrated in Figure 4.

The residues of Thr19 and Phe36 are located in the loop region and form hydrogen bonds with the interior H_2O molecules.[1,20] The amide protons for these two residues have intermediate exchange rates in both H_2O and THF, suggesting that THF does not disrupt the tertiary structure or the H-bond between the NH and the interior H_2O's.

These results suggest that the energetics of water–peptide and peptide–peptide H-bonds are quite comparable in purely aqueous solution and hydrated organic solvent, thus allowing transient unfolding of the helix. Indeed, Doig and Williams[35] have shown that the formation of a hydrogen bond is highly favorable in both aqueous and nonpolar solvents with a mean value of –26 kJ/mol in water and –32 kJ/mol in tetrachloromethane. It was suggested that hydrogen-bond formation in nonpolar solvents is driven by electrostatic attraction between the polar peptide units and the

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Table I. Amide Proton-Exchange Rates and Protection Factors (P) of Ferricytochrome c

residue	k_{THF} (37 °C) (h^{-1})	k_{D_2O} (24 °C) (min^{-1})	R_1 (k_{D_2O}/k_{THF}) (pH 8.9)	P_1 (in THF) (pH 8.9)	P_2 (in D_2O) (pD 9.3)	P_3 (native, in D_2O) (pD 7.0)[c]
Gly6	0.88 (87)[a]	0.98 (100)[b]	2.2×10^2	5.1×10^2	1.1×10^6	
Lys7	0.17 (74)	3.8 (100)	4.5×10^3	2.7×10^3	6.1×10^4	1.5×10^5
Lys8	0.45 (76)	0.64 (81)	2.8×10^2	1.0×10^3	3.6×10^5	3.0×10^4
Ile9	0.27 (51)	0.51 (96)	3.8×10^2	1.7×10^3	2.7×10^5	9.0×10^5
Phe10	0.043 (34)	<0.004 (29)	<19	1.1×10^4	$>1.1 \times 10^7$	1.4×10^8
Val11	0.64 (42)	0.23 (100)	72	7.1×10^2	3.9×10^5	1.0×10^6
Gln12	0.33 (46)	1.1 (100)	6.6×10^2	1.4×10^3	2.5×10^4	2.0×10^5
Lys13	0.56 (57)	0.71 (91)	2.5×10^2	8.1×10^2	2.3×10^5	6.3×10^5
Cys14	0.79 (62)	0.87 (88)	2.2×10^2	5.7×10^2	1.7×10^6	1.3×10^6
Thr19	1.0 (58)	0.87 (75)	1.7×10^2	4.5×10^2	1.5×10^6	1.9×10^5
Leu32	0.63 (61)	0.20 (95)	63	7.2×10^2	1.0×10^6	1.3×10^7
His33	0.20 (46)	0.66 (89)	6.6×10^2	2.3×10^3	4.0×10^6	1.6×10^6
Phe36	0.32 (61)	1.3 (66)	8.1×10^2	1.4×10^3	3.5×10^4	1.9×10^5
Ile57	0.68 (95)	0.44 (20)	1.3×10^2	6.7×10^2	3.2×10^5	1.5×10^4
Lys60	1.1 (56)	1.2 (72)	2.2×10^2	4.1×10^2	1.2×10^5	1.6×10^6
Thr63	1.2 (86)	0.33 (57)	55	3.8×10^2	2.2×10^6	
Leu64	4.8 (69)	4.1 (100)	1.7×10^2	94	3.4×10^4	2.5×10^6
Glu66	1.7 (55)	0.62 (77)	73	2.7×10^2	3.2×10^4	2.8×10^4
Leu68	0.23 (71)	0.32 (100)	2.8×10^2	2.0×10^3	2.8×10^5	7.4×10^7
Glu69	0.40 (59)	0.98 (100)	4.9×10^2	1.1×10^3	9.2×10^4	1.8×10^6
Asn70	0.24 (61)	1.1 (64)	9.1×10^2	1.9×10^3	5.1×10^4	3.9×10^4
Lys73	0.28 (84)	1.1 (81)	7.8×10^2	1.6×10^3	2.3×10^5	
Ile75	1.2 (95)	1.7 (100)	2.8×10^2	3.8×10^2	5.2×10^4	2.4×10^5
Ile85	0.89 (52)	0.90 (53)	2.0×10^2	5.1×10^2	1.6×10^5	2.7×10^4
Glu90	0.12 (67)	0.13 (84)	2.2×10^2	3.8×10^3	1.4×10^6	
Arg91	0.77 (53)	1.1 (95)	2.8×10^2	5.9×10^2	1.0×10^5	1.2×10^6
Glu92	0.44 (61)	1.1 (95)	5.0×10^2	1.0×10^3	1.6×10^5	3.5×10^6
Asp93	1.3 (66)	0.71 (99)	1.1×10^2	3.5×10^2	1.5×10^5	1.5×10^6
Leu94	0.49 (87)	0.031 (82)	13	9.2×10^2	2.6×10^6	$>2.0 \times 10^8$
Ile95	<0.001 (19)	<0.008 (43)	$\sim 1.6 \times 10^3$	$>4.5 \times 10^5$	$>8.9 \times 10^6$	$>2.0 \times 10^8$
Ala96	0.47 (32)	$<1 \times 10^{-4}$ (9)	<0.04	9.6×10^2	$>7.1 \times 10^8$	$>2.0 \times 10^8$
Tyr97	<0.002 (15)	$<8 \times 10^{-4}$ (15)	~ 80	$>2.3 \times 10^5$	$>5.7 \times 10^7$	$>2.0 \times 10^8$
Leu98	<0.002 (13)	$<5 \times 10^{-4}$ (1)	~ 50	$>2.3 \times 10^5$	$>1.8 \times 10^8$	$>2.0 \times 10^8$
Lys99	0.14 (68)	<0.006 (44)	<8.5	3.2×10^3	$>1.8 \times 10^7$	$>2.0 \times 10^8$
Lys100	0.41 (73)	2.6 (82)	1.3×10^3	1.1×10^3	8.8×10^4	1.6×10^5

[a,b] The values in parentheses represent the percentage of decrease for NH–C_αH crosspeaks in 2D TOCSY spectra of ferricytochrome c after incubating with THF for 52 h or with D_2O for 3 h. [c] The protection factors of ferricytochrome c in the native state at pD 7.0 are reproduced from ref 1.

introduction of a number of low-frequency vibrations in the bound complex. However in water, hydrogen-bond formation is largely driven by the release of bound water molecules. The magnitude of the binding energy can be considered to arise from a number of factors including the difference in solvation between a free peptide group and a C=O·H—N group, a change in the number of vibrational modes, and electrostatics.

Because of the change of hydrophilic interactions on the exterior of cytochrome c in nonaqueous solvent, a large difference in NH-exchange rates is expected for the exterior residues of the protein. However, most of these residues exchange too rapidly to be observed in the slowest exchange condition and cannot be observed by 2D NMR spectroscopy. Thus, only these amide protons with sufficiently slow exchange rates in the native state can be observed.

Speculations on Structure, Dynamics, and Catalysis in Mixed Organic Solvents. Proteases such as chymotrypsin have been shown to catalyze both synthesis and hydrolysis of peptide bonds and to have altered substrate specificity under nonaqueous conditions.[4,5] Remarkably, chymotrypsin and other serine proteases do not denature and even retain activity in nearly 100% organic solvents such as acetone, THF, and even octane[2]—thus the half-life for inactivation of chymotrypsin in octane is several hours at 100 °C and yet is only minutes in water at 60 °C.[2] Stability of the enzyme is greatly enhanced. The proteins, which must be suspended in these anhydrous organic solvents, still retain approximately 50 or fewer bound water molecules. This is considerably less than a simple monolayer which would consist of some 500 molecules. Thus the remaining waters cannot be a true monolayer and must be clustered at specific locations on the protein, perhaps at charged groups or in or near the active site.

Zaks and Klibanov[2] have shown that the enzymatic activity of chymotrypsin decreases significantly upon a decrease in hydrophobicity of the solvent; as an example, the reactivity in octane exceeds that in pyridine by more than 10^4. Water participates (directly or indirectly) in all noncovalent interactions maintaining the native, catalytically active enzyme conformation. There is a certain number of water molecules required for the enzymatic activity. Hence, removal of these essential waters should drastically distort that conformation and inactivate the enzyme. It is likely that hydrophilic solvents strip the essential water from chymotrypsin molecules, thereby diminishing enzymatic activity. Recently, using solid-state NMR methods, Burke et al.[36] have shown that up to ca. 42 ± 5% of the active sites of α-chymotrypsin could be disrupted by lyophilization. This loss of structural integrity was caused by further dehydration, i.e. stripping of water retained by the enzyme during drying, as opposed to freezing.

Klibanov and co-workers have shown that at low water content in organic solvent addition of water generally increases enzyme activity[2,3,6] and have suggested that this could be due to increased conformational flexibility of the enzyme in more aqueous-like environments. Affleck et al.[37] have shown that addition of less than 1% (vol/vol) water leads to a substantial increase in the transesterification activity of subtilisin Carlsberg. In this study, the subtilisin showed enhanced flexibility of an active-site bound spin label with increasing water content. Other proteins behave similarly in very low water content organic solvents.

These results have been interpreted in terms of a stepwise perturbation in the flexibility of a protein as a function of water

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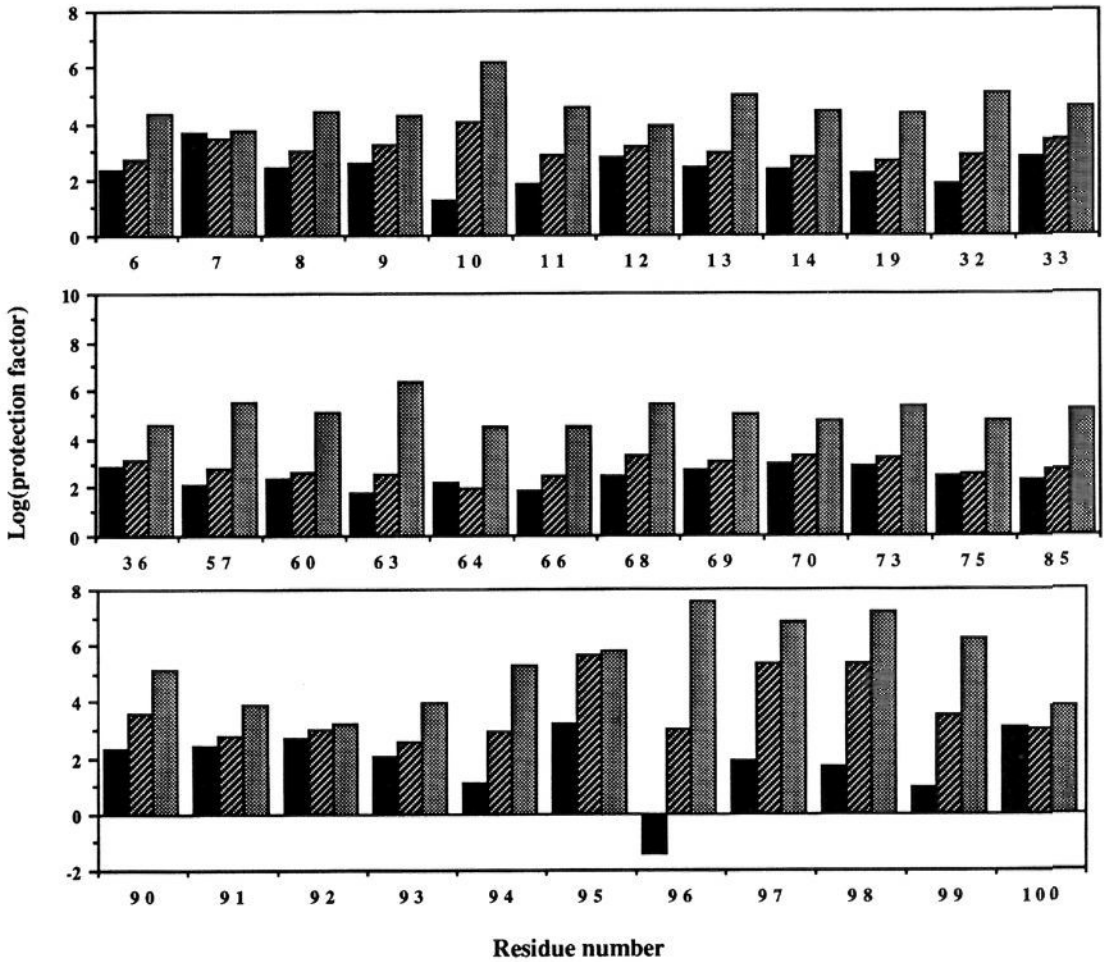

Figure 3. Plot of logarithm of ratios R_1 (solid bar) and protection factors P_2 (striped bar) and P_3 (stippled bar) against the corresponding residue number for ferricytochrome *c* in THF containing 1% D_2O and in D_2O (pD 9.3). R_1 is $= k_{D_2O}/k_{THF}$ (corrected for the temperature difference). P_1 and P_2 are the protection factors of cytochrome *c* in THF and D_2O (pD 9.3), respectively. Some of the bars represent minimal values (see Table I).

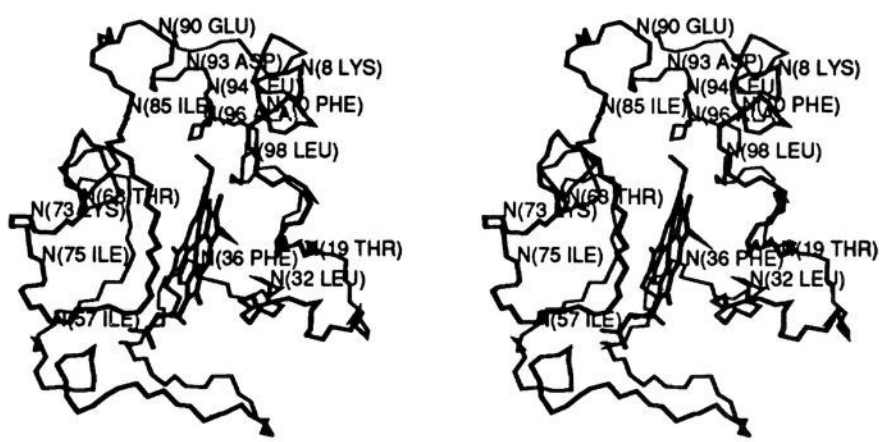

Figure 4. Stereoview of the backbone of ferricytochrome *c* identifying some of the important residues with large or unusual protection factors for hydrogen exchange in both aqueous solution and THF media. Coordinates[40] for tuna cytochrome *c* were used in the modeling.

content in organic media.[2,38] In the absence of added water, lyophilized proteins are believed to be conformationally rigid and to have little enzymatic activity. As water is added (as few as 50 molecules per protein), the protein becomes more flexible, which permits conformational changes required for reasonable enzymatic activity. As more water is added (similar to our THF/1% D_2O solvent), this flexibility increases. At quite high water levels, enzymatic activity often falls, as most proteins will denature in mixed aqueous organic solvents. Our own results strongly support this picture. Thus the decreased NH-exchange protection factors in THF/1% D_2O can be interpreted in terms of *increased* flexibility of the protein in this solvent. Of course our results do not address protein dynamics at very low levels of water. Indeed we do find that at 0.5% water content exchange is slowed considerably while the exchange rates for residues such as Leu32, Glu69, and Arg91 in 2% D_2O/THF increase a further 1.8–3.6-fold (unpublished). This is consistent with the observation that increased water content increases protein flexibility.[37]

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Our hydrogen-exchange results on cytochrome *c* suggest that water does indeed serve to "lubricate" the local dynamics of the protein in THF/1% D_2O. However, we find that the slightly hydrated organic solvent further *enhances* the dynamic flexibility of all regions of the native structure relative to purely aqueous solutions. Our results thus appear to conflict with previous data which suggest that organic solvents either restrict the local dynamics of proteins or at least have little effect upon them at higher water content.[2,37-39] Perhaps these differences can be explained if cytochrome *c* behaves anomalously and is partially denatured by the organic solvents at quite low levels of added water. Clearly it will be important to extend the hydrogen-exchange experiment to other proteins which will allow us to correlate measured activity with dynamic flexibility (currently in progress).

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