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On Protein Denaturation in Aqueous−Organic Mixtures but Not in Pure Organic Solvents

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Abstract: FTIR spectroscopy was used to quantitatively assess the secondary structure of proteins in aqueous−organic mixtures ranging from pure water to a pure solvent. For every such solution/suspension, the α-helix content of the protein was independently calculated from the amide I and amide III spectral regions (which gave essentially identical results). In all cases studied (two unrelated enzymic proteins—lysozyme and subtilisin; three dissimilar water-miscible solvents—acetonitrile, tetrahydrofuran, and 1-propanol), the protein secondary structure was much more native-like in pure organic solvents than in most water−solvent mixtures, e.g., 60% (v/v) organic solvents. In fact, placing lyophilized (or crystalline) proteins in the anhydrous solvents tested had no appreciable effect on the α-helix content, whereas the latter declined markedly in the 60% (v/v) solvents. This behavior was found to be kinetically controlled, i.e., to be due to inherent restrictions on protein conformational mobility in anhydrous, in contrast to aqueous−organic, media.

Introduction

Biochemists often state that organic solvents denature proteins, e.g., "Proteins can be denatured...by certain miscible organic solvents such as alcohol or acetone...".[1] Such conclusions, however, rarely come from studies where proteins are actually examined in "organic solvents such as alcohol or acetone"; instead, miscible organic solvents are usually added to aqueous solutions of proteins.[2] Although it is tempting to presume that if proteins are denatured in aqueous−organic mixtures, they will certainly experience that fate, and likely to a greater extent, in pure (neat) organic solvents, this extrapolation may not be correct. The difficulty of testing it experimentally stems from the fact that proteins are insoluble in most organic solvents,[3] and conventional biophysical techniques for protein characterization are designed for protein solutions, not suspensions.

The burgeoning area of nonaqueous enzymology,[4] whereby enzymes suspended in neat organic solvents exhibit catalytic activity, would seem to contradict the notion of protein denaturation in such media. However, one could argue that because enzymatic activity in organic solvents usually constitutes but a small fraction of that in water, it is not inconsistent with severe, albeit incomplete, denaturation exceeding in fact that in aqueous−organic mixtures. The problem with assessing

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

(1) Lehninger, A. L.; Nelson, D. L.; Cox, M. M. *Principles of Biochemistry*, 2nd ed.; Worth: New York, 1993; p 180. Our comments should not be construed as a criticism of this excellent textbook.

(2) For example, see: Lapanje, S. *Physicochemical Aspects of Protein Denaturation*; Wiley: New York, 1978; Chapters 1, 2, 3, and 6.

(3) Notable exceptions include dimethyl sulfoxide, formamide, and a few other polar, hydrophilic organic solvents (Singer, S. J. *Adv. Protein Chem.* **1961**, *17*, 1−68. Chin, J. T.; Wheeler, S. L.; Klibanov, A. M. *Biotechnol. Bioeng.* **1994**, *44*, 140−145). While proteins are indeed denatured when dissolved in them, such protein-dissolving organic solvents are not suitable reaction media for nonaqueous enzymology.[4] Consequently, they were not used in the present study.

(4) (a) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141−144. (b) Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194−211. (c) Chen, C. S.; Sih, C. J. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 695−707. (d) Gupta, M. N. *Eur. J. Biochem.* **1992**, *203*, 25−32. (e) Koskinen, A. M. P., Klibanov, A. M., Eds. *Enzymatic Reactions in Organic Media*; Blackie: London, 1996.

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this argument is that the proteins and solvents used in the examination of protein denaturation in aqueous−organic mixtures[2] are distinct from those employed with enzymes suspended in organic solvents,[4] thus making direct comparisons impossible.

Recent advances in Fourier-transform infrared (FTIR) spectroscopy have made it a method of choice for quantitatively investigating the structure of *solid*, e.g., lyophilized, proteins.[5] In the present work, we extend this methodology to protein *suspensions* in organic solvents. Since FTIR characterization of protein *solutions* is well established,[6] we have been able to assess protein structure throughout the entire range of aqueous−organic mixtures, i.e., from pure water to a pure organic solvent, regardless of solubility. These measurements reveal that although proteins are denatured in aqueous−organic mixtures, their secondary structure remains essentially intact in the corresponding pure organic solvents.

Results and Discussion

Two regions of the IR spectrum of a protein, amide I (1600−1700 cm^{-1}) and amide III (1215−1335 cm^{-1}), have been widely used to quantify the individual elements of the secondary structure in aqueous solution.[6c,7] The same has been recently accomplished for solid, e.g., lyophilized, protein samples.[5c,8] While in aqueous solution both the α-helix and β-sheet contents are indicative of the intactness of protein structure, for protein powders the latter parameter is suspect because it is inflated by the intermolecular β-sheet formation.[5c] Therefore, in the present study we employed the percentage of α-helices, independently determined from the amide I and amide III spectral regions whenever possible, to gauge the degree of denaturation of proteins in various solvent systems. Hen egg-white lysozyme, a typical and thoroughly investigated protein,[9] was selected as the main model; acetonitrile, extensively used in nonaqueous enzymology,[4,10,11] was employed as the organic (co)solvent in much of this work.

The IR spectrum of lysozyme in aqueous solution in the amide I and amide III regions is depicted in Figure 1A.[12] Gaussian deconvolution of this spectrum revealed a protein α-helix content of 34% (34 ± 2% from amide I and 34 ± 1% from amide III). The same FTIR analysis of the enzyme lyophilized from that aqueous solution showed some dehydration-induced, reversible[5c,13] denaturation; the α-helix content declined to 26−27% (27 ± 2% from amide I and 26 ± 3% from amide III). However, when this lyophilized lysozyme sample was suspended in anhydrous acetonitrile, its IR spectrum

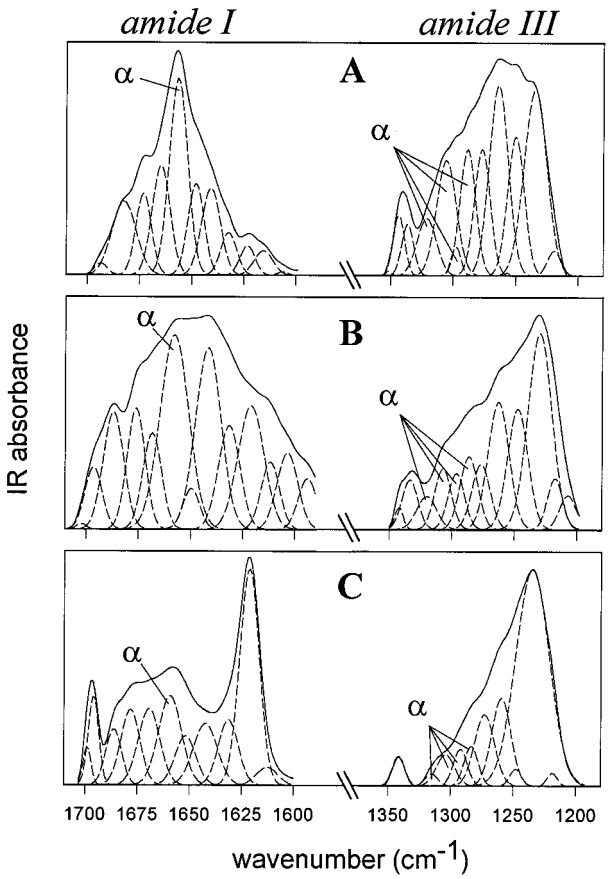

Figure 1. IR spectra of lysozyme dissolved in water at pH 1.9 (A), suspended in pure acetonitrile as a pH 1.9 lyophilized powder (B), and dissolved in a mixture of 60% acetonitrile and 40% (v/v) pH 1.9 water (C) in the amide I and amide III regions. The solid curves represent the spectra after Fourier self-deconvolution in the amide I region, and the original spectra in the amide III region. (The results of the Gaussian curve-fitting, shown superimposed, are nearly identical.) The dashed peaks represent the individual Gaussian bands. Note that the spectra in the amide I and III regions are not drawn to the same scale (the IR absorbance in the amide III region is 10 times smaller than in the amide I region). For other experimental details, see the Materials and Methods.

Table 1. α-Helix Contents of Lysozyme, Lyophilized from pH 1.9, in Different Organic Solvents and Their Mixtures with Water[a]

	α-helix content, %	
solvent[b]	amide I	amide III
100% acetonitrile	25 ± 2	24 ± 3
60% acetonitrile + 40% H_2O	13 ± 2	11 ± 2
100% tetrahydrofuran	22 ± 3	*c*
60% tetrahydrofuran + 40% H_2O	15 ± 3	16 ± 1
100% 1-propanol	23 ± 1	c
60% 1-propanol + 40% H_2O	12 ± 3	c

[a] In all experiments, 50 mg of lysozyme was suspended in 1 mL of the solvent. At this concentration, lysozyme was soluble in 60% acetonitrile, 60% tetrahydrofuran, and 60% 1-propanol, and insoluble (i.e., yielded suspensions) in the 100% solvents. [b] All the solvent percentages are v/v. The water was adjusted to pH 1.9 prior to mixing with acetonitrile. [c] Not determined because correction for the solvent was impossible due to strong solvent bands in the amide III spectral region.

(5) (a) Prestrelski, S. J.; Arakawa, T.; Carpenter, J. F. *Arch. Biochem. Biophys.* **1993**, *303*, 465−473. (b) Prestrelski, S. J.; Tedischi, N.; Arakawa, T.; Carpenter, J. F. *Biophys. J.* **1993**, *65*, 661−671. (c) Griebenow, K.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10969−10976.

(6) (a) Braiman, M. S.; Rothschild, K. J. *Annu. Rev. Biophys. Chem.* **1988**, *17*, 541−570. (b) Surewics, W. K.; Mantsch, H. H. *Biochim. Biophys. Acta* **1988**, *952*, 115−130. (c) Arrondo, J. L. R.; Muga, A.; Castresana, J.; Goni, F. M. *Prog. Biophys. Mol. Biol.* **1993**, *59*, 23−56. (d) Jackson, M.; Mantsch, H. H. *Crit. Rev. Biochem. Mol. Biol.* **1995**, *30*, 95−120.

(7) (a) Singh, B. R.; Fuller, M. P.; Schiavo, G. *Biophys. Chem.* **1990**, *46*, 155−166. (b) Singh, B. R.; Fu, F.-N.; Ledoux, D. N. *Struct. Biol.* **1994**, *1*, 358−360. (c) Fu, F.-N.; DeOliveira, D. B.; Trumple, W. R.; Sakar, H. K.; Singh, B. R. *Appl. Spectrosc.* **1994**, *48*, 1432−1441.

(8) Dong, A.; Prestrelski, S. J.; Allison, S. D.; Carpenter, J. F. *J. Pharm. Sci.* **1995**, *84*, 415−424.

(9) Blackburn, S. *Enzyme Structure and Function*; M. Dekker: New York, 1976; Chapter 10.

(10) Schmitke, J. L.; Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 3360−3365.

(11) Including X-ray crystallographic studies in anhydrous acetonitrile: Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8653−8657. Fitzpatrick, P. A.; Ringe D.; Klibanov, A. M. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 675−681.

(12) The secondary structure of lysozyme in aqueous solution at pH 1.9, 3.1, 4.0, and 5.1 is the same.[13] Subsequent lyophilization results in significant secondary structural changes, but the structure of the lyophilized powders is the same irrespective of the pH of the aqueous solution.[13] However, the extent of lysozyme denaturation in acetonitrile−water mixtures is pH-dependent and less pronounced for lysozyme obtained at neutral pH values. We chose pH 1.9 in this work to demonstrate the effect of protein denaturation at different acetonitrile concentrations in water most clearly.

(13) Costantino, H. R.; Griebenow, K.; Mishra, P.; Langer, R.; Klibanov, A. M. *Biochim. Biophys. Acta* **1995**, *1253*, 69−74.

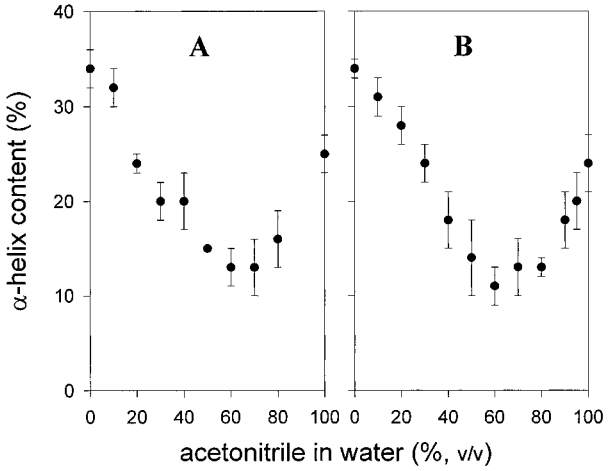

Figure 2. Dependence of the α-helix content of lysozyme on the concentration of acetonitrile in the medium. The α-helix content was independently calculated from the amide I (A) and amide III (B) spectral regions. Each data point represents the mean value of at least four separate measurements; standard deviations are indicated by error bars. Because correction for the water IR absorbance band was not possible at 90% and 95% acetonitrile in the amide I region, these data points are missing. Water added to acetonitrile in all cases had pH 1.9. The lysozyme concentration was 50 mg/mL, which yielded solutions at 0−60% (v/v) acetonitrile and suspensions at higher acetonitrile contents. For other experimental details, see the Materials and Methods.

(Figure 1B) indicated no further denaturation; the α-helix content (the first entry in Table 1) was, within experimental error, the same as for the powder.

The conclusion that neat acetonitrile causes no appreciable denaturation of the protein was confirmed using crystalline (as opposed to lyophilized) lysozyme. The α-helix content of the crystalline protein sample, 27 ± 2% (amide III) to 30 ± 2% (amide I), remained the same, within the error of experiment, when the crystals were suspended in neat acetonitrile—27 ± 2% from amide III and 28 ± 2% from amide I.

Next, we measured the α-helix content of lysozyme in various water−acetonitrile mixtures, and the results obtained are depicted in Figure 2. Several noteworthy comments can be made. Parts A and B of the figure (corresponding to independent calculations from the amide I and III spectral regions, respectively) look very similar, thus further validating our α-helix determination methodology. The dependence of the α-helix content on the fraction of acetonitrile in the solvent mixture is an inverted bell-shaped curve; i.e., lysozyme's secondary structure is compromised (some α-helicity lost) in water−acetonitrile mixtures but not in either pure water or acetonitrile. For example, in 60% (v/v) acetonitrile (the highest acetonitrile fraction where lysozyme is still soluble under our conditions), the α-helix content is just half of that in the pure organic solvent (second entry in Table 1). Even visually the character of the IR spectrum, as well as its Gaussian deconvolution, in 60% (v/v) acetonitrile (Figure 1C) is quite distinct from those in water (Figure 1A) and acetonitrile (Figure 1B).

To shed light on why lysozyme may be more denatured in water−acetonitrile mixtures than in neat acetonitrile (Figure 2), we assessed the secondary structure of the protein in 90% (v/v) acetonitrile prepared in two different ways. In one, lysozyme was directly suspended in this water−solvent mixture. In the other, the enzyme was first dissolved in water, and then acetonitrile was added to the final concentration of 90% (v/v). The α-helix content was found to be[14] 18 ± 3% in the former case and only 10 ± 2% in the latter. That there is a significant difference in the protein secondary structure depending on the mode of preparation indicates that the situation is under a kinetic, rather than thermodynamic, control. If the protein suspended in 90% acetonitrile is kinetically trapped, then it will be even more so in neat acetonitrile. This is consistent with our earlier hypothesis[15] that enzymes retain catalytic activity in anhydrous solvents due to their structural rigidity in such media (compared to water), resulting in high kinetic barriers preventing the native-like conformation from unfolding.

As mentioned above, beyond 60% (v/v) acetonitrile, lysozyme is not completely soluble in the solvent mixture at 50 mg/mL. Thus, one might consider the following explanation to the ascending portion of the curve in Figure 2. If one assumes that the dissolved protein is more prone to solvent-induced denaturation than the suspended protein (due to the loss of stabilizing protein−protein contacts), then at higher acetonitrile (beyond 60%) concentrations there will be less dissolved lysozyme. In other words, the α-helix content increases because the fraction of the dissolved (and more denatured) protein decreases.

We tested, and ruled out, this hypothesis by comparing lyophilized lysozyme placed in 60%, 70%, and 95% (v/v) acetonitrile. In the first two systems the α-helix content is, within the error of experiment, the same—11 ± 2% vs. 13 ± 3%, respectively—whereas the solubilities (at 50 mg/mL protein) vary drastically—100% and <2%, respectively. As far as the second two mixtures are concerned, the solubilities at 50 mg/mL lysozyme in both 70% and 95% acetonitrile were found to be below 2%, while the α-helix contents differed significantly—13 ± 3% and 20 ± 3%, respectively.[14] These observations demonstrate that the ascending part of the curves in Figure 2 reflects intrinsic properties of suspended lysozyme, not differences in solubility.

It was of interest to verify this conclusion by comparing the secondary structure of dissolved vs suspended lysozyme in the same solvent system (as opposed to 60% and 70% acetonitrile above). To this end, we took advantage of the fact that another organic solvent, methanol, dissolves high concentrations of lysozyme.[16] First, we prepared a saturated solution of lysozyme in methanol (approximately 35 mg/mL) and measured its IR spectrum. The α-helix content was calculated (from the amide I region) to be 17 ± 3%. Second, we placed 80 mg of the same lyophilized lysozyme in 1 mL of methanol, measured its IR spectrum, and then subtracted that of the dissolved protein. Gaussian deconvolution of the resulting differential spectrum yielded an α-helix content of 12 ± 1% for the suspended enzyme. Thus, the latter is indeed no less denatured than the dissolved enzyme which is devoid of protein−protein contacts. Hence, in this system these contacts have no appreciable stabilizing effect on the secondary structure.

It was essential to ascertain whether the behavior depicted in Figure 2 would be expressed with solvents other than acetonitrile. Consequently, we tested two additional, dissimilar water-miscible solvents, tetrahydrofuran and 1-propanol. In each case, the secondary structure of lysozyme was examined by FTIR spectroscopy in the pure solvent and in 60% (v/v) solvent. As can be seen in Table 1, in 60% tetrahydrofuran the α-helix content of lysozyme was one-third lower than in the neat solvent. For 1-propanol, the difference was even greater—nearly 2-fold.

The serine protease subtilisin Carlsberg has been one of the most researched enzymes in nonaqueous enzymology.[4,10,11] Therefore, we employed it herein to test the generality of the

(14) Using the amide III band spectral region. Solvent subtraction in the amide I region proved to be impossible at this acetonitrile concentration.

(15) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194−3201.
(16) Chin *et al.* Reference 3.

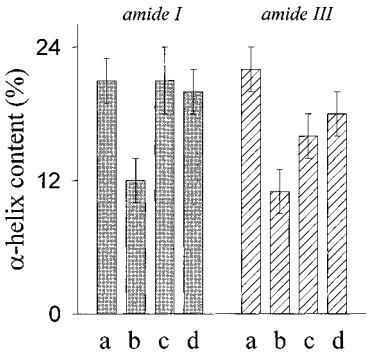

Figure 3. α-Helix content of subtilisin dissolved in water at pH 3.0 (a), suspended in 60% acetonitrile + 40% (v/v) pH 3.0 water (b), suspended in pure acetonitrile (c), and as a pH 3.0 lyophilized powder (d). The percentage of α-helices was independently calculated from the amide I (stippled bars) and amide III (lined bars) spectral regions. Errors shown are the standard deviations from the mean of at least four separate measurements. The enzyme concentration was 50 mg/mL (a−c) and 1 mg/200 mg of KBr (d). For other experimental details, see the Materials and Methods.

conclusions reached with lysozyme. Subtilisin's secondary structure was measured by means of FTIR analysis in various solvent systems.[17] As seen in Figure 3, the α-helix content of subtilisin is halved upon transition from pure water (bar a) to 60% (v/v) acetonitrile (bar b). It increases by approximately two-thirds when the lyophilized enzyme is suspended in neat acetonitrile (bar c) instead. Note that the α-helix content of subtilisin in pure acetonitrile is the same as that of the dry enzyme (bars c and d, respectively); i.e., the anhydrous solvent has no denaturing effect.

In closing, this work provides the first direct biophysical evidence that, somewhat counterintuitively, proteins are more denatured in aqueous−organic mixtures than in the corresponding pure organic solvents. This phenomenon can be understood if two effects, simultaneously at play, are considered. On the one hand, as the organic solvent content in the medium is raised, the tendency of a protein to denature increases. On the other hand, as the water content in the medium declines, the protein conformational mobility, and hence its ability to acquire the thermodynamically dictated conformation, diminishes.[18] Consequently, although in pure organic solvents the propensity of a protein to denature is undoubtedly even greater than in, e.g., 60% solvent (Table 1), its capacity to actually undergo such denaturation is severely impaired. This explains why enzymes, paradoxically, may be more catalytically active in pure organic solvents than in aqueous−organic mixtures, i.e., why addition of the natural solvent water is detrimental rather than beneficial. Also, the interplay of the two effects results in an inverted bell-shaped dependence of the kind shown in Figure 2 and explains how proteins with their conformations kinetically trapped in anhydrous solvents defy the notion that the latter should be more denaturing than their mixtures with water. Finally, as with other kinetically, but not thermodynamically, controlled systems, the history of enzymes placed in anhydrous media becomes important for their behavior.[19]

(17) Griebenow, K.; Klibanov, A. M. *Biotechnol. Bioeng.*, in press.

(18) Because water, due to its ability to form multiple hydrogen bonds including those with proteins, acts as a molecular lubricant in protein systems: Kuntz, I. D.; Kauzmann, W. *Adv. Protein Chem.* **1974**, *28*, 239−245. Finney, J. L.; Poole, P. L. *Comments Mol. Cell. Biophys.* **1984**, *2*, 129−151. Rupley, J. A.; Careri, G. *Adv. Protein Chem.* **1991**, *41*, 37−172. Gregory, R. B., Ed. *Protein-Solvent Interactions*; M. Dekker: New York, 1995.

(19) Klibanov, A. M. *Nature* **1995**, *374*, 596.

Materials and Methods

Materials. Hen egg-white lysozyme (EC 3.2.1.17; crystallized, dialyzed, and lyophilized three times) and subtilisin Carlsberg (alkaline protease from *Bacillus licheniformis*, EC 3.4.21.14) were purchased from Sigma Chemical Co. and used without further purification. Acetonitrile, tetrahydrofuran, methanol, and 1-propanol, all from Aldrich Chemical Co., were of analytical grade or better. KBr powder for IR spectroscopy was from Spectra Tech.

Protein Lyophilization. Proteins were dissolved in deionized water at 10 mg/mL, and the pH was adjusted to the desired value. All aqueous solutions of lysozyme (pH 1.9 and 3.0) and subtilisin (pH 3.0) to be lyophilized were frozen in liquid nitrogen and applied to a Labconco Model 8 freeze-drier for 48 h at a pressure of 10 μm of Hg and a condenser temperature of −50 °C. Lyophilized protein powders were stored in a freezer at −20 °C in sealed vials over Drierite (Hammond Drierite Co.).

Lysozyme Crystallization. Lysozyme was crystallized according to the literature procedure.[20] To 30 mL of a lysozyme solution (8%, pH 1.9) was added the same volume of a NaCl solution (10%, pH 1.9), the solutions were mixed, and crystallization was performed at 20 °C to result in needle-like crystals within 24 h. For FTIR measurements, the crystals were collected by suction filtration, dehydrated by pressing them between layers of filter paper and air-drying for 1.5 h, and then used immediately.

Determination of Protein Concentration. The concentration of dissolved lysozyme was determined by measuring its absorbance at 280 nm (A_{280}). A calibration curve was established using lysozyme standards of known concentrations. Samples were placed in the solvents and agitated for 30 min as described below, and undissolved particles were removed by centrifugation (10 min, 8000 rpm). The A_{280} of the supernatant was measured after a 1:10 dilution with water in a quartz cuvette of 10-mm path length.

In the case of lysozyme lyophilized from an aqueous solution of pH 3.0 and suspended in methanol,[16,21] the protein was placed in 5-mL screw-cap scintillation vials, followed by addition of the solvent. The resulting suspensions (6−80 mg of lysozyme/mL of methanol) were shaken at 30 °C and 300 rpm for 16 h. Lysozyme was fully soluble up to some 30 mg/mL under these conditions. At 40 mg of lysozyme per mL of methanol minor amounts of undissolved protein were observed, and at 80 mg/mL a pronounced suspension ensued. Removal of the undissolved particles by centrifugation was not feasible in the last two mixtures due to their extreme viscosity. Consequently, both were used directly to determine the concentration of dissolved lysozyme by UV absorbance; A_{303} values were corrected for the light scattering (measured at 320 nm). For the 40 mg/mL lysozyme, approximately 35 mg/mL was found to be dissolved. For the 80 mg/mL suspension, the concentration of the dissolved protein was found to be similar, 32 mg/mL.

FTIR Spectroscopy. FTIR studies were conducted with a Nicolet Magna-IR System 550 optical bench as described previously.[5c] A total of 256 scans at 2 cm^{-1} resolution using Happ-Ganzel apodization were averaged to obtain each spectrum. For all experiments involving solutions or suspensions, a Spectra Tech liquid cell equipped with CaF_2 windows was used. Lyophilized protein powders and lysozyme crystals were measured as KBr pellets (1 mg of protein per 200 mg of KBr).[5] Each protein sample was measured at least four times. When necessary, spectra were corrected for the solvent background to obtain protein vibrational spectra. The latter were analyzed in the amide I and amide III spectral regions. When the amide I band was analyzed, small water vapor bands present were eliminated from the spectra.

Lyophilized proteins (50 mg) were suspended/dissolved in 1 mL of the solvents used. In the case of mixtures of organic solvents with water, the pH of the water was first adjusted to that from which the proteins had been lyophilized. After a 30-s ultrasonication, the samples were stirred for 30 min. In one experiment, lysozyme was dissolved in water, the pH was adjusted to 1.9, and then acetonitrile was added to its final concentration of 90% (v/v); the protein concentration in this case was 24 mg/mL. Lysozyme crystals (80 mg) were soaked in

(20) Alderton, G.; Fevold, H. L. *J. Biol. Chem.* **1946**, *164*, 1−5.

(21) Bromberg, L. E.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1262−1266.

six consecutive 2-mL portions of acetonitrile, recovered by centrifugation, and decanted to replace the interstitial water with acetonitrile. The IR cuvette was filled with the suspension/solution, and the IR spectra were recorded. Solvent background spectra were obtained by measuring the same solvent system in the same cuvette with the same spacers. Note that FTIR spectroscopy is among the few spectroscopic methods allowing one to obtain spectra of inhomogeneous dispersions.[6a,d] The FT calculation excludes IR intensities that do not follow the correct optical path, and stray light is thus excluded from the contribution to IR intensities.[6a]

Suspensions of solid proteins in acetonitrile were measured using 50 μm-thick spacers. FTIR measurements in tetrahydrofuran, methanol, and 1-propanol, as well as in 90% and 95% (v/v) acetonitrile, were conducted using 25-μm spacers. In all other cases, 15-μm spacers were used.

All spectra except those obtained in KBr were corrected for the solvent backgrounds in an interactive manner using the Nicolet OMNIC 2.1 software.[5c] Subtraction of the H_2O background was performed as described earlier.[5c,22] It was confirmed that the subtraction of the strong water IR absorbance band in the amide I band area with a maximum at 1643 cm^{-1} was possible using the aforementioned equipment and 15-μm spacers: spectra of lysozyme in aqueous solution at pH 1.9 recorded with a path length of 3 μm (determined from the IR absorbance value of the water band of 1.22 at 1643 cm^{-1} for the 10-μm path length[23]) were virtually identical, including the second derivative spectra, to those obtained with 15-μm spacers. For proteins suspended in anhydrous solvents, the solvent spectra were subtracted. Subtraction of the backgrounds in the case of organic solvent−water mixtures was performed in an interactive manner following the previously established criteria for subtraction of water backgrounds[5c] and elimination of the IR bands of the organic solvent.[17] Since acetonitrile does not have significant IR bands in the amide I and III regions, the subtraction is straightforward. Tetrahydrofuran, methanol, and 1-propanol have no significant IR bands in the amide I region, but relatively strong ones (compared to those of proteins) in the amide III region. Therefore, correction for the solvents in the latter spectral region is impossible in the pure solvents. For reasons unknown, correction for the water absorbance band in the amide I region was not reproducible for suspensions of lysozyme in water−acetonitrile mixtures containing 90% or more acetonitrile (Figure 2A). However, the subtraction of the water−solvent background in the amide III region was possible in these cases using the disappearance of the acetonitrile bands as a subtraction criterion. This is due to the fact that the IR absorbance of water in the amide III region is quite small.[7]

The IR spectra of lysozyme lyophilized from an aqueous solution of pH 3.0 and placed in methanol[16,21] were corrected using the methanol background spectrum. Gaussian deconvolution in the amide I region (see below) afforded an α-helix content of 18 ± 3% for a 20 mg/mL solution of lysozyme in methanol. The same α-helix content, 17 ± 3%, was calculated for the saturated solution (1 mL of methanol added to 40 mg of lysozyme) containing 35 mg of dissolved lysozyme/mL of methanol. We then subtracted the spectrum of the saturated lysozyme solution from that of the suspension in order to both correct for the methanol background and subtract the contribution of the dissolved lysozyme. Gaussian devonvolution of the resulting differential spectrum, representing exclusively the undissolved protein, afforded an α-helix content of 12 ± 1%.

FTIR Data Analysis. (a) Second Derivatization. All spectra were analyzed by second derivatization in the amide I and amide III regions for their component composition.[5c,7,24] Second-derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}).[5c]

(b) Fourier Self-Deconvolution (FSD)[6b,25] was applied to the unsmoothed spectra to enable quantification of the secondary structure in the amide I region by Gaussian curve-fitting[6c,26] using the program OMNIC 2.1. The parameters chosen (a value of 16 for the full width at half-maximum (FWHM) and the enhancement factor $k = 2.0$ in the case of subtilisin and $k = 2.2$ in the case of lysozyme) are in the range of those published.[17,26−28] Note that FSD alters the band shapes, but preserves the integrated band intensities.[25a,27a] The conservative values chosen for FSD in our analyses eliminate the risk of overdeconvolution which could result in distorted band areas.[25b,26]

(c) Gaussian Curve-Fitting. The frequencies of the band centers found in the second-derivative spectra in the amide I and III regions were used as starting parameters for the Gaussian curve-fitting (performed using the program GRAMS 386 from Galactic Industries, Inc.). The secondary structure contents were calculated from the areas of the individual assigned bands and their fraction of the total area in the amide I and III spectral regions.[5c,7,17,26] Gaussian curve-fitting was performed (i) in the amide III region using the original (not resolution-enhanced) spectra[5c,13] and (ii) in the amide I region after band-narrowing of the protein vibrational spectra by FSD.[17,26,28] The results obtained in both regions were in agreement with each other (Figures 2 and 3). In all cases, a linear baseline was fitted in addition to the Gaussian bands. In most instances, the discrepancies between component frequencies obtained by second derivatization (not shown) and the Gaussian curve-fitting were below 3 cm^{-1}. The secondary structure content was determined from at least four independently obtained spectra, the values were averaged, and the standard deviations were calculated.

We verified that the parameters chosen for FSD did not influence the result of the Gaussian curve-fitting in the amide I spectral region. To this end, we selected three representative samples of lysozyme lyophilized from pH 1.9 - dissolved in water at pH 1.9, dissolved in 60% (v/v) acetonitrile, and suspended in pure acetonitrile. The corrected spectra of these samples were resolution-enhanced in the amide I region using FWHM values of 8, 12, 16, 20, 24, 28, and 32 cm^{-1}. For each of the FWHM parameters, the enhancement factors k selected were 1.2, 1.6, 2.0, 2.2, 2.6, and 3.0. These ranges include all combinations typically found in the literature for FSD prior to Gaussian curve-fitting (FWHM values of 13−30 cm^{-1} and k values of 2.0−2.8).[8,17,26−28] Several parameter combinations (e.g., FWHM = 16, k = 3.0) resulted in overdeconvoluted spectra, as reflected by side lobes and noise. Such spectra were not analyzed by Gaussian curve-fitting. The results obtained for the α-helix contents of the three lysozyme samples depending on the parameters used for FSD are summarized in Table 2. They show that the α-helix content determined in the amide I region does not depend significantly on the parameters used for FSD when overdeconvolution is avoided. We averaged out the α-helix contents determined for all the different parameter sets used and found the value of 32 ± 3% for lysozyme in aqueous solution, which is similar to that (34 ± 2%) obtained using an FWHM of 16.0 and k of 2.2. Likewise, for lysozyme in 60% acetonitrile, the same α-helix contents of 13 ± 2% and 13 ± 2%, respectively, were obtained using these two methods of analysis. For lysozyme suspended in pure acetonitrile, the values were also similar—24 ± 3% and 25 ± 2%. Thus, Gaussian curve-fitting is essentially independent of the parameters used for FSD in our cases, in contrast to concerns expressed recently,[6d,8] as long as overdeconvolution is avoided.

Band Assignments. (a) Amide I. The band assignment in the amide I region followed those in the literature.[8,17,28,29] The assignment

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Table 2. Dependence of the Calculated α-Helix Contents of Lysozyme Lyophilized from pH 1.9, Placed in Water−Acetonitrile Solvent Systems, on the FWHM of the Lorentzian Function Used for Fourier Self-Deconvolution at Different Resolution Enhancement Factors *k*[a]

FWHM, cm^{-1}	α-helix content, %[b]		
	water[c]	60% acetonitrile + 40% water[c]	100% acetonitrile
8	30 ± 3	nd[d]	nd[d]
12	32 ± 2	11 ± 2	24 ± 1
16	34 ± 3	12 ± 1	23 ± 3
20	35 ± 3	14 ± 2	25 ± 2
24	33 ± 2	14 ± 1	23 ± 0
28	33 ± 2	nd[d]	nd[d]
32	32 ± 3	nd[d]	nd[d]

[a] In all experiments, 50 mg of pH 1.9 lyophilized lysozyme was suspended in 1 mL of the solvent. At this concentration, lysozyme was soluble in water and 60% acetonitrile and insoluble (yielded a suspension) in 100% acetonitrile. For other experimental details, see the Materials and Methods. [b] The α-helix contents were determined by Gaussian curve-fitting in the amide I spectral region after band narrowing by Fourier self-deconvolution using the *k* values[8] of 1.2, 1.6, 2.0, 2.2, and 2.6 for each FWHM. The individual α-helix contents were averaged, and the standard deviations were calculated. For other experimental details, see the Materials and Methods. [c] The water was adjusted to pH 1.9 prior to mixing with acetonitrile. Solvent percentages are in v/v. [d] Not determined.

of the bands for lysozyme in aqueous solution (pH 1.9) was essentially the same as in the work of Dong *et al.*[29] The main band at 1657 ± 1 cm^{-1} was assigned to α-helices (Figure 1). Bands at 1693 ± 1, 1640 ± 1, and 1632 ± 1 cm^{-1} were assigned to β-sheets. All other bands (1683 ± 1, 1673 ± 1, 1666 ± 1, and 1648 ± 1 cm^{-1}) were assigned to unordered structural elements (β-turns, random coil, extended chains).[30] The secondary structure content determined by Gaussian curve-fitting in the amide I region using these assignments (34 ± 2% α-helix and 22 ± 1% β-sheet) were the same, within the error limits, as those determined from the amide III spectral region at pH 1.9.[13]

When the spectra of lysozyme lyophilized from pH 1.9 in nonaqueous media were analyzed (Figure 1B,C), new bands at 1621 and 1698 cm^{-1} were assigned to β-sheets. Bands at such frequencies are often assigned to intermolecular β-sheets.[8,28] Otherwise, the frequencies of the Gaussian bands found for lysozyme in organic solvents were virtually the same as for the aqueous solution. The averaged frequencies of the Gaussian bands determined for lysozyme in the acetonitrile−water mixtures used (Figure 2) were: 1696 ± 2, 1685 ± 2, 1677 ± 3, 1668 ± 2, 1657 ± 1, 1649 ± 2, 1640 ± 2, 1631 ± 2, and 1621 ± 1 cm^{-1}. Except for the above-mentioned two new bands, the rest were assigned as for the aqueous solution.

Analysis of the IR spectrum of subtilisin Carlsberg in aqueous solution at pH 3.0 afforded Gaussian bands at 1695 ± 2, 1683 ± 3, 1671 ± 2, 1665 ± 1, 1659 ± 1, 1646 ± 2, 1633 ± 2, and 1624 ± 2 cm^{-1}. When the main band at 1659 cm^{-1} and the minor band at 1665 cm^{-1} were assigned to α-helices (as proposed for acetylcholinesterase[28]), Gaussian deconvolution afforded the α-helix content of 21 ± 2%.[31] The β-sheet content (calculated from the areas of the bands at 1695, 1633, and 1624 cm^{-1}) was 17 ± 2%, similar to 14% calculated from the X-ray data.[31] All other bands were assigned to unordered secondary structural elements. Analysis of the subtilisin spectra in pure acetonitrile, in 60% acetonitrile + 40% water (pH 3.0), and in the dry state afforded components with frequencies very similar to those in aqueous solutions. Consequently, they were assigned the same way.

(b) Amide III. The band assignments in the amide III region was as published previously.[5c,7,13,33] For lysozyme, bands at 1319 ± 3, 1308 ± 2, 1300 ± 2, and 1290 ± 2 cm^{-1} were assigned to α-helices, those at 1235 ± 4 and 1221 ± 3 cm^{-1} to β-sheets, and the remaining bands at 1276 ± 4, 1260 ± 2, and 1247 ± 2 cm^{-1} to unordered secondary structures. Note that the averages were obtained from Gaussian curve-fitting of lysozyme in aqueous solution, in lyophilized form, and in various mixtures of acetonitrile with water (10−100%). The results obtained in this work for lysozyme in aqueous solution (34 ± 1% α-helix and 20 ± 1% β-sheet) and in the lyophilized form (26 ± 3% α-helix and 39 ± 5% β-sheet) in the amide III region were, within the error limits, the same as those reported earlier for lysozyme at pH 1.9 and 2.0.[13,33]

For subtilisin in aqueous solution at pH 3.0, bands at 1328 ± 2, 1314 ± 2, 1304 ± 1, and 1294 ± 2 cm^{-1} were assigned to α-helices, the band at 1234 ± 1 cm^{-1} was assigned to β-sheets, and the remaining bands between 1290 and 1245 cm^{-1} were assigned to unordered secondary structures.[31] Analysis of the spectra of subtilisin in the lyophilized form, as well as suspensions of the lyophilized powder in pure acetonitrile and in 60% acetonitrile, afforded components at similar frequencies.[34] They were assigned the same way. One additional component at approximately 1220 cm^{-1} was assigned to β-sheets.

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