Assessing the Structural Integrity of a Lyophilized Protein in Organic Solvents

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Abstract: The structure of a model protein, bovine pancreatic trypsin inhibitor (BPTI), in organic solvents has been examined using hydrogen isotope exchange/high-resolution NMR methodology. When lyophilized deuterated BPTI is suspended in acetonitrile, tetrahydrofuran, ethyl acetate, or butanol, each containing 1% ^1H_2O, several protein amide protons that are buried and strongly hydrogen bonded in aqueous solution are found to exchange with the solvent significantly within 24 h. In contrast, in water most of these protons do not exchange appreciably even after a week under otherwise similar conditions. The isotope exchange rates of the corresponding amide protons of BPTI are similar in these nonaqueous solvents, as well as in acetonitrile containing methanol or butanol (instead of water) as a H donor. When solid BPTI is prepared by different methods, such as rotary evaporation, acetone precipitation, or lyophilization from a dimethyl sulfoxide solution, and subsequently suspended in acetonitrile containing 1% water, the exchange intensities of the amide protons vary greatly among the preparations. These data combined suggest that the structure of BPTI in the four aforementioned organic solvents is partially unfolded, but not more so than in lyophilized powder, i.e., that these solvents cause little additional protein denaturation beyond that brought about by lyophilization. Using the same methodology, the BPTI structure also has been studied in several protein-dissolving solvents containing 1% water. In dimethyl sulfoxide, dimethylformamide, or methanol, the same amide protons exchange almost completely within 24 h, while in glycerol (known to stabilize proteins and to function as a water mimic) they do not. These results demonstrate that some protein-dissolving organic solvents strongly denature BPTI and that intermolecular contacts in the suspended protein are important in maintaining the protein conformation in organic solvents. These findings, if general, explain the considerable but much reduced (compared to water) enzymatic activity in nonaqueous media.

Introduction

To rationalize mechanistically enzymatic catalysis in organic solvents,[1] one has to understand the structure and dynamics of proteins placed in such an unnatural milieu. Recently, the *crystal* structures of the proteases subtilisin Carlsberg[2] and γ-chymotrypsin[3] in two anhydrous solvents, acetonitrile and hexane, respectively, have been elucidated by X-ray crystallography and found to be nearly identical to those in water. Available spectroscopic data on *lyophilized powders* of proteins are controversial: while some studies[4,5] point to a significant reversible conformational change upon lyophilization, others[6] suggest the same structure as in water. At any rate, it is unknown whether addition of organic solvents to lyophilized proteins causes any additional conformational changes.

With respect to protein dynamics in organic solvents, a variety of studies indicates that lyophilized (as well as crystalline[2b]) proteins are less flexible when suspended in organic solvents than in aqueous solution.[7,8] For example, a solid-state NMR investigation of α-lytic protease has revealed that both flipping rates and librational motions of enzyme's tyrosine residues are much greater in aqueous crystals than in lyophilized powders suspended in anhydrous solvents.[8] Furthermore, the conformational flexibility of the enzyme increases upon adding small quantities of water to the solvent[8] (a similar conclusion was independently made from the titration of accessible lysine residues in polyphenol oxidase suspended in a nonaqueous solvent[9]).

Recently, a paper appeared[10] describing a 2D NH-exchange NMR spectroscopic examination of cytochrome *c* suspended in tetrahydrofuran containing 1% water. The authors conclude that the structure of the protein in this medium is the same as, and the conformational flexibility is greater than, in water,[10] which contradicts both the results of the aforementioned studies and the general notion on the role of solvent water in proteins.[11] The present work has been undertaken in order to address these

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issues further and to gain additional insight into protein structure in organic media.

Results and Discussion

One of the salient features of amide hydrogen isotope exchange in proteins is the wide range of exchange rates.[12] Buried and strongly hydrogen-bonded amide protons exchange orders of magnitude slower than those exposed to the solvent. Hence, the isotope exchange rates of individual amide protons can shed light on the local structure and dynamics in a protein.

The exchange rate constants of several slowly exchanging amide protons in bovine pancreatic trypsin inhibitor (BPTI), a stable[13] model protein, have been reported.[14] In aqueous solution, four amide protons are so protected that they do not exchange appreciably (<1%) even after 3 weeks at pH 3.5 and 36 °C.[14b] Out of these, the protons of Tyr21, Phe22, and Tyr23 (belonging to the buried central β strand of BPTI[15]) are well-resolved in the one-dimensional ^{1}H NMR spectrum[16] (Figure 1A). Three other well-resolved amide protons, those of Phe33, Tyr35, and Phe45 (belonging to the peripheral β strands of the protein[15]), exchange faster, but still no appreciable isotope exchange is observed after days under identical conditions.[14b]

In our investigation[5] of the structure of solid BPTI lyophilized from p^{2}H 3.5, these six protons were found to exchange significantly within 24 h at 37 °C. We concluded that BPTI partially unfolded upon lyophilization, thereby exposing these buried and hydrogen-bonded amide protons and thus accelerating their exchange. We reasoned that a similar strategy could be adopted for investigating the structural integrity of BPTI in nonaqueous solvents. If BPTI is partially unfolded in organic media, some of the six buried protons may become exposed and hence exchange with the solvent. Subsequent dissolution of the BPTI sample in aqueous solution at p^{2}H 3.5[17] would trap these slowly exchanging protons and allow their detection by ^{1}H NMR spectroscopy.

All exchangeable protons of BPTI were replaced with deuterons using 8 M urea-d_4 solution at 75 °C. The ^{1}H NMR spectrum of the resultant BPTI (^{2}H-form) in ^2H_2O confirmed (compare Figures 1B and 1A) that all the backbone amide protons had been labeled with deuterons. BPTI(^{2}H) was lyophilized from a ^2H_2O solution at p^{2}H 3.5 and suspended in acetonitrile[18] containing 1% (v/v) ^1H_2O[19] at 37 °C. Following the removal of the organic solvent 24 h later, the protein sample was redissolved in ^2H_2O at p^{2}H 3.5[17] and its ^{1}H NMR spectrum (Figure 1C) was recorded.

The peaks of the NH protons belonging to Tyr21, Tyr23, Phe33, Tyr35, and Phe45[20] residues are clearly seen in Figure 1C, demonstrating that isotope exchange occurred within 24 h. Table 1 lists the intensities for the amide protons. A control exchange experiment with BPTI(^{2}H) incubated in ^1H_2O at pH 3.5 and 37 °C for 24 h, in agreement with the literature data,[14b] yielded no signals of these slowest exchanging protons (data not shown). Thus the dramatic increase in the exchange rates of the five buried and strongly hydrogen-bonded amide protons of lyophilized BPTI suspended in the organic solvent, compared to those for the protein dissolved in water, can be due to either (i) partial unfolding of the protein resulting in the exposure of these protons to the solvent or (ii) a marked enhancement in the conformational flexibility of the native structure of the protein (as concluded in ref 10).

In order to distinguish between these two alternative explanations,[21] the isotope exchange in BPTI was monitored in two other, unrelated solvents—tetrahydrofuran[10] and ethyl acetate, each containing 1% water—under otherwise identical conditions. It can be seen in Figure 2 that the intensities of the six amide protons are invariant of the solvent system (also see columns I through III in Table 1). This behavior is inconsistent with the enhanced flexibility hypothesis (ii) because the latter dictates that the conformational flexibility and, hence, the hydrogen isotope exchange rates should be solvent dependent. On the other hand, these experimental observations are consistent with hypothesis (i) provided that the partial unfolding of the protein is brought about by lyophilization (which is indeed the case[5]), rather than by the subsequent placement in organic solvents.

In addition, if the enhanced conformational flexibility hypothesis[10] were correct, it could only be due to the presence of 1% water in the solvent because protein flexibility in anhydrous organic solvents is greatly reduced compared to that in aqueous solution.[7b,d,8] Thus, if 1% water were replaced with an organic solvent capable of engaging in hydrogen isotope exchange, the flexibility of BPTI in such an anhydrous system should be lower than in aqueous solution, thereby resulting in no appreciable exchange for the six amide protons.[22] To this end, the isotope exchange in BPTI was monitored in two anhydrous solvent systems—acetonitrile containing 2.2% (v/v) methanol and acetonitrile containing 5.1% (v/v) butanol—under otherwise the same conditions as in the acetonitrile/1% water system (the volume fractions of the alcohols were selected in such a way that their molar concentrations in acetonitrile equalled that of water in acetonitrile containing 1% (v/v) ^1H_2O, i.e., 0.56 M). Figure 1D depicts the ^{1}H NMR spectrum of the BPTI sample following a 24-h incubation in the former solvent. Figure 2 shows that the six NH protons for both anhydrous solvent systems exchange to a similar extent as in acetonitrile containing 1% water (also compare columns IV and V with column I in Table 1). Once again, these observations, while contradicting hypothesis (ii) above, are consistent with the hypothesis of

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(17) The hydrogen isotope exchange of amide protons in BPTI at this pH is at the minimum.[14b]

(18) Acetonitrile was chosen because it is a typical aprotic organic solvent frequently used in nonaqueous enzymology,[1] it remains monophasic with 1% (v/v) water, and the crystal structure of a protein[2] in it has been determined.

(19) When the hydrogen isotope exchange experiment was conducted in acetonitrile containing 0.1% (v/v) ^1H_2O, five of the six well-resolved NH protons could be detected. These five protons were identical to those detected in the case of 1% ^1H_2O. Also, the ^{1}H NMR spectra obtained in these two experiments were qualitatively very similar. However, much lower intensities of the amide protons were observed in the experiment with 0.1% water than in that with 1%. This greatly increased both the time required for recording the spectra and the experimental error. Hence a 1% volume fraction was employed in all subsequent experiments.

(20) No appreciable isotope exchange of the Phe22 amide proton was detected in acetonitrile containing 1% water.

(21) It also may be postulated that the increased isotope exchange rates arise from a distinct microenvironment around these protons (compared to that in water) in lyophilized BPTI suspended in acetonitrile. This distinct microenvironment may be due to lyophilization-induced change in the effective pH, ionic strength, or another factor.[6a,c] However, this explanation was previously considered and experimentally ruled out for the exchange in the lyophilized state.[5] Since the same lyophilized powder is suspended in acetonitrile containing 1% ^1H_2O, this explanation does not apply herein either.

(22) In aqueous solution under similar conditions the six NH protons do not exchange to any appreciable degree (<1%) within 24 h.

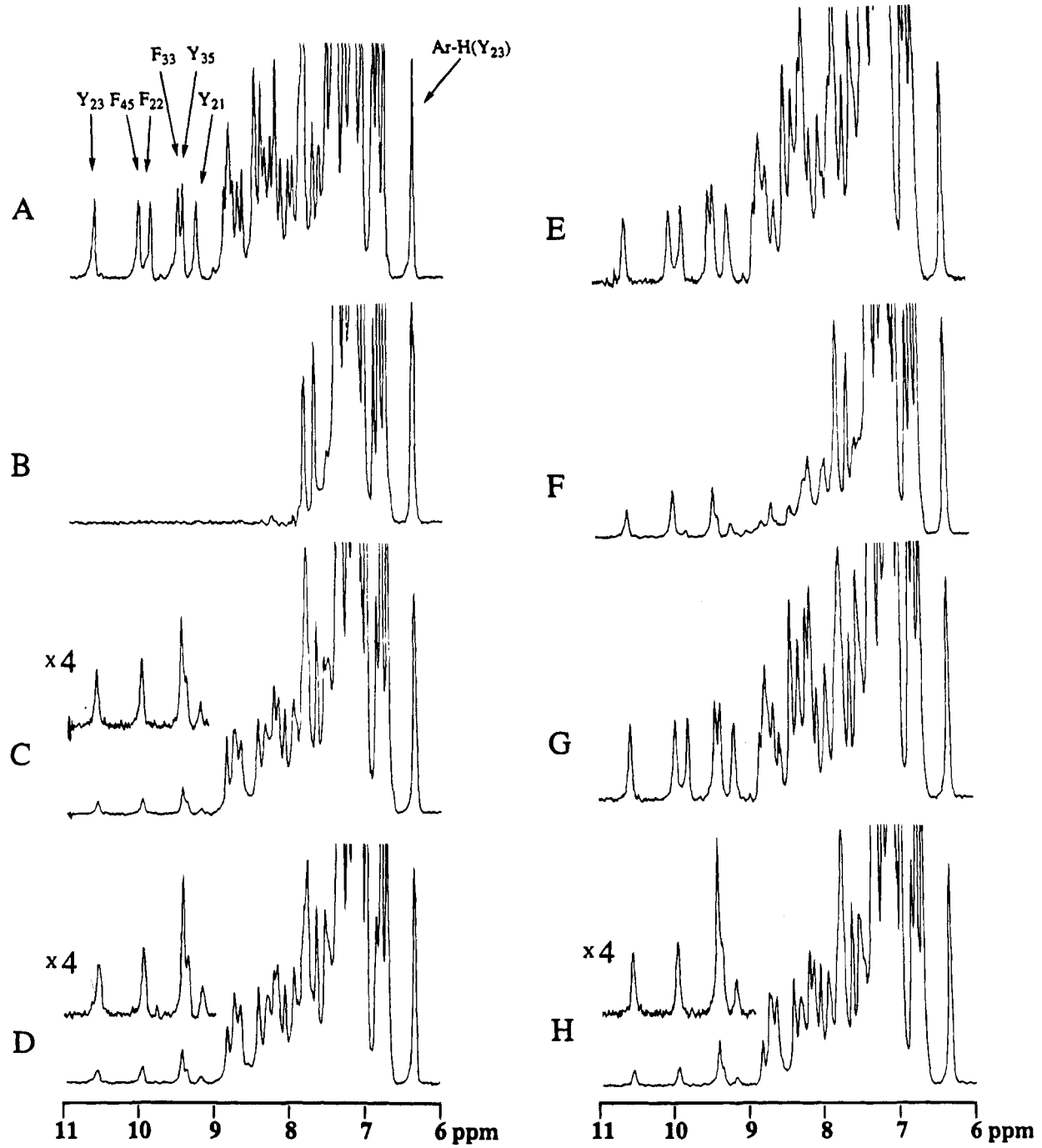

Figure 1. The ^1H NMR spectra of BPTI samples in ^2H_2O at p^{2}H 3.5. Spectra are plotted in such a way that the intensity of non-exchangeable signal at 6.35 ppm (Ar-H_2 of Tyr23) remains constant. (A) BPTI(^{1}H) lyophilized from pH 3.5; (B) BPTI(^{2}H) lyophilized from p^{2}H 3.5; (C) lyophilized BPTI(^{2}H) incubated in acetonitrile containing 1% (v/v) ^1H_2O; (D) lyophilized BPTI(^{2}H) incubated in acetonitrile containing 2.2% (v/v) methanol; (E) lyophilized BPTI(^{2}H) incubated in dimethyl sulfoxide containing 1% (v/v) ^1H_2O; (F) lyophilized BPTI(^{2}H) incubated in glycerol containing 1% (v/v) ^1H_2O; (G) lyophilized BPTI(^{2}H) incubated in methanol containing 1% (v/v) ^1H_2O; and (H) lyophilized BPTI(^{2}H) incubated in butanol containing 1% (v/v) ^1H_2O. Inserts in parts C, D, and H show the magnified (4-fold) amide proton region of interest. The chemical shifts of the identified amide protons in spectra A and C to H were found to be identical using the dedicated Varian NMR software. Note that the intensity of protons in the region of 7.8 to 8.8 ppm is much lower in spectrum F (exchange in glycerol) than in spectra C, D, E, G, and H (exchange in other organic solvents). Spectrum F was recorded with 3−5 times lower concentration of BPTI than in the other instances (see Experimental Section), and consequently the time required for the NMR experiment (~11 h) was significantly greater than in the rest of the experiments (~2 h). Several intermediate-range exchanging amide protons resonate in this region and presumably back-exchange with the ^2H_2O solvent during the time of sample preparation and recording of the ^{1}H NMR spectrum. This phenomenon would result in the reduced intensity for the intermediate-range exchanging amide protons in spectrum F. Y = tyrosine, F = phenylalanine, Ar-H = aromatic protons.

lyophilization-induced unfolding of BPTI and little subsequent unfolding caused by the organic solvent.

To further elucidate the mechanism of BPTI's hydrogen isotope exchange in organic solvents, we prepared solid deuterated BPTI by three different methods presumably resulting in distinct conformations of the ensuing protein—rotary evaporation, acetone precipitation, and lyophilization from a dimethyl sulfoxide solution—and monitored the exchange in acetonitrile containing 1% ^1H_2O. We reasoned that the conformational flexibility of BPTI in a given medium should be independent of the mode of protein preparation. Thus if hypothesis (ii) is correct, the exchange pattern of the six NH protons in different

Table 1. Intensities of Amide Protons of Lyophilized Deuterated BPTI[a] following Incubation in Various Organic Solvents at 37 °C for 24 h

amide proton	chemical shift, ppm	extent of hydrogen isotope exchange,[b] %									
		I	II	III	IV	V	VI	VII	VIII	IX	X
Tyr21	9.21	9.5 ± 3.2	12 ± 1.0	11 ± 4.1	12 ± 4.9	9.3 ± 0.87	100[c]	96 ± 2.1	20 ± 0.58	100[c]	15 ± 4.0
Phe22	9.83	<1.0[d]	<1.0[d]	<1.0[d]	<1.0[d]	<1.0[d]	100[c]	92 ± 6.4	12 ± 2.5	100[c]	<1.0[d]
Tyr23	10.55	22 ± 4.0	28 ± 5.8	23 ± 3.8	21 ± 1.7	25 ± 5.0	93 ± 2.8	100[e]	33 ± 6.4	100[c]	23 ± 1.0
Phe33[e]	9.46	41 ± 5.5	48 ± 2.0	40 ± 6.0	44 ± 5.0	44 ± 5.7	100[c]	100[e]	56 ± 6.0	100[c]	47 ± 6.1
Tyr35[e]	9.39	18 ± 2.6	22 ± 1.2	21 ± 5.7	20 ± 5.5	17 ± 5.5	100[c]	100[e]	25 ± 4.7	100[c]	20 ± 3.1
Phe45	9.97	24 ± 4.6	26 ± 2.9	22 ± 6.4	28 ± 4.5	31 ± 5.7	100[c]	100[e]	67 ± 5.9	100[c]	28 ± 1.7

[a] The protein was lyophilized from a ^2H_2O solution at p^{2}H 3.5 (see Experimental Section for details). [b] The extent of exchange was calculated as follows: the ratio of intensity (peak area) of each NH proton to that of the signal of non-exchangeable protons of Tyr23 at 6.35 ppm for BPTI(^{1}H) (Figure 1A) was set to 100%. This ratio, obtained for each amide proton in the exchange experiment, was compared to the above 100% values. Roman numerals correspond to the following organic solvent systems (all ratios are in v/v): I, acetonitrile−H_2O (99:1); II, tetrahydrofuran−H_2O (99:1); III, ethyl acetate−H_2O (99:1); IV, acetonitrile−methanol (97.8:2.2); V, acetonitrile−butanol (94.9:5.1); VI, dimethyl sulfoxide−^1H_2O (99:1); VII, dimethylformamide−^1H_2O (99:1); VIII, glycerol−^1H_2O (99:1); IX, methanol−^1H_2O (99:1); and X, butanol−^1H_2O (99:1). Mean values and standard deviations were obtained from at least two independent measurements. [c] All measurements provided the same value. [d] This value represents the lowest intensity that can be quantified by our method. [e] The intensities for these protons were obtained by multiplying the height proportions of the individual peaks in the unresolved signal by its peak area.

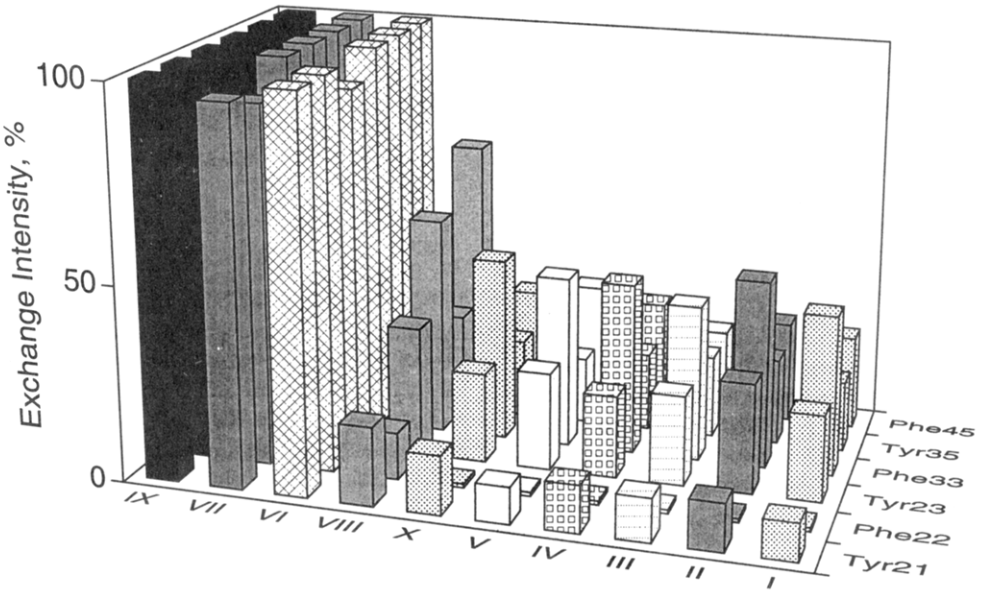

Figure 2. Bar chart representing the variation in isotope exchange intensity of the six amide protons in lyophilized deuterated BPTI following a 24-h incubation in various nonaqueous solvents. Roman numerals correspond to the organic solvent systems listed in Table 1. Mean values of hydrogen isotope exchange intensities (from Table 1) are plotted. The order of the solvents was selected to provide the clearest visual perception of the relative rates of exchange of the six NH protons in different solvents. Note that solvents I through V and X do not dissolve BPTI, and solvents VI through IX do (and of the latter group, VI, VII, and IX are protein-denaturing, and VIII is not).

preparations should be similar to that of lyophilized BPTI in the same organic medium. On the other hand, if the protein structure is different in these BPTI preparations, then the pattern of isotope exchange should also vary among them.

Figure 3 depicts the intensities of the six protons of deuterated BPTI, prepared by different methods, suspended in acetonitrile containing 1% ^1H_2O. One can see that the exchange patterns differ significantly for the various modes of preparation (Table 2), thus ruling out the conformational flexibility hypothesis. At the same time, since the structure of a solid protein may depend upon the manner of its preparation, these data are compatible with the partially unfolded structure hypothesis.

It is tempting to compare the extent of BPTI unfolding in organic solvents with that in the lyophilized state.[5] However, such a direct comparison is not justified because the dielectric constants and the water activities in organic solvents containing 1% water and in a humid gaseous atmosphere are far different. Nevertheless, the similarity of the extent of exchange in several unrelated organic solvents strongly suggests that, as mentioned above, these solvents cause little additional denaturation of BPTI beyond that brought about by lyophilization.

All these observations appear to contradict the conclusions of Wu and Gorenstein[10] that the conformational flexibility of a protein is greater in tetrahydrofuran containing 1% water than in aqueous solution. Our conclusions in this regard, however, are consistent with a variety of other literature data. EPR studies have shown that the conformational flexibility (1) of alcohol dehydrogenase in organic solvents is lower than in water,[7d] (2) of α-chymotrypsin drops dramatically with decreasing solvent dielectric constant,[7b] and (3) of subtilisin Carlsberg in tetrahydrofuran rises upon addition of water.[7c] Likewise, a solid-state NMR investigation of α-lytic protease has revealed that its conformational mobility as a function of the medium decreases in the following order: aqueous solution > hydrous organic solvents > anhydrous organic solvents.[8] Finally, molecular dynamics calculations involving BPTI[23] and α-chymotrypsin[7b] indicate that both proteins are more flexible in water than in organic solvents. Perhaps cytochrome *c* behaves anomalously.[10]

Proteins are insoluble in most organic solvents, including those studied thus far herein. However, a few nonaqueous solvents, such as dimethyl sulfoxide and *N*,*N*-dimethylforma-

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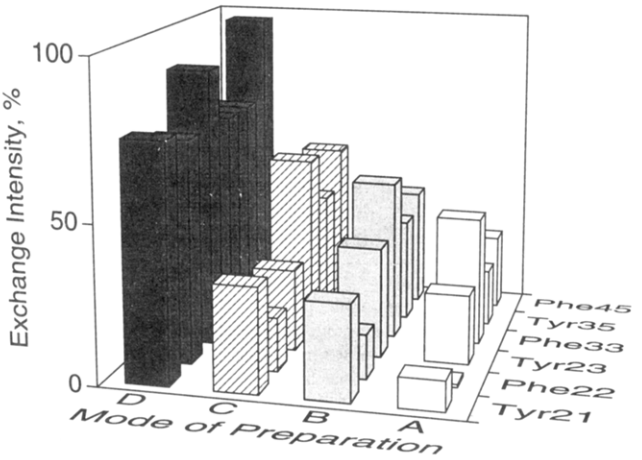

Figure 3. Bar chart representing the variation in isotope exchange intensity of the six amide protons following incubation of different preparations of deuterated BPTI in acetonitrile containing 1% (v/v) ^1H_2O. Deuterated BPTI was prepared using the following methods: (A) lyophilization from aqueous solution; (B) rotary evaporation of aqueous solution; (C) acetone precipitation from aqueous solution; and (D) lyophilization from a solution in dimethyl sulfoxide. (See Experimental Section for details.) Mean values of hydrogen isotope exchange intensities (from Table 2) are plotted.

Table 2. Intensities of Amide Protons of Deuterated BPTI, Prepared by Different Methods, following a 24-h Incubation in Acetonitrile Containing 1% ^1H_2O at 37 °C[a]

	extent of hydrogen isotope exchange, %,[b] as a function of the mode of protein preparation			
amide proton	lyophilization from ^2H_2O	rotary evaporation	acetone precipitation	lyophilization from dimethyl sulfoxide
Tyr21	9.5 ± 3.2	30 ± 5.1	33 ± 3.1	75 ± 4.6
Phe22	<1.0[c]	14 ± 4.9	17 ± 2.9	71 ± 7.5
Tyr23	22 ± 4.0	35 ± 6.0	26 ± 7.8	89 ± 7.8
Phe33[d]	41 ± 5.5	51 ± 1.0	57 ± 1.7	70 ± 12
Tyr35[d]	18 ± 2.6	33 ± 3.6	40 ± 5.0	70 ± 12
Phe45	24 ± 4.6	38 ± 6.1	52 ± 6.2	97 ± 2.9

[a] See footnote *b* to Table 1 for details on calculations of the intensities and the Experimental Section for details of the protein preparations. [b] Mean values and standard deviations were obtained from at least two independent measurements. [c] This value represents the lowest intensity that can be quantified by our method. [d] The intensities for these protons were obtained by multiplying the height proportions of the individual peaks in the unresolved signal by its peak area.

mide, can dissolve significant concentrations of common proteins.[24] We have decided to employ our hydrogen isotope exchange/high-resolution NMR methodology to compare protein structures in these two types of organic solvents.

Deuterated BPTI lyophilized from p^{2}H 3.5 was dissolved in dimethyl sulfoxide containing 1% ^1H_2O, incubated there for 24 h at 37 °C, lyophilized, and redissolved in ^2H_2O. As seen in Figure 1E, the isotope exchange for five of the six NH protons is total in this case (also see Figure 2 and Table 1). This striking increase in the amide proton exchange rates compared to those in the nondissolving organic solvents suggests that BPTI is far more unfolded in dimethyl sulfoxide. This is in agreement with several literature reports that this solvent readily denatures proteins.[24,25] To ascertain whether other organic solvents that dissolve BPTI also cause its denaturation, the exchange experiment was carried out in dimethylformamide containing 1% ^1H_2O. As seen in Figure 2 and Table 1, the resultant exchange intensities are essentially the same as in the dimethyl sulfoxide experiment, thus indicating major protein unfolding in dimethylformamide as well.

From the results described in the preceding sections, one can conclude that nonaqueous solvents that dissolve BPTI cause greater unfolding than those solvents that do not. Two alternative mechanisms are possible for this behavior: (a) protein−protein interactions, existing in protein suspensions but not in solution,[26] stabilize proteins against denaturation, and/or limit the accessibility of the protein interior to the solvent, or (b) some solvents, such as dimethyl sulfoxide and dimethylformamide, have a much stronger propensity to denature proteins than others, such as acetonitrile, tetrahydrofuran, and ethyl acetate.

To test these two possibilities, the hydrogen isotope exchange of BPTI was studied in glycerol in which BPTI is soluble and thus protein−protein contacts are absent. Also, being a water mimic with respect to its ability to form multiple hydrogen bonds,[27] glycerol stabilizes proteins.[28] Therefore, if mechanism (a) is solely responsible for the observed differences between protein-dissolving and -nondissolving solvents, then the exchange in glycerol should be, as in dimethyl sulfoxide and dimethylformamide, nearly 100%. On the other hand, mechanism (b) dictates less exchange in glycerol than in all the other solvents tested. As can be seen in Table 1, column VIII, the extent of BPTI's hydrogen isotope exchange is greater in glycerol than in nondissolving solvents (columns I−III) but far below that in protein-dissolving solvents (columns VI and VII). These observations suggest that both aforementioned mechanisms in fact play a role.

We found that BPTI is soluble in methanol but not in a homologous alcohol, butanol (at 1mg/mL). When isotope exchange was studied in BPTI dissolved in methanol containing 1% ^1H_2O, all six NH protons exchanged completely (Figure 1G and column IX in Table 1) within 24 h, suggesting that the protein is strongly denatured in methanol. In contrast, for BPTI suspended in butanol containing 1% ^1H_2O, five of the six NH protons exchanged to a significant extent (Figure 1H and column X in Table 1) but much less than 100%. Moreover, the isotope exchange rates in butanol are similar to those in acetonitrile, each containing 1% ^1H_2O (Figure 2). These results, once again, underscore the important role of protein−protein contacts.

In conclusion, this work demonstrates that solvents which do not dissolve proteins, such as acetonitrile, cause no appreciable denaturation of BPTI beyond that brought about by prior lyophilization. This is consistent with the X-ray crystallographic data[2,3] showing that protein crystal structures in water and in acetonitrile and hexane (which also does not dissolve proteins) are virtually identical. Protein-dissolving organic solvents do cause major additional denaturation of BPTI, the extent of which depends on the nature of the solvent. The fact that BPTI is partially unfolded in the lyophilized state[5] suggests that enzymes are less active in nonaqueous media than in aqueous solution at least in part due to their denaturation upon lyophilization.[29] This denaturation is not reversed by organic

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solvents. Thus efforts to preserve the native conformation in the lyophilized state, e.g., by co-lyophilization with lyoprotectants,[29] or certain salts,[30] should lead to a superior enzymatic activity in organic solvents.

Experimental Section

Materials. Bovine pancreatic trypsin inhibitor (BPTI) and urea-d_4 were from Sigma Chemical Co. Deuterated water (^2H_2O, 99.8 atom % ^2H) was from Cambridge Isotope Laboratories. 3-(Trimethylsilyl)-propionic-2,2,3,3-d_4 acid, sodium salt (TSP), was from Aldrich Chemical Co. Reagent-grade organic solvents were from Mallinckrodt Specialty Chemical Co. Anhydrous organic solvents were prepared by drying over molecular sieves (3 Å) for at least 48 h. All solvent systems consisting of an organic solvent containing 1% (v/v) ^1H_2O were prepared from the corresponding anhydrous solvent and deionized and distilled water which had been adjusted to pH 3.5 with glacial acetic acid. The water contents of these solvent systems were confirmed using an optimized Karl Fischer titration.[31]

^1H NMR Analysis of BPTI Samples. The ^1H NMR spectra were obtained on a Varian VXR 500 NMR spectrometer operating at a ^1H frequency of 500 MHz. The spectra were recorded with a digital resolution of 1 Hz/pt and referenced to the TSP signal (0 ppm). The residual H^2HO signal at 4.8 ppm was irradiated with a decoupler pulse for 3 s. The NMR probe temperature was set to 27 °C to eliminate irradiation artifacts. At least 1024 transients were acquired with a pulse repetition time of 6 s, and the free induction decays were Fourier-transformed using a line broadening factor of 2 Hz. The spectra were analyzed utilizing a Sun Spark station equipped with the Varian NMR software. The integrals for the amide protons were obtained after incorporating a suitable phase and baseline correction routine.

Preparation of Deuterated Lyophilized BPTI. The protein (100 mg) and urea-d_4 (5 g) were dissolved in 6.8 mL of ^2H_2O. The solution was incubated at 75 °C for 10 min, cooled by addition of 100 mL of ^2H_2O, and subjected to repeated ultrafiltrations at 10 °C using ^2H_2O. The resultant 1 mL of BPTI(^2H) solution was diluted with ^2H_2O to 1.0 mg/mL, and the p^2H was adjusted to 3.5, followed by lyophilization. The water content of the powder was found[31] to be 8.9 ± 1.7% (w/w).

Preparation of Rotary-Evaporated BPTI(^2H). A solution of 100 mg of BPTI(^2H) in 1 mL of ^2H_2O at p^2H 3.5 was evaporated on a Büchi rotary evaporator at the water bath temperature of 37 °C. The powder thus obtained was finely ground with a mortar and pestle and gave a yield of BPTI(^2H) of 91%. The water content of the protein powder was found[31] to be 15 ± 0.9% (w/w).

Preparation of Acetone-Precipitated BPTI(^2H). Acetone-d_6 (9 mL) was gradually added (500 μL aliquots over a period of 90 min) to a solution of 100 mg of BPTI(^2H) in 1 mL of ^2H_2O at p^2H 3.5 with vigorous stirring at 4 °C. After an additional hour of stirring, the suspension was centrifuged at 5900 × *g* for 20 min. The supernatant was decanted, and the protein powder was dried under a stream of purified argon. Acetone-precipitated BPTI(^2H) was recovered with a 99% yield. The water content of the protein powder was found[31] to be 15 ± 1.7% (w/w).

Preparation of BPTI(^2H) Lyophilized from Dimethyl Sulfoxide. A solution of 50 mg of BPTI(^2H) (lyophilized from ^2H_2O at p^2H 3.5 as described above) in 50 mL of dimethyl sulfoxide containing 1% (v/v) ^2H_2O was incubated at room temperature for 3 h, followed by lyophilization. The lyophilized powder was finely ground with a mortar and pestle and stored under a positive pressure of purified argon until further use. The water content of the protein powder was found[31] to be 9.8 ± 0.9% (w/w).

Hydrogen Isotope Exchange of BPTI(^2H) Suspended in Nondissolving Solvents. Lyophilized BPTI(^2H) (1 mg/mL) was suspended in an organic solvent containing 1% (v/v) (0.56 M) ^1H_2O or the same molar concentration of methanol or butanol. Following 24 h of stirring at 37 °C, the mixture was centrifuged at 5900 × *g* for 20 min, the supernatant was decanted, and the residue was dried under a stream of purified argon. The powder thus obtained was dissolved in 700 μL of ^2H_2O containing 150 μM TSP, and the solution was then filtered through a membrane with 0.22-μm pores into an NMR tube and the p^2H adjusted to 3.5. The final solution for the NMR analysis contained between 2.5 and 3.5 mM BPTI.

Hydrogen Isotope Exchange of Lyophilized BPTI(^2H) in Dimethyl Sulfoxide, Dimethylformamide, and Methanol. Lyophilized BPTI(^2H) was dissolved at 1 mg/mL in one of these solvents, each containing 1% (v/v) ^1H_2O. The isotope exchange was allowed to proceed for 24 h at 37 °C, followed by removal of the organic solvent to recover the protein. In the case of the dimethyl sulfoxide solution, the solvent was removed by lyophilization (10 μm Hg pressure, −50 °C, 48 h). The dimethylformamide and methanol solutions of BPTI were evaporated within 1 h using a vacuum of some 1 mm Hg and a bath temperature not exceeding 37 °C. The protein samples were dissolved in ^2H_2O, and their ^1H NMR spectra were recorded as described above.

Hydrogen Isotope Exchange of Lyophilized BPTI(^2H) in Glycerol. To a solution of 5 mg of lyophilized BPTI(^2H) in 50 μL of ^1H_2O was added with stirring 4.9 mL of glycerol. After incubation for 24 h at 37 °C, the solution was added dropwise to 45 mL of a vigorously stirred 10 mM aqueous sodium acetate buffer (pH 3.5) at 4 °C. The mixture was stirred for an additional 10 min and then subjected to repeated ultrafiltrations using the same aqueous buffer at 4 °C. This ultrafiltration procedure required approximately 8 h.[32] The resultant 1 mL of this solution was lyophilized, the protein powder thus obtained was dissolved in 700 μL of ^2H_2O, and its ^1H NMR spectrum was recorded. The concentration of BPTI in the NMR tube was between 0.5 and 1 mM, and at least 8000 transients were recorded.

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(32) When 18 mg of BPTI (that provides ~3.5 mM protein concentration in the NMR tube) in 180 μL of ^1H_2O and 17.8 mL of glycerol was used (see the legend to Figure 1), 160 mL of the aqueous buffer was needed. This increases the time required for ultrafiltration to approximately 24 h. This scaled-up procedure was not used in the rest of this study because a reasonably short workup time was desirable.

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