Effect of Water on the Structure of a Model Polypeptide

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ABSTRACT: Vibrational spectroscopy in conjunction with X-ray and gravimetric methods has been used to study the structural stability of a hydrated polypeptide prepared in a genetically engineered strain of *Escherichia coli*. The sample adopts a β-sheet conformation in the solid state with a well-defined crystalline stem length and fold surfaces believed to be decorated with carboxylic acid groups. Ionization and subsequent hydration of these acid groups are found to have a major effect on the crystal packing and chain conformation. We have also established that the structural changes accompanying hydration of this model polypeptide occur in a stepwise fashion. First, because of their high accessibility, the carboxylic acid or carboxylate groups on the lamellar surfaces can readily interact with water molecules. In the second step, water penetrates into the regions between the hydrogen-bonded sheets; however, the resulting expansion in the intersheet distance can occur without altering the chain conformation. Lastly, when the water content is high, the hydrogen-bonded sheets are disrupted, leading to a change in chain conformation from β-strands to helical or disordered chains.

Introduction

Because of its small molecular size and propensity for forming strong hydrogen bonds, water is an important determinant of the chain conformation and structural stability of globular proteins. Water induces proteins to minimize accessible hydrophobic surface area, and the relative strength of protein−protein versus protein−water interactions determines the ability of chains to fold into ordered three-dimensional structures. The conformation and packing of biological polymers with ionic groups such as poly(L-lysine hydrochloride) or sodium poly(L-glutamate) are known to be particularly affected by the presence of water.[1−3] These polymers can exist in different conformations, e.g., β-sheet, α-helix, or disordered forms, depending on the relative humidity. Interaction of lysozyme, which contains fewer ionic groups as compared to poly(L-lysine) or sodium poly(L-glutamate), with water has also been extensively studied. In this case, evidence for conformational change as a function of water content is not as clear.[4−6] For this protein hydration-induced conformational changes have been directly correlated to the onset of increasing enzyme activity.[7] For synthetic polymers, such as nylons, water also has a major effect on the glass transition temperature and mechanical properties.[8] In Nylon 6, it has been found that a few water molecules in the interlamellar region may change the overall crystalline state,[9] and solid−solid state phase transitions in Nylon 66 have also been observed.[10,11]

Although it is known that water can influence strongly the three-dimensional states of proteins and polymers, it is necessary to specify the number and location of water molecules required to perturb macromolecular structures. Polymers seldom attain a high degree of crystallinity and will generally contain both amorphous and crystalline regions. In most instances water will preferentially interact with functional groups in the amorphous regions of a semicrystalline polymer, because of the restricted mobility of water to permeate crystalline regions. From the microscopic viewpoint, hydration may also be a stepwise process because hydration of ionizable and ionic groups is expected to be completed before that of other polar sites and because nonpolar sites are the last to be hydrated.[4,6,12]

The exact mechanism of the structural changes induced by water in polymers is not clear. Vibrational spectroscopy is an ideal tool to analyze the amount of water present and to characterize the chain conformations of polymers. The bands used for analyzing chain conformations are in general well understood, and it is also possible to follow both water uptake and water location by using D_2O instead of H_2O. Specific vibrational frequencies affected by hydrogen−deuterium exchange may be exploited to determine sites where exchange occurs most rapidly. Based on spectroscopic studies, it is possible to (1) utilize the kinetics of hydration in order to characterize the structure of polymers and proteins, (2) identify conformational and packing changes as a function of water content, (3) identify surface-bound water, and (4) clarify the relationship between surface structure and crystalline packing.

The polymer system described herein is a model polypeptide (**1**) prepared via bacterial expression of an artificial gene.[13]

$$-((\mathrm{AlaGly})_3\mathrm{GluGly})_{36}- \qquad (1)$$

The repeating alanylglycine diads were chosen as the basis of the design because alanylglycine-rich polypeptides are known to exist as β-sheet structures in the solid state.[14] The glutamic acid residue was thought to be sufficiently bulky as to be excluded from the crystalline interior and would therefore be expected to be confined either to the fold surface or to the amorphous phase. Glycine has the conformational flexibility needed for completion of the reverse turn and should allow the chain to fold back on itself, forming antiparallel β-sheets between triplet alanylglycine diads. The observed solid-state structure of this polymer is consistent with chain folding at glutamic acid, leading to lamellar crystals approximately 30 Å thick, bearing surfaces decorated with carboxylic acid groups.[13] Therefore, in addition to its regular tertiary and secondary structure, this polypeptide offers the possibility of

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studying the effects of strong interactions between water and accessible ionizable groups. It is also possible to change the surface functionality from carboxylic acid groups (COOH) to sodium carboxylate groups (COONa).

The use of such model polypeptides provides a unique opportunity to characterize the kinetics of the hydration process by following the H → D exchange rate, in both the amorphous and crystalline regions. By carrying out simultaneous gravimetric measurements, it is also possible to determine the exact amount of the water present. By following the vibrations characteristic of the carboxylic acid, one can then characterize the changes in the environment of these highly polar groups. This combination of information provides an opportunity to investigate hydration effects on protein and polymer structures at a level of detail not available previously. We show herein that the hydration of **1** is a three-step process. First, water penetrates into the most easily accessible amorphous (or fold surface) regions containing the most strongly interacting functional groups. Further hydration induces separation of the hydration-bonded sheets, and then finally one observes changes in the secondary structures of the polypeptide chains.

Experimental Section

The polypeptide was prepared via bacterial expression of the corresponding artificial gene, as described previously.[13] The chain conformation in the crystalline regions can be controlled by the procedure selected for sample preparation. If the polymer is dissolved in aqueous formic acid solution (70% v/v) and precipitated with methanol, the powder obtained has the silk II, or β chain, conformation. On the other hand, dissolution in aqueous lithium bromide solution (60%) and precipitation by dialysis against progressively diluted lithium bromide solutions affords the silk I structure. The chain conformation for samples prepared in this manner is still undefined although distinct spectroscopic features have been observed. In this study, we have examined only samples with the β conformation, with crystalline features close to those proposed for *Bombyx mori* silk fibroin and poly(L-alanylglycine).[15,16] Fibroin has the following orthogonal unit cell dimensions: *a* axis, 9.4 Å (inter-β-strand or hydrogen-bonding direction); *b* axis, 9.2 Å (intersheet spacing); *c* axis, 6.9 Å (peptide repeat).

Ionization of the sample is accomplished by treating the polypeptide with dilute sodium methoxide–methanol solution (~0.1% w/v). No changes in the vibrational spectra or wide-angle X-ray diffraction pattern are found after this treatment except for the disappearance of the absorption for carboxylic acid groups and the corresponding appearance of the carboxylate group absorption in the infrared spectrum. Therefore, the change in surface functionality from COOH to COONa does not affect either the chain conformation or the structure of the hydrogen-bonded sheets.

Hydration or deuteration is achieved by absorption of water or deuterium oxide vapor at room temperature. It is possible to vary the equilibrium H_2O or D_2O content by altering the relative humidity (RH) of the sample environment using various saturated salt solutions (5% RH, NaOH; 12% RH, LiCl; 22% RH, CH_3COOK; 32% RH, $CaCl_2$; 44% RH, K_2CO_3; 56% RH, NaBr; 76% RH, NaCl; 86% RH, KCl; 94% RH, Na_2SO_4).[17] Water uptake is obtained by measuring the weight gain of the sample between two AgCl windows directly on a balance or by measuring the weight loss of the sample on a Perkin-Elmer TGS-2 thermogravimetric analyzer (TGA). When the difference between these two measurements exceeded 10%, the TGA data were used for the analysis.

All infrared spectra (2 cm^{-1} resolution) were obtained using a Nicolet IR-38 spectrometer. To prevent loss of water during transfer and measurement, samples were sealed between two AgCl windows. Raman spectra (4 cm^{-1} spectral resolution) were taken on a Bruker FRA 106 spectrometer with a Nd:YAG laser (1064 nm wavelength) as the irradiation source. A backscattering collection geometry was used. Samples were sealed in capillaries and the dead space above the sample was made as small as possible to reduce loss of water due to heating by the laser. Wide-angle X-ray diffraction (WAXD) powder patterns were recorded on a Statton camera to follow crystal packing changes. The same precautions as in the Raman experiment were taken to prevent loss of water during exposure.

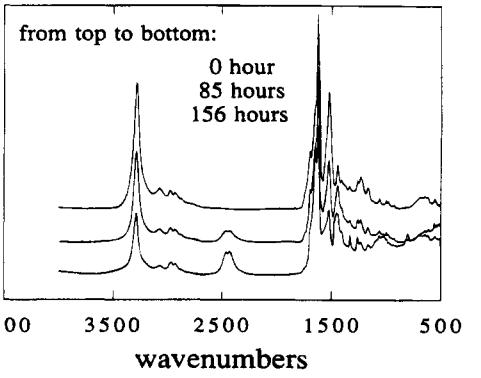

Figure 1. Infrared spectra of progressively deuterated samples in the 500–4000 cm^{-1} region.

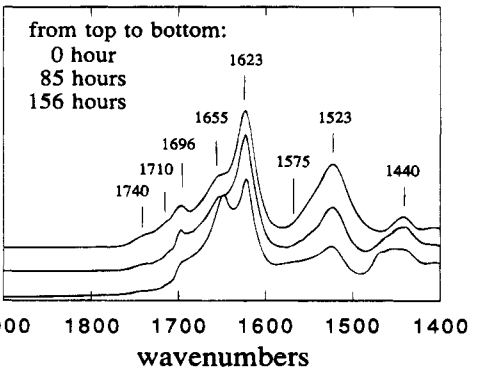

Figure 2. Infrared spectra of progressively deuterated samples in the Amide I and Amide II regions.

Intensities of vibrations were calculated using a Lab Calc software package, based on the deconvoluted area associated with each absorption. For the Amide I region (1710–1610 cm^{-1}) three peaks were considered. Three vibrations were also considered in the region between 1610 and 1500 cm^{-1}. A combination of Gaussian and Lorentzian band shapes was used. Frequency, width at half-height, intensity, and percentage of Gaussian or Lorentzian band were varied to obtain the best fit. The resulting χ-square values were on the order of 10^{-3}–10^{-4}. Estimations of error are based on three independent measurements. The underlying water absorption in the Amide I region may introduce errors which are difficult to account for.

Results and Discussion

The dramatic changes in the infrared spectra obtained for the model polypeptide when D_2O is introduced are shown in Figures 1 and 2. Virtually all vibrations involving internal coordinates affected by the H → D substitution will exhibit changes in frequency and intensity. The vibrational bands in the 1500–1700 cm^{-1} region are well assigned. The strong infrared-active vibration at 1623 cm^{-1} can be assigned to the Amide I vibration associated with the extended chain conformation associated with the β-sheet.[18] For the crystalline structure containing this chain conformation, we also expect an additional component due to crystal field splitting at 1696 cm^{-1} as observed.[18,19] For this model polypeptide with its extended chain conformation, the dominant Amide II vibration is centered at 1523 cm^{-1}.

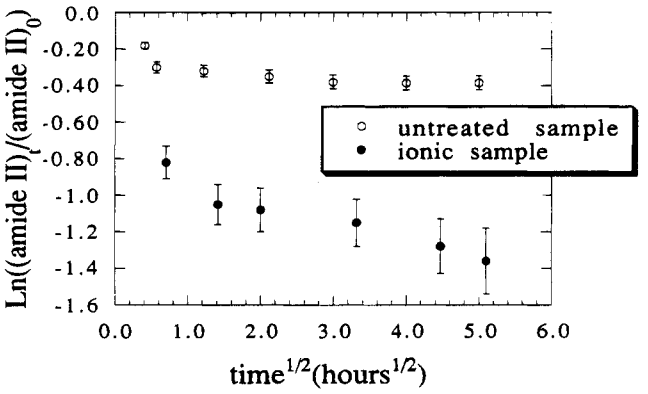

Figure 3. Rate of deuteration of samples in the carboxylic acid and carboxylate forms calculated from the rate of decrease of total Amide II intensity.

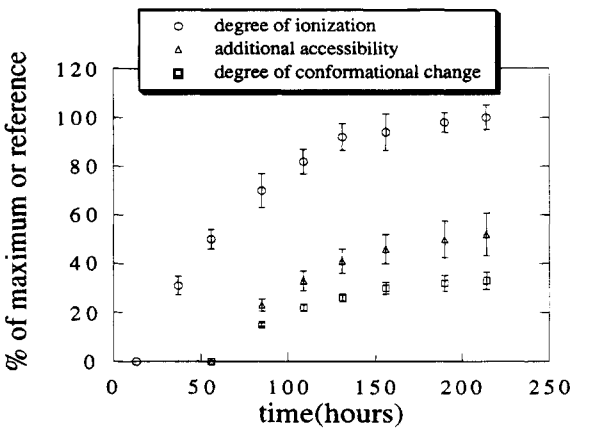

Figure 4. Comparison of rates of changes as a function of time for the Amide II and Amide I bands.

The Amide I vibration is dominated by C=O stretching and therefore is only slightly perturbed by the H → D substitution. On the other hand, the Amide II vibration has a significant contribution from the N–H in-plane-bending internal coordinate. Therefore, as H → D exchange takes place when D_2O is introduced, we expect to observe significant changes in the 1500 cm^{-1} region. The Amide II vibration is expected to be shifted to the 1450 cm^{-1} region. The observed changes are shown in Figure 2. Other chain conformations such as the β-turn also exhibit well-defined vibrations in the 1500–1700 cm^{-1} region. The broad band centered at 1655 cm^{-1} for hydrated samples can be assigned either to a disordered chain conformation, to α-helix, or to turn structures.[18,19] This band has also been assigned to the silk I structure.[20] Other bands of interest include the weak vibrations at 1710 and 1740 cm^{-1} which are assignable to the carboxylic acid of the glutamic acid residue.[21]

The difference in accessibility between crystalline and disordered phases in polymers has been well recognized,[9,10,22–24] and the protons of acid groups on the lamellar surfaces should be exchanged most easily. The absorptions associated with carboxylic acids at 1710 and 1740 cm^{-1} decreased with the introduction of D_2O, with a corresponding growth of the hydrated carboxylate band at 1575 cm^{-1}. Upon the introduction of D_2O vapor, the amide groups on the fold surface are also expected to be deuterated, while amide groups in the β-sheet crystalline region should remain unchanged. This expectation is confirmed by following the deuteration kinetics as shown in Figure 3. For the sample containing carboxylic acid, the intensity of the Amide II band decreases within the first 10 h and then remains constant for up to 3 days as shown by the top curve of Figure 3. During this period, no conformational changes is detected since the Amide I band remains unchanged. For this sample, approximately three of the eight constituent amino acids are located at the fold and should be easily accessible.[13] The plateau shown in Figure 3 represents approximately 32% deuteration; thus although the sample may not be fully crystalline, the fraction of amide protons readily accessible to D_2O vapor suggests that the degree of crystallinity is high. For the sample containing carboxylate, the introduction of D_2O causes far more significant changes in the infrared spectra as shown in Figure 3. The amide protons of this sample seem to be much more accessible as compared to those in the original material since they can be exchanged at a much faster rate. For the sample in the carboxylate form the fast rate of H → D exchange did not diminish as a function of time for up to 50 h.

For the sample in the carboxylate form exposed to D_2O vapor for longer than 50 h, the Amide II vibration decreased dramatically in intensity, indicating that its structure has "opened up", making more of the amide groups accessible to the H → D substitution. This is also true for the sample containing carboxylic acids, but in this case, the required time of exposure is much longer. This increase in accessibility may involve changes in both chain packing and conformation. It is possible, however, to distinguish changes in the environment of amide groups versus changes in chain conformation. The conformational change is readily identified by the relative intensity changes for the Amide I bands, while the decrease in the Amide II band intensity can be used to monitor the accessibility of the amide units. As mentioned previously, changes in the carboxylic acid bands are also readily analyzed.

The relative intensities of three types of vibrations observed for progressively deuterated sample are shown in Figure 4. Data as a function of time are expressed as a percentage of the band intensity measured at the longest time of D_2O exposure. The degree of ionization of the carboxylic acid is calculated using the 1575 cm^{-1} band. As mentioned earlier, based on the Amide II intensity measurements, approximately 32% of the amide units can be deuterated readily. Because of the slow diffusion, the crystalline regions are not expected to be deuterated easily. The slow change in Amide II intensity beyond 50 h reflects this restricted deuteration of amide units in crystalline regions of the sample. The accessibility of NH groups in the crystalline phase is calculated in terms of the incremental percentage change from the plateau value, 68%, shown in Figure 3. Finally, the degree of conformational change is calculated from the decrease in intensity of the 1623 cm^{-1} crystalline Amide I band.[25,26] It is clear from these data that changes in different parts of the structure occur at very different rates.

The intensity of the band at 1655 cm^{-1} attributed to disordered or folded structures increases at the expense of the crystalline β-sheet band as the hydration process progresses. If a change in chain conformation is the only mechanism for increasing the number of accessible NH groups beyond those on the fold surface, then the Amide I and II bands should evolve at the same rate. Instead, it is evident that the rate of increase in accessibility represented by the Amide II vibration is higher than that of the conformational change represented by the changes in the Amide I region. In this analysis, small errors associated with the band decon-

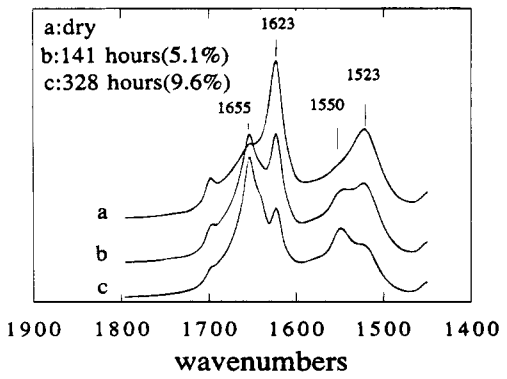

Figure 5. Effect of hydration on the conformation of the sample in the carboxylate form manifested in the Amide I and Amide II region of the infrared spectrum.

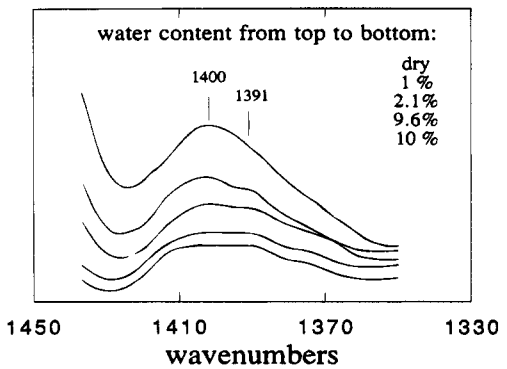

Figure 6. Effect of hydration on the symmetric COO^- band in the infrared spectrum.

volution procedure exist, and the water band in the Amide I region was not taken into account. Nevertheless, our data still serve to indicate that the hydration-induced structural changes proceed in a stepwise fashion. We propose that water first interacts with ionic and polar groups predominantly in the interlamellar region. Subsequent hydration of the ionic groups then causes changes in the underlying crystal packing (e.g., in the intersheet spacing, which is fixed only through relatively weak interactions between sheets). Only in the later stages of hydration are the interchain spacing and chain conformation changed.

To test this hypothesis, the sample in the carboxylate form proved to be especially instructive. As mentioned previously, the X-ray and vibrational spectroscopic data indicate that neither the chain conformation nor the intersheet packing is altered by conversion of the surface-bound side chains from the protonated state to the sodium salt form, thus it is not necessary to be concerned about the degree of dissociation of the carboxylic acid groups during hydration. The effects of hydration on the carboxylate of the polypeptide are shown in Figure 5. In addition, the disordered Amide II band at 1550 cm^{-1},[19] not observable in the deuteration experiments described above (Figure 2), can now be observed.

Figure 6 shows that the symmetric COO^- stretching vibration at 1400 cm^{-1} gradually shifts to lower frequency (1391 cm^{-1}) when the sample in the carboxylate form is progressively hydrated and that interaction between water and the carboxylate groups is readily detected even at low levels (e.g., 1%) of hydration. Similar spectral changes have been observed for hydrated ionomers.[27,28] In the absence of water, the sodium–carboxylate can be regarded as a contact ion

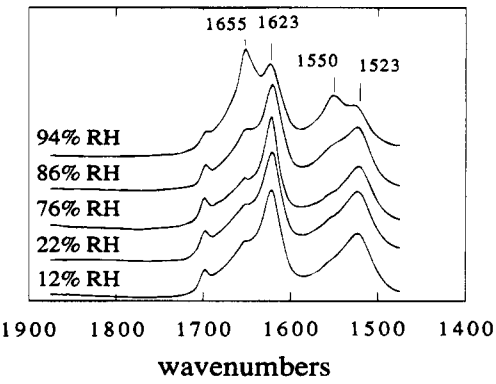

Figure 7. Infrared Amide I and Amide II region of samples equilibrated with various humidity environments, showing evidence for conformational changes only at high relative humidity.

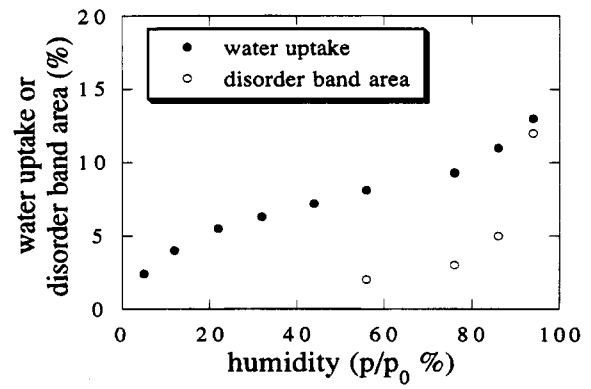

Figure 8. Water content and relative increase in the "disorder" band of the hydrated samples in Figure 7.

pair due to strong electrostatic interactions. With water present around the ions, the anion–cation interaction is perturbed,[27] and a hydration-mediated dissociation equilibrium between the ions is gradually established.

Based on the spectroscopic evidence presented above, it is clear that water penetrates into the interlamellar regions of the polymer quite readily. For the polypeptide in the carboxylic acid form, hydration of the acid groups is slower than that measured for the sample in the carboxylate form. For the latter sample, water can potentially interact with two ionic centers, COO^- and Na^+, making the interlamellar regions much more accessible as compared to the sample in the carboxylic acid form. The hydrated ions, either because of increasing steric interactions or through long-range electrostatic interactions, change the interlamellar regions and in turn perturb the crystalline core. It has been demonstrated both experimentally and theoretically that a few water molecules on the lamellar surface can perturb the crystalline structure of nylons.[9,10]

The experimental results shown so far have principally dealt with the kinetics of structural perturbation when water is absorbed into the system. It is also important to relate the total amount of water uptake to changes in the structure. Samples in the carboxylate form were subjected to hydration at a variety of partial vapor pressures to examine the extent to which water molecules penetrate into different morphological regions during hydration.[23] The results obtained from infrared spectroscopy, the equilibrium water content from gravimetric measurements, and the intersheet distance determined via WAXD are shown in Figures 7–9, respectively.

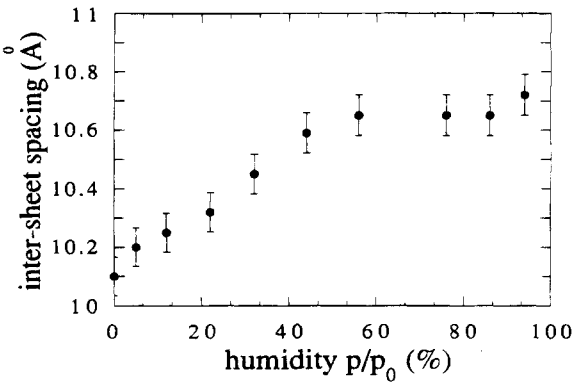

Figure 9. Changes in intersheet spacing induced by hydration of the sample in Figures 7 and 8.

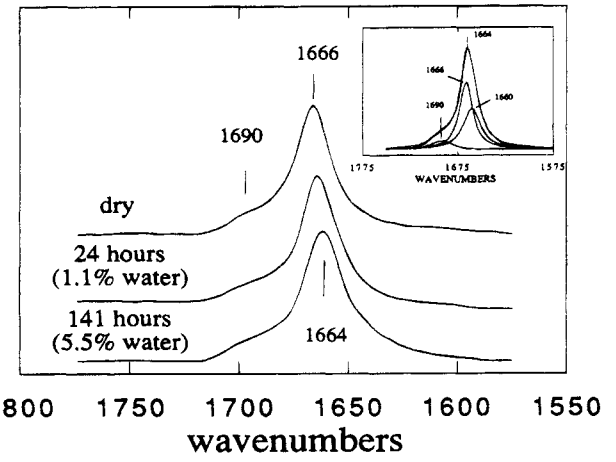

Figure 10. Raman spectra of progressively hydrated ionic samples.

For samples exposed to low partial vapor pressure, no chain conformational changes can be observed even for samples exposed for long times (Figure 7). No changes in the conformation-sensitive infrared bands are found. In these same samples, however, the intersheet distance (Figure 9) has increased and the COO^- symmetric stretch band has already shifted to a lower frequency, indicating hydration of the ionic groups. The changes in chain conformation occur *after* the intersheet spacing has expanded, i.e., after the crystalline region has become accessible to water. That water could access the crystalline regions has been confirmed by previous deuteration experiments. We infer that the conformational disordering effect of water occurs most probably through competition for hydrogen bonding with the amide groups.

It is also interesting to speculate how "disordered" the chain conformation really is. This issue can be resolved by comparing the infrared and Raman spectra of hydrated samples. Raman spectra of a series of hydrated samples in the carboxylate form are shown in Figure 10. There is only one Raman-active Amide I band (at 1666 cm^{-1}) for the β-sheet conformation, and the small broad band centered at about 1690 cm^{-1} in Figure 10 is assigned to amides on the lamellar fold surface.[19] With hydration, the 1666 cm^{-1} band shifts to lower frequency and broadened slightly, centered at 1664 cm^{-1} for samples containing about 5% water. This frequency shift is due to the increase in intensity of the band at 1660 cm^{-1}, as seen from the band convolution shown in the inset. This band (1660 cm^{-1}) in the Raman spectrum should have the same structural origin as the 1655 cm^{-1} band in the infrared spectrum, since both of their intensities increase upon hydration. The frequency difference between the Raman and infrared Amide I bands has been calculated for "disordered" polypeptides previously.[29] It was argued that because of steric interactions along the chain, the probability of finding truly random chain conformations is quite small, and the distribution of ϕ and ψ in the regions associated with well-characterized chain conformations in fact is narrow. Therefore, even for "disordered" polypeptides, individual peptide units may still maintain dihedral angles close to those of a few allowed stable states. Assuming nearest-neighbor interactions only, it was found that the frequency difference between the Raman and infrared maxima should be of the order of 5 cm^{-1} for "disordered" polypeptides with local β-sheet dihedral angles, which was close to the experimental values.[29] What we observe here (1660 vs 1655 cm^{-1}) is also close to the calculation. Therefore, we conclude that the conformational change upon hydration involves the loss of long-range correlation in dihedral angles but that the peptide units maintain dihedral angles close to those of the β-sheet.

Conclusions

Kinetic and equilibrium information derived from vibrational spectroscopy and wide-angle X-ray diffraction has been used to establish that the hydration of polypeptide **1** is a stepwise process. Polypeptides such as **1**, with well-defined β-sheet conformations and uniform lamellar structures with fold surfaces decorated with functional groups, serve as good model systems for examination of the mechanism by which water affects polymer structure at different levels. Each step of the structural change occurring as a function of water content can be characterized. At low water contents, changes in intersheet spacing are caused by hydration of the ionic groups on the fold surface, without involving water directly in the crystalline region. Consequently, crystalline regions become increasingly accessible to water and susceptible to changes in chain conformation. The extent of intersheet expansion is a function of water content. Comparison of infrared and Raman spectra obtained for hydrated samples suggests an increase in long-range disorder with retention of local order, i.e., a structure with local β-sheet dihedral angles, in which long-range correlation has been lost due to the interaction with water. Because this polypeptide has a relatively thin lamellar structure (thickness about 30 Å), unfavorable interactions on the lamellar surface can account for a considerable part of the overall energy. Upon hydration, the surface interactions become comparable to the cohesive energy in the crystalline phase and drive the observed structural changes. We propose that thicker lamellae with similar structures will display slower structural changes upon hydration. Experiments to test this hypothesis are underway.

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