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Protein Structure in the Lyophilized State: A Hydrogen Isotope Exchange/NMR Study with Bovine Pancreatic Trypsin Inhibitor

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Abstract: The structure of a stable model protein, bovine pancreatic trypsin inhibitor (BPTI), in the lyophilized form has been investigated using the hydrogen isotope exchange/high-resolution NMR methodology. Six amide protons of BPTI that are buried in the protein interior and strongly hydrogen-bonded in aqueous solution are found to exchange with water vapors within hours in the lyophilized state; in aqueous solution, most of these protons do not exchange appreciably even after a week under otherwise identical conditions. When BPTI is lyophilized in the presence of the lyoprotectant sorbitol, no significant hydrogen isotope exchange of these protons in the solid state is detected. On the basis of these and other observations it is concluded that the structure of BPTI is partially (and reversibly) denatured on lyophilization. This conclusion, if true for other proteins, may explain the drastically reduced enzymatic activity in nonaqueous media compared to that in water.

The use of enzymes in organic solvents (instead of water) has the potential of enhancing the synthetic utility of biocatalysis.[1] However, the reactivity of most enzymes in organic solvents is far lower than in aqueous solution.[2] This may be due to a reduced conformational flexibility of enzymes in nonaqueous media compared to that in aqueous solution[3] or partial unfolding of enzymes by nonaqueous solvents and/or upon prior lyophilization.[4] To examine the last possibility, in this work we have investigated the structure of a model protein, bovine pancreatic trypsin inhibitor (BPTI), in the lyophilized state using hydrogen isotope exchange, followed by high-resolution NMR spectroscopy. The data obtained suggest that the protein is partially reversibly unfolded in the lyophilized state.

Despite several investigations dealing with the structure of proteins in the lyophilized state, this issue remains controversial. Results of IR spectroscopic studies[5,6] and those measuring the overall hydrogen isotope exchange[5,7] on hydration have led to a conclusion that the structure of lysozyme in the lyophilized state is the same as in aqueous solution. In contrast, Raman spectroscopy data point to conformational changes in lysozyme on hydration.[8] Likewise, dehydration-induced conformational transitions in several labile proteins have been demonstrated with IR spectroscopy.[9] All these methods, however, are not capable of pinpointing the putative changes in proteins to specific amino acid residues. The methodology of hydrogen isotope exchange monitored by NMR, employed by us herein, can do so.

Results and Discussion

Amide hydrogen exchange rates in proteins are primarily controlled by the strength of hydrogen bonding[10] and range from too fast to be observed to extremely slow. In BPTI, four amide protons are so protected that in water they do not exchange appreciably (<1%) even after 3 weeks at pH 3.5 and 36 °C[11] (Figure 1). Three of them—those of Tyr21, Phe22, and Tyr23 (belonging to the buried central β strand of BPTI[12])—are well resolved in the one-dimensional ^{1}H NMR spectrum.[13] Three other well resolved protons, those of Phe33, Tyr35, and Phe45 (belonging to the peripheral β strands of the protein[12]), exchange somewhat faster, but still no appreciable isotope exchange is observed after days under identical conditions.[11b] We reasoned that if BPTI is unfolded in the lyophilized state, some of these buried protons may become exposed and exchange with water vapors. Subsequent dissolution of the lyophilized sample at pH 3.5 would trap the slowly exchanging protons and afford their detection by ^{1}H NMR.

All exchangeable protons of BPTI were replaced with deuterons using 8 M urea-d_4 solution at 75 °C. Comparison of the ^{1}H NMR spectrum of the resultant BPTI (^{2}H-form) with that of BPTI(^{1}H) (Figure 2B,A) confirms that all the backbone amide groups had been labeled with deuterons. BPTI(^{2}H), lyophilized from p^{2}H 3.5 aqueous solution, was subsequently exposed to ^1H_2O vapors in a controlled humidity chamber at 37 °C for 24 h. The resultant solid protein sample was dissolved in ^2H_2O at p^{2}H 3.5,[14] and its ^{1}H NMR spectrum was recorded.

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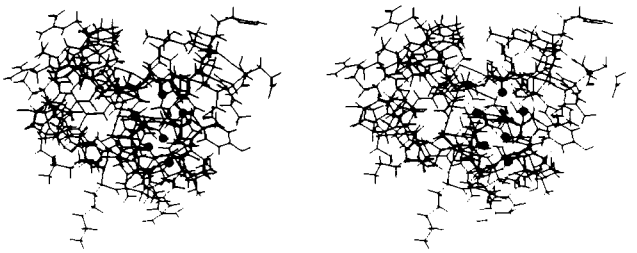

Figure 1. Stereoview of the crystal structure of BPTI[12] showing the location of the six buried amide protons under investigation herein. They are represented by black spheres and belong to Tyr23, Tyr35, Phe45, Phe22, Phe33, and Tyr21 (from left to right). Solvent accessibility calculations utilizing a spherical probe with a radius of 1.4 Å which mimics a water molecule, performed using the INSIGHT software from Biosym Technologies (San Diego, CA), reveal that these six protons are completely inaccessible to the solvent. The crystal structure of BPTI was viewed using INSIGHT.

Figure 2C depicts the NMR spectrum of a BPTI(^{2}H) sample hydrated at 67% relative humidity (rh). The NH protons belonging to Tyr21, Phe22, Tyr23, Phe33, Tyr35, and Phe45 residues are clearly seen demonstrating that isotope exchange occurred within 24 h (Figure 2C). Even in a drier atmosphere—at 11% rh—the last four of these six protons appreciably exchanged within 24 h, albeit to a lesser extent than at 67% (data not shown). A control, solution-state 24-h exchange experiment with BPTI-(^{2}H) in ^1H_2O (pH 3.5) at 37 °C, in agreement with the literature data,[11b] did not yield the signals of the six slowest exchanging protons (data not shown). Note that all the slowest exchanging protons are also protected from the isotope exchange in the BPTI crystals even after 3 months of soaking the crystals in ^2H_2O at pH 8.2, as determined by neutron-diffraction techniques.[12]

That the exchange rates of certain NH protons in lyophilized BPTI are much faster than in solution (and in hydrated crystals) can be due to either (i) enhancement of the local cooperative fluctuations in the native structure, or (ii) partial unfolding of the protein accompanied by exposure of the amide protons, or (iii) a distinct (compared to that in water) microenvironment of the amide protons in the native structure in the lyophilized state. This microenvironment may arise from a lyophilization-induced change in the effective p^{2}H, ionic strength, or some other factors.[5,7]

Changes in the local cooperative fluctuations (scenario (i)) cannot account for the increased rates because such motions, in fact, are drastically reduced in the lyophilized state compared to aqueous solution.[3a,5,6] The fundamental difference between the remaining two possibilities is that the exchange occurs either from the unfolded conformation (scenario (ii)) or from the native one (scenario (iii)). We reasoned, therefore, that the actual mechanism could be arried at by using lyoprotectants to potentially stabilize the native conformation in the lyophilized state.[4,9] If the increased exchange rates stem from scenario (iii), then lyophilization with a lyoprotectant (e.g., sorbitol) should not stop the exchange. In contrast, if the exchange proceeds from the unfolded structure, colyophilization with sorbitol should arrest the exchange.

We have found that, in fact, on hydration of solid BPTI(^{2}H) colyophilized with deuterated sorbitol at 37 °C and 67% rh for 24 h, no exchange was observed for all six protons (Figure 2D). One may argue that no exchange occurs in this experiment because upon colyophilization sorbitol forms a physical barrier restricting the passage of water molecules to the protein. To test this, exchange experiments with deuterated *N*-acetylglycine *N'*-methylamide (AGMA), an accepted peptide model for exchange studies[15] lyophilized with and without deuterated sorbitol were

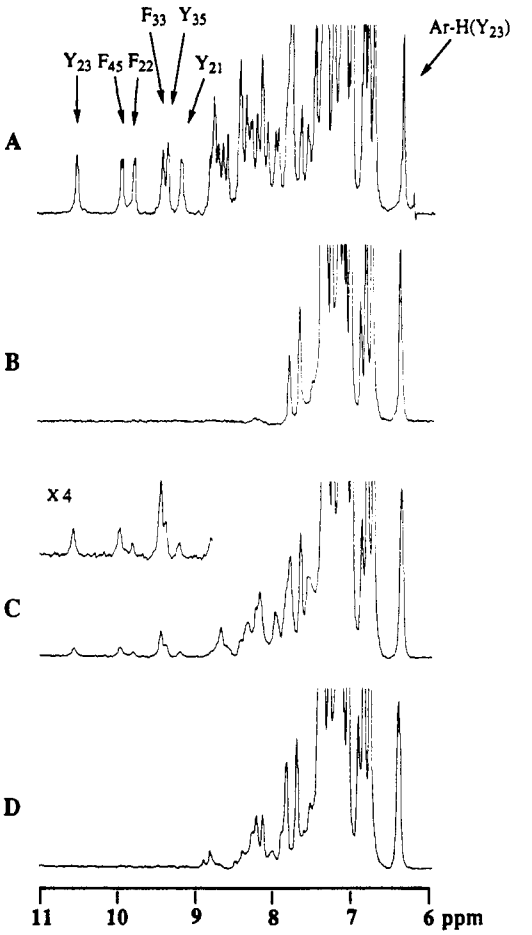

Figure 2. The ^{1}H NMR spectra of BPTI samples recorded in ^2H_2O at p^{2}H 3.5. Spectra are plotted in such a way that the intensity of the nonexchangeable signal at 6.35 ppm (Ar-H of Tyr23) remains constant: (A) BPTI(^{1}H) lyophilized from pH 3.5; (B) BPTI(^{2}H) lyophilized from p^{2}H 3.5; (C) BPTI(^{2}H) lyophilized from p^{2}H 3.5 and hydrated at 37 °C and 67% rh for 24 h; and (D) BPTI(^{2}H) colyophilized with deuterated sorbitol from p^{2}H 3.5 and hydrated at 37 °C and 67% rh for 24 h. The insert in part C shows the expanded (4-fold) amide proton region of interest. The chemical shifts of the six identified protons in spectrum A and those in spectrum C were found to be identical using the dedicated Varian NMR software. Note that a broad peak at approximately 8.8 ppm is observed in spectrum D but not in C. Several intermediate-range exchanging amide protons resonate in this region, and they presumably back-exchange with the ^2H_2O solvent during the time of sample preparation and recording of the ^{1}H NMR spectrum. The large molar concentration of sorbitol present in the BPTI sample colyophilized with sorbitol (spectrum D) reduces the time required for shimming the magnet (to 10–30 min) compared to that for the BPTI sample without sorbitol (1–2 h; spectrum C). This would result in the detection of the protons at 8.8 ppm in spectrum D but not in C. To test this hypothesis, we performed a hydration experiment with a deuterated BPTI sample colyophilized with deuterated sorbitol under exactly the same conditions as before (see Experimental Section). However, the ^{1}H NMR spectrum of this hydrated sample was recorded both 15 min after dissolving the sample in ^2H_2O and then again 2 h later. Indeed, while the first spectrum exhibited the expected peak at 8.8 ppm, the second one did not. Abbreviations: Y = tyrosine, F = phenylalanine, Ar-H = aromatic protons.

performed. The intensities of both amide protons in AGMA colyophilized with deuterated sorbitol significantly exceeded those in individually lyophilized AGMA (see Experimental Section). Further, no more than 1% exchange was observed when deuterated AGMA was mixed with sorbitol (^{1}H-form) in DMSO-d_6 within the time of recording the spectrum. These data suggest that sorbitol does not restrict (and actually facilitates) the passage of water molecules to AGMA in the lyophilized state and hence is expected not to form a physical barrier around BPTI molecules either. This observation rules out the distinct microenvironment

(14) For BPTI, at this pH isotope exchange with solvent protons is minimal.[11b]

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Table 1. Intensities of Amide Protons of Deuterated Lyophilized BPTI, Determined By ^{1}H NMR Spectroscopy, Following a 24-h Hydration at 37 °C and 67% Relative Humidity[a]

amide proton	location in BPTI	chemical shift (ppm)	intensity (%)[b]
Tyr21	central β strand[c]	9.21	6.7 ± 0.8[d]
Phe22	central β strand[c]	9.83	6.8 ± 3.2
Tyr23	central β strand[c]	10.55	12 ± 1.8
Phe33[e]	peripheral β strand[f]	9.46	31 ● 4.8
Tyr35[e]	peripheral β strand[f]	9.39	16 ± 1.6
Phe45	peripheral β strand[g]	9.97	14 ± 4.5

[a] The protein was lyophilized from p^{2}H 3.5. The weight gain on hydration was 18 ± 3.6%. [b] Intensities were calculated as follows: the ratio of intensity (peak area) of each amide proton to that of the non-exchangeable signal of aromatic protons of Tyr23 at 6.35 ppm for BPTI(^{1}H) (Figure 2A) was set to 100%. This ratio obtained for each amide proton in the hydration experiment was compared to the above 100% values. [c] Extending from Arg17 to Asn24.[12] [d] Mean values and standard deviations were obtained from measurements done in triplicate. [e] The intensities for these protons were obtained by multiplying the height proportions of the individual peaks in the unresolved signal by the total peak area. [f] Extending from Leu29 to Tyr35.[12] [g] Extending from Asn43 to Lys46.[12]

hypothesis in the lyophilized state. Hence, only the remaining scenario, (ii), can account for the increased exchange rates.

In conclusion, the dramatic enhancement in the exchange rates of amide protons, compared to those in solution and in the crystalline state, suggests a partially (and reversibly[16]) unfolded conformation in the lyophilized state. Given that BPTI is one of the more stable proteins,[17] others might be even more liable to such an unfolding. This conclusion, apart from its implications for lyophilized protein pharmaceuticals,[18] supports our earlier hypothesis[4] that enzymes are less active in organic solvents than in water primarily due to their prior denaturation during lyophilization which is not subsequently reversed by the organic solvents.[2] Consequently, the majority of enzyme molecules in the lyophilized (but not crystalline[19]) state, suspended in non-aqueous solvents, may be catalytically inactive.[20] They can be activated, however, e.g., by adding the lyoprotectant sugars to enzyme aqueous solution prior to lyophilization.[4]

Experimental Section

Chemicals. Bovine pancreatic trypsin inhibitor (BPTI), urea-d_4, and acetyl glycine were from Sigma Chemical Co. Triethylamine (99.9%), methylamine hydrochloride (99%), and DMSO-d_6 (99.9 atom % ^{2}H) were from Aldrich Chemical Co. ^2H_2O (99.8 atom % ^{2}H) was from Cambridge Isotope Laboratories. Potassium iodide, lithium chloride, and organic solvents were reagent grade from Mallinckrodt Specialty Chemical Co.

Preparation of Completely Deuterated BPTI. BPTI (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. The resultant 1 mL of BPTI(^{2}H) solution was diluted with ^2H_2O to 1.0 mg/mL, and the p^{2}H was adjusted to 3.5, followed by lyophilization. This powder was used for hydration experiments.

Hydration and ^{1}H NMR Analysis of Lyophilized Deuterated BPTI Powder. A saturated aqueous solution of KI (67% rh) or LiCl (11% rh)[21] was freshly prepared in a glass chamber at 37 °C. The humidity in the chamber was measured using a VWR hygrometer and found to be within 2% rh of the expected value. The lyophilized deuterated BPTI powder (18 mg) was hydrated in such a glass chamber for 24 h. The ^{1}H NMR spectra were recorded at ambient temperature using a Varian VXR 500 MHz spectrometer with a 6 s pulse delay and were processed with a 4-Hz line broadening factor. Two batches of ^2H_2O (500 and 200 μL) were used to dissolve the hydrated BPTI sample for better recovery. The solution was filtered through a membrane with 0.22-μm pores, and the p^{2}H was adjusted to 3.5. The concentration of the hydrated samples in the NMR tube ranged from 2.5 to 3.5 mM.

Hydration of BPTI Colyophilized with Sorbitol. Deuterated sorbitol was prepared by lyophilizing a 1 mg/mL solution of sorbitol in ^2H_2O. The colyophilized solid was prepared from BPTI(^{2}H) (100 mg) and deuterated sorbitol (100 mg) in 1 mL of ^2H_2O at p^{2}H 3.5. This powder was hydrated at 37 °C for 24 h in a glass chamber containing saturated aqueous solution of KI (67% rh)[21] and analyzed by ^{1}H NMR spectroscopy as before. The weight gain on hydration was 21%.

Hydration Experiments with *N*-Acetylglycine *N'*-Methylamide. AGMA was synthesized from acetylglycine and methylamine hydrochloride using the method of Koyama and Shimanouchi.[22] AGMA was purified using silica gel column chromatography and deuterated by incubation of a 100 mg/mL solution in ^2H_2O at p^{2}H 4.5 for 2.5 h, followed by lyophilization. The hydrogen isotope exchange of the lyophilized powder (24 mg) was carried out at 4 °C in a glass chamber of saturated aqueous solution of $CuCl_2$ (68% rh)[21] for 24 h. After hydration, the sample was dried under vacuum and dissolved in 1 mL of DMSO-d_6, and the ^{1}H NMR spectrum was immediately recorded. The colyophilized solid of AGMA and sorbitol was prepared by lyophilizing 1 mL of ^2H_2O containing 100 mg each of deuterated BPTI and deuterated sorbitol at p^{2}H 4.5. This colyophilized powder was hydrated and analyzed in a same manner as AGMA powder. The degree of exchange of AGMA in the lyophilized state was calculated using the signal intensity of the unexchangeable acetyl protons as internal standard. The intensity of the two amide protons of AGMA increased from 21 ± 5% (N*H*Ac) and 18 ± 3% (N*H*Me) for the individually lyophilized AGMA to 64 ● 9% (N*H*Ac) and 59 ● 10% (N*H*Me) for the AGMA colyophilized with deuterated sorbitol.

(16) Because the ^{1}H NMR spectra of the original and lyophilized/redissolved BPTI samples were found to be identical.

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