Fourier Transform Infrared Studies of Proteins Using Nonaqueous Solvents. Effects of Methanol and Ethylene Glycol on Albumin and Immunoglobulin G†

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ABSTRACT: An infrared/attenuated total reflection (ATR) technique has been utilized to study the structural changes in proteins induced by nonaqueous solvents, without the need of dissolving the protein in the nonaqueous solvent. For the two proteins studied, methanol and ethylene glycol caused similar changes in albumin, i.e., an increase in helix secondary structure. However, the two solvents had dissimilar effects on immunoglobulin G (IgG). Changes in the pH of aqueous solutions of IgG produced a third effect. By dissolving some IgG in ethylene glycol and then adsorbing IgG from this solution onto an ATR crystal, the time behavior of the adsorption process could be studied and a mechanism for the structural changes proposed.

The behavior of proteins in nonaqueous solvents has been used both to study structural changes in proteins induced by different solvents and to compare behavior in nonaqueous and aqueous solutions in order to elucidate the role of water in protein folding, unfolding, and stabilization (Singer, 1962). However, due to the lack of solubility of proteins in nonaqueous solvents, most of these studies either have been theoretical or have used a mixture of solvent and water. This has led to very few infrared studies (Purcell & Susi, 1984) of proteins in nonaqueous solvents. Attenuated total reflectance infrared spectroscopy offers the advantage of not requiring the protein to be dissolved in the nonaqueous solvents. In this paper, we have deposited protein films on an infrared-attenuated total reflection (ATR) crystal from aqueous solution. We could then replace the aqueous protein solution by using any desired solvent and thus could expose the protein film to nonaqueous solvents. In this manner, we have studied the behavior of albumin and γ-globulin (IgG) exposed either to methanol or to ethylene glycol and, in addition, have studied the adsorption of IgG onto the ATR crystal from a very dilute ethylene glycol solution. We have compared the spectral results in nonaqueous solutions to the spectra of aqueous solutions, related the spectral changes to changes in the secondary structure of the protein, and postulated a mechanism for how these changes occur.

Experimental Procedures

Crystallized and lyophilized, essentially globulin-free, human serum albumin and lyophilized, essentially salt-free immunoglobulin G (IgG) were used as received from Sigma Chemical Co. (albumin, A-8763, lot no. 54F-9300, and IgG, I-4506, lot no. 103-8920). The purity of each was verified by SDS gel electrophoresis.

Infrared data were collected on either a Digilab FTS-10M or a Mattson Sirius 100 Fourier transform infrared (FT-IR) system, each equipped with a narrow-band mercury–cadmium–telluride (MCT) detector. A total of 2000 scans at 8 cm^{-1} resolution using triangular apodization were coadded to ensure an adequate signal to noise ratio. Spectra of the proteins were obtained by digital subtraction of the solvent spectrum from the spectrum of the protein plus solvent. Fourier self-deconvolution, as described by Kauppinen et al. (1981), was used to resolve intrinsically overlapped bands in the spectrum of the protein.

Protein Adsorbed from Saline. A solution of protein (3 wt % albumin or 1 wt % IgG in 0.15 N saline) was circulated through a Spectra Tech attenuated total reflection (ATR) Circle Cell with a glass flow body and a Ge crystal. The solution was circulated for a period of 2 h to allow the protein to adsorb onto the ATR crystal. Saline was then circulated through the cell to remove any nonadsorbed protein, and infrared spectra were recorded of the adsorbed protein exposed to saline.

The nonaqueous solvent (methanol or ethylene glycol) was allowed to flow through the cell, displacing the saline. Fresh solvent was used through the system to ensure more complete removal of water. Infrared spectra were obtained on the protein exposed to the nonaqueous solvent. Fresh saline was then allowed to flow through the cell, and infrared spectra were recorded of the protein reexposed to saline.

IgG Adsorbed from Ethylene Glycol. A solution of 20 mg of IgG in 50 mL of ethylene glycol was prepared. The IgG did not all go into solution. The solution was filtered, and it is estimated that after filtering the solution concentration was much less than 0.1 wt %. At this concentration, no protein infrared signal would be observed from the dissolved protein, only from proteins absorbing on the ATR crystal. The filtrate was circulated for 3 h through a Harrick Scientific ATR flow cell with a Ge crystal. Infrared spectra were recorded over this period of time. The nonadsorbed protein was displaced from the cell by allowing fresh ethylene glycol to flow through the cell, and infrared spectra were recorded.

IgG pH Studies. Sample solutions of 2% by weight of IgG in 0.15 N saline (pH 2.4 and 7.4) were prepared. The pHs of the solutions were adjusted to final pHs of 2.2 and 7.2 by using HCl and NaOH, respectively. Each sample was run in a Spectra Tech Microcircle ATR cell. The spectra were obtained by subtracting the spectrum of 0.15 N saline at each respective pH.

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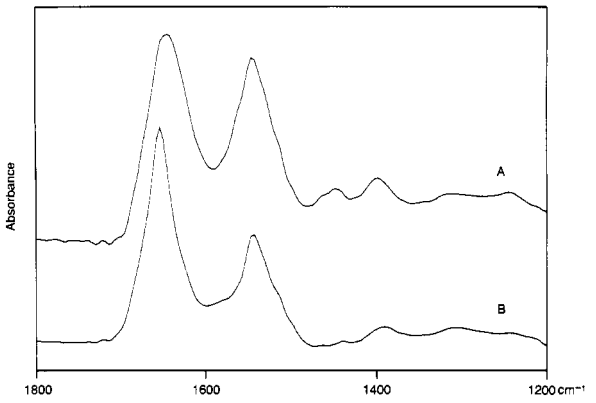

FIGURE 1: Spectra of an adsorbed human serum albumin film (A) exposed to water and (B) exposed to methanol.

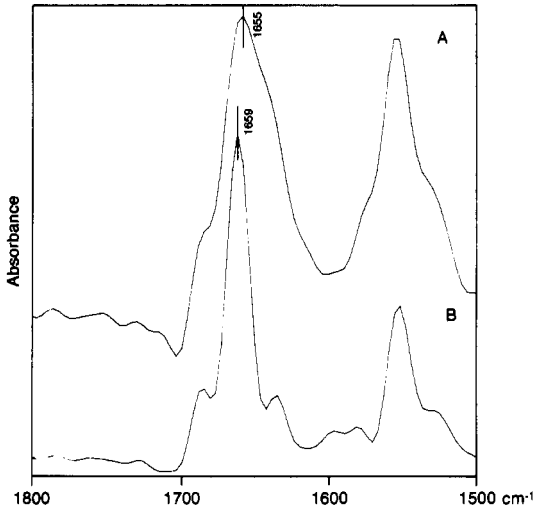

FIGURE 2: Deconvoluted spectra of an adsorbed human serum albumin film in the amide I and II regions (A) exposed to water and (B) exposed to methanol. Deconvolution parameters: width = 28 cm^{-1}, K = 1.8, and apodization = Bessel.

RESULTS

Albumin. Spectra of an adsorbed albumin film exposed to saline and exposed to methanol are shown in Figure 1. To better observe the spectral changes, deconvoluted spectra of adsorbed albumin in the amide I and amide II frequency regions are shown in Figure 2. Figure 2A shows the spectrum of adsorbed albumin exposed to or in contact with saline, while Figure 2B gives the spectrum of the same albumin film after the saline was forced out of the ATR cell with methanol. Thus, Figure 2B is a spectrum of the adsorbed albumin film in contact with methanol. The spectrum of Figure 2B is normalized with respect to the peak height of the amide I band ($\sim$1650 cm^{-1}) of Figure 2A. With the normalization in mind, three differences can be detected for the amide I band between the two spectra of Figure 2. Upon exposure to methanol, there is (1) a shift to higher frequencies (1655–1659 cm^{-1}), (2) a pronounced bandwidth narrowing, and (3) an increase in both peak height intensity and band area. For the amide II vibration ($\sim$1550 cm^{-1}), the only observable change upon exposure to methanol is some of the same band sharpening or resolution as seen for the amide I band ($\sim$1650 cm^{-1}).

The deconvoluted spectra of the same two adsorbed albumin films in the amide III frequency region are shown in Figure 3. The important changes in this region concern the intensity ratio of the amide III complex due to helix structure (around 1300 cm^{-1}) to that of the amide III band due to "random" or disordered structures (1245 cm^{-1}). Here it can be observed

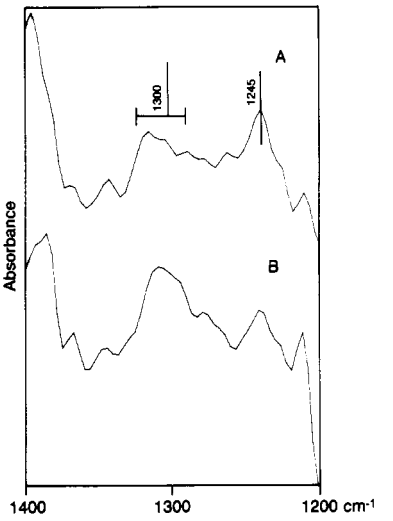

FIGURE 3: Deconvoluted spectra of an adsorbed human serum albumin film in the amide III region (A) exposed to water and (B) exposed to methanol. Deconvolution parameters: width = 16 cm^{-1}, K = 2.0, and apodization = Bessel.

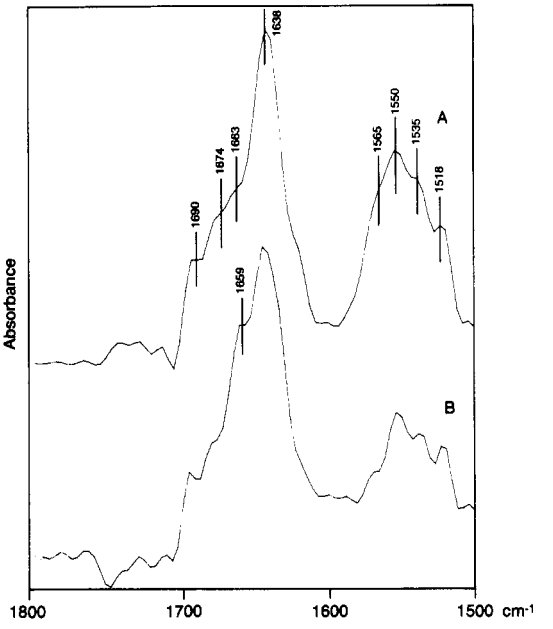

FIGURE 4: Deconvoluted spectra of an adsorbed IgG film in the amide I and II regions (A) exposed to water and (B) exposed to methanol. Deconvolution parameters: width = 20 cm^{-1}, K = 2.2, and apodization = Bessel.

that this ratio is much higher after exposure to methanol than after exposure to saline.

Virtually identical behavior in the amide I, II, and III spectral regions has been observed for adsorbed albumin exposed to ethylene glycol (Jakobsen et al., 1986) after exposure to water or saline.

γ-Globulin. Figure 4 shows the deconvoluted spectra (in the amide I and II frequency regions) of adsorbed γ-globulin exposed to saline (Figure 4A) and exposed to methanol (Figure 4B). In the amide I region of the adsorbed film exposed to saline, four bands can be observed: (1) the intense major peak at 1638 cm^{-1} and (2) three high-frequency shoulders at 1663, 1674, and 1690 cm^{-1}. In the amide II region of this spectrum, the strongest peak is at 1550 cm^{-1} with weaker low-frequency shoulders at 1536, 1518, and 1500 cm^{-1}. An asymmetry on the high-frequency side of the 1550 cm^{-1} band indicates another component with a frequency in the 1560–1570 cm^{-1} region. In the amide I region of the adsorbed γ-globulin

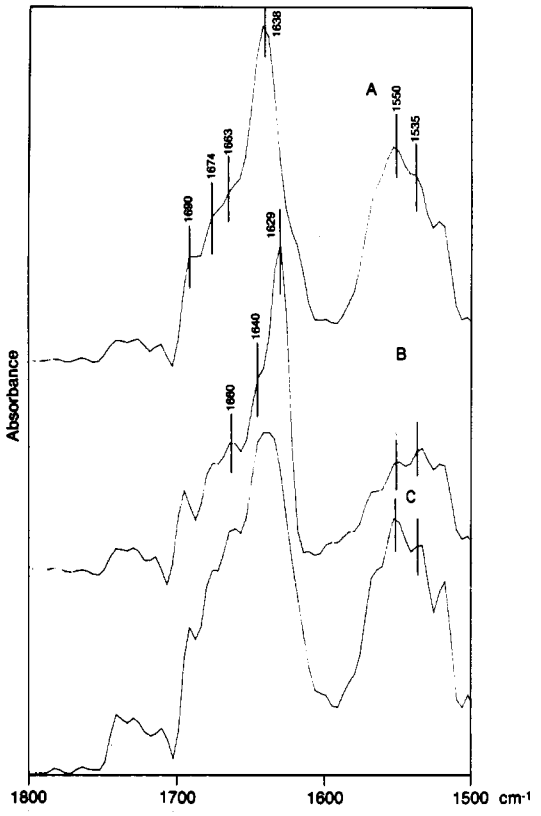

FIGURE 5: Deconvoluted spectra of an adsorbed IgG film in the amide I and I regions (A) exposed to water, (B) exposed to ethylene glycol, and (C) reexposed to water. Deconvolution parameters: width = 20 cm^{-1}, K = 2.2, and apodization = Bessel.

exposed to methanol (Figure 4B), there is only one significant difference between this spectrum (Figure 4B) and the spectrum exposed to saline (Figure 4A). This is that the 1663 cm^{-1} band of γ-globulin exposed to saline has either shifted to or been replaced by a more intense band at 1659 cm^{-1} when the γ-globulin is exposed to methanol. In the amide II region of the spectrum (Figure 4B), exposure to methanol brings about a better resolution of the bands at 1565, 1550, 1535, 1518, and 1500 cm^{-1}, but no new bands or frequency shifts are observed on going from saline to methanol. The intensity ratio of the most intense band in the amide I region to the most intense band in the amide II region increases on going from saline to methanol. Although normalized in Figure 4, both the amide I and amide II intensities (exposed to methanol) increase as compared to the intensities of the adsorbed film exposed to saline; therefore, an increase in the amide I and amide II intensity ratio indicates that for exposure to methanol the amide I intensity increases more than the amide II intensity.

The infrared spectra of adsorbed γ-globulin exposed to saline and to methanol have been shown in the amide III region in another publication (Jakobsen et al., 1985). The major difference between these two spectra was that the γ-globulin exposed to methanol showed increased broad infrared absorption in the 1300 cm^{-1} region, indicating formation of a helix conformation amide III vibration.

When γ-globulin is exposed to ethylene glycol instead of methanol, the deconvoluted results are shown in Figure 5 for the amide I and amide II spectral regions. Here Figure 5A shows the spectrum of γ-globulin in contact with saline, Figure 5B the spectrum of γ-globulin exposed to ethylene glycol, and Figure 5C the γ-globulin spectrum after reexposure to saline. Two major changes occur in the amide I vibration upon contact with ethylene glycol. As with the methanol case (Figure 4), the 1663 cm^{-1} amide I component (Figure 5A) is replaced by

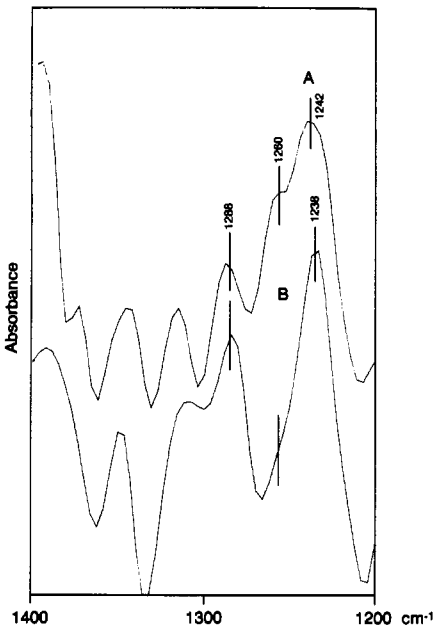

FIGURE 6: Deconvoluted spectra of an adsorbed IgG film in the amide III region (A) exposed to water and (B) exposed to ethylene glycol. Deconvolution parameters: width = 24 cm^{-1}, K = 2.0, and apodization = Bessel.

a more intense band near 1660 cm^{-2} (Figure 5B). However, different from the methanol case, the strongest amide I component at 1638 cm^{-1} does not stay at the same frequency when γ-globulin is in contact with ethylene glycol. In the ethylene glycol contact case, it shifts to 1629 cm^{-1} (with a weak shoulder remaining near 1640 cm^{-1}). Figure 5C shows that upon reexposure to saline, the changes are to a large extent reversible. As can be seen in this figure, changes also occur in the amide II spectral region upon exposure to ethylene glycol. These changes mainly center in a reversal of the 1550 and 1535 cm^{-1} intensities and in better resolution of the amide II region bands in the ethylene glycol case. As with methanol (Figure 4), the amide I/amide II intensity ratio increases upon exposure to ethylene glycol (Figure 5). This intensity ratio increase is even greater than with methanol possibly due to the narrowing of the central amide I band (1629 cm^{-1}) upon exposure to ethylene glycol. Such band narrowing is not as apparent in the methanol case.

Deconvoluted spectra of these γ-globulin films in the amide III spectral region are shown in Figure 6. Several differences can be noted between the spectra of γ-globulin exposed to ethylene glycol (Figure 6B) as compared to saline (Figure 6A). Upon exposure to ethylene glycol, the intensity of the 1288 cm^{-1} amide III band increases, there is a possible decrease in the intensity of the band near 1260 cm^{-1}, and there is a definite increase in the intensity of the amide III complex near 1240 cm^{-1}. There is also a small frequency decrease (1242 to 1238 cm^{-1}) in this amide III complex. The intensity increase in the complex near 1240 cm^{-1} could be partially caused by a band narrowing of the amide III band much as observed for the amide I peak at 1629 cm^{-1}.

As described under Experimental Procedures, it was possible to dissolve a very small amount of γ-globulin in ethylene glycol, and this protein solution was flowed through the ATR cell. Protein adsorption onto the ATR crystal was monitored with time of solution flow. Figure 7 shows the deconvoluted spectra in the amide I and III regions, for this adsorbed film after 10 min of solution flow time (Figure 7A) and after 180 min of flow time (Figure 6B). In these normalized spectra, it can be seen that the 1629 cm^{-1} component is present even after

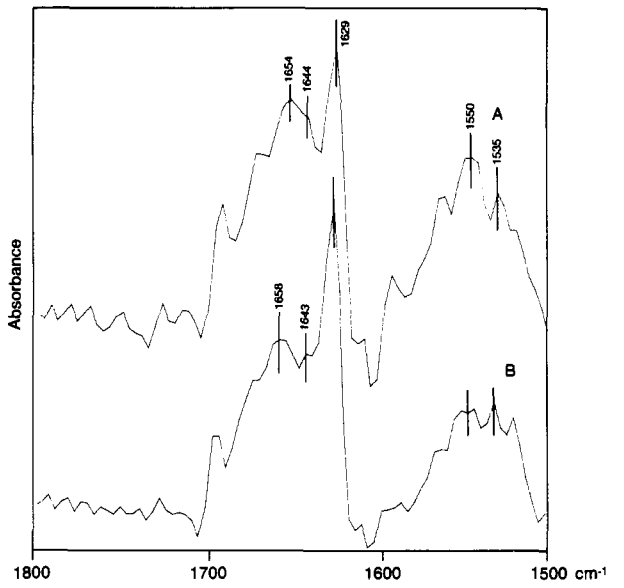

FIGURE 7: Deconvoluted spectra of an IgG solution in ethylene glycol in the amide I and II regions (A) after 10 min of flow time and (B) after 180 min of flow time. Deconvolution parameters: width = 24 cm^{-1}, K = 2.2, and apodization = Bessel.

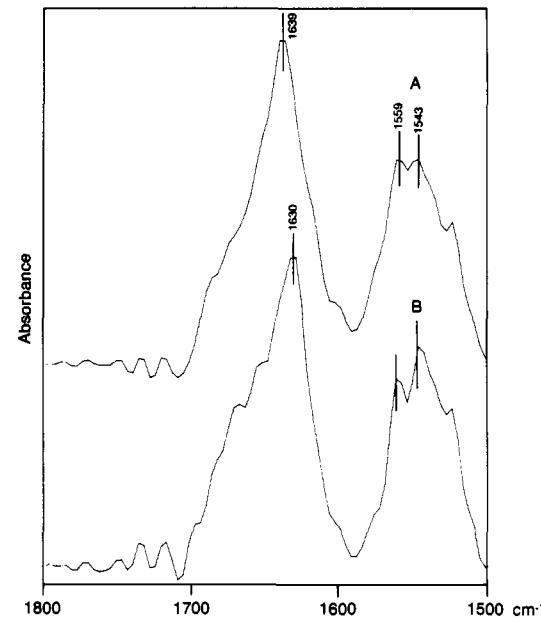

FIGURE 8: Deconvoluted spectra of an IgG solution in the amide I and II regions (A) at pH 7.2 and (B) at pH 2.2. Deconvolution parameters: width = 22 cm^{-1}, K = 1.8, and apodization = Bessel.

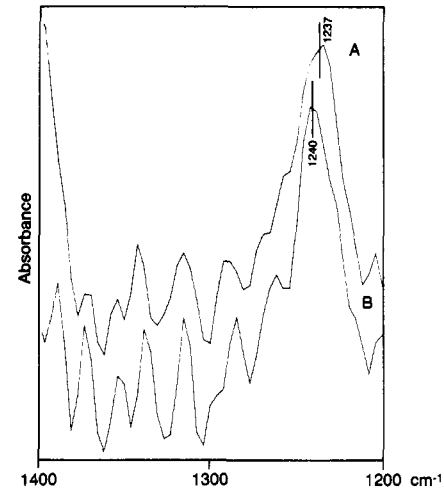

FIGURE 9: Deconvoluted spectra of an aqueous IgG solution in the amide III region (A) at pH 7.2 and (B) at pH 2.2. Deconvolution parameters: width = 18 cm^{-1}, K = 1.8, and apodization = Bessel.

only 10 min of flow time and that this 1629 cm^{-1} component increases in intensity (relative to the other amide I bands) with time of flow. At 10-min flow time, deconvolution also resolves a band with components at 1654 and 1644 cm^{-1}. By 180 min of flow time, these two bands appear to be better resolved and have shifted to 1658 and 1643 cm^{-1}, and the intensity ratio has changed even more in favor of the 1658 cm^{-1} component.

In the amide II region of the spectra of these two flow times, the 1550 cm^{-1} band is stronger than the 1535 cm^{-1} band at 10 min of flow time, but this intensity ratio has reversed at 180-min flow time. This is similar to the behavior observed when ethylene glycol was flowed past an adsorbed γ-globulin film (Figure 5B).

To compare the infrared results to circular dichroism results (Doi & Jirgenson, 1970), infrared spectra of aqueous solutions of γ-globulin at pH 7.2 and at pH 2.2 were run and are shown in Figure 8 for the amide I and amide II spectral regions and Figure 9 for the amide III spectral region. The major change in the amide I spectral region is a shift of the strongest band from 1639 cm^{-1} at pH 7.2 to 1630 cm^{-1} at pH 2.2. In the amide II region, the 1559/1543 cm^{-1} intensity ratio changes as the pH changes while the amide III bands shifts from 1237 to 1240 cm^{-1} and shows a small intensity increase on going from pH 7.2 to pH 2.2. These changes are very similar to some of the amide I, II, and III changes between adsorbed γ-globulin exposed to water and then exposed to ethylene glycol.

Discussion

Albumin. Two major changes were observed in the infrared spectrum of adsorbed albumin after exposure to either methanol or ethylene glycol (Jakobsen et al., 1986). The first was the previously described changes in the amide I band, and the second was the change in the intensity ratio of the amide III infrared bands. The narrowing of the bandwidth of the amide I vibration and its shift to a higher frequency have been attributed (Jakobsen et al., 1986) to a decrease in the amount of water bound to the albumin molecules and an increase in the helix order. In fact, it was shown that for albumin, the amount of bound water could be directly related to the bandwidth of the amide I vibration.

However, the increase in the peak height and the band area of the amide I vibration cannot be explained by a decrease in the amount of bound water. It is much more likely that the intensity increase signifies a change in the secondary structure of albumin, and from the frequency of the vibration (1659 cm^{-1}), the conformational change appears to be an increase in helix secondary structure, although the frequency of the vibrational could also indicate an increase in "random" or disordered structure. The spectrum of Figure 3B clarifies that the change is an increase in helix conformation. The ratio of the helix amide III vibration near 1300 cm^{-1} to that of the disordered structure amide III frequency near 1245 cm^{-1} has increased after exposure to methanol (Figure 3B), also indicating an increase in the amount of helix structure. The combination of the amide I and amide III frequencies definitively establishes the change as an increase in helix structure and eliminates the possibility of an increase in disordered structure. Thus, in terms of secondary structure, the effect of either methanol or ethylene glycol on adsorbed albumin is to increase the amount of helix structure in the albumin molecule.

Table I: Deconvoluted Amide I Frequencies (cm^{-1}) of Adsorbed γ-Globulin

assignment	exposed to saline	exposed to methanol	exposed to ethylene glycol
turns or β-sheet	1690	1693	1694
β-sheet or turns	1674	1678	1675
disordered	1663		
α-helix		1659	1660
ordered, intramolecular β-sheet	1638	1641	1640 sh
disordered, intermolecular β-sheet			1629

γ-Globulin. γ-Globulin has a largely β-sheet secondary structure with smaller amounts of turns and disordered or random secondary structure (Amzel & Poljak, 1979). This protein could also contain very short α-helical stretches (Amzel & Poljak, 1979). Thus, in Figure 4A (γ-globulin exposed to saline), the strong peak at 1639 cm^{-1} must be one component of the β-sheet amide I vibration. Not only does the band intensity indicate a β-sheet conformation assignment but also the observed frequency (1639 cm^{-1}) falls within the range reported (Carey, 1982; Parker, 1983) for this conformation. The second component of the β-sheet vibration could be either of the high-frequency shoulders at 1674 or 1690 cm^{-1}. For ribonuclease A (Olinger et al., 1986), which also has a significant β-sheet content, it was demonstrated by use of deuteriation kinetics that a band near 1690 cm^{-1} was the second component of the β-sheet amide I vibration. However, as will be detailed later, by comparison of the frequencies of adsorbed γ-globulin and those of γ-globulin in solution, it could be logical to assign the 1674 cm^{-1} band of adsorbed γ-globulin to the second component of the β-sheet vibration. For other proteins (Yang et al., 1985), a band in the 1674–1679 cm^{-1} range was assigned as the β-sheet vibration. While, for γ-globulin, we favor assigning the 1674 cm^{-1} band as the β-sheet frequency and the 1690 cm^{-1} band as a turn vibration, it is obvious that such a choice is arbitrary. However, either assignment (1674 or 1690 cm^{-1} for the β-sheet) does not materially affect the central points of this paper concerning structural changes in γ-globulin. Whichever frequency is assigned as the β-sheet vibration would leave the other band for assignment as a turn vibration, although vibrations of amino acid side chains are a possibility at either frequency. This leaves the shoulder at 1663 cm^{-1}, and by the process of elimination as well as the frequency (Carey, 1982; Parker, 1983), the 1663 cm^{-1} band is assigned to disordered species within the γ-globulin molecules. The frequencies and assignments for the amide I bands of adsorbed γ-globulin exposed to saline are listed in Table I.

In the amide II spectral region of γ-globulin exposed to saline, assignments in terms of secondary structure are nowhere near as certain as in the amide I region. The 1550 and 1535 cm^{-1} bands are likely amide II vibrations, but the 1518 and 1500 cm^{-1} bands, as well as the shoulder in the 1560–1570 cm^{-1} range, are more likely assigned to amino acid side chain vibrations. The 1518 and 1500 cm^{-1} bands are likely vibrations of those amino acids containing aromatic rings while the 1560–1570 cm^{-1} shoulder could be due to amino acids containing carboxylate side chains.

As stated under Results, only one significant change occurs in the amide I region when the adsorbed γ-globulin film is exposed to methanol. This change is that the 1663 cm^{-1} band of γ-globulin exposed to saline either shifts to or is replaced by a band at 1659 cm^{-1}, and this 1659 cm^{-1} band is more intense (relative to the other amide I bands) than the 1663 cm^{-1} band. If, upon exposure to methanol, γ-globulin retains its disordered secondary structure, the 1659 cm^{-1} frequency would probably indicate a shift of the 1663 cm^{-1} band to 1659 cm^{-1}. However, formation of new secondary structure would indicate that the 1659 cm^{-1} band is replacing all or part of the 1663 cm^{-1} part and is indicative of this new structure. If it is a new type of secondary structure, the frequency (1659 cm^{-1}) would only fit a helix structure. Thus, the frequency of this band (1659 cm^{-1}) and the increase in relative intensity indicate formation of helix structure, and confirmation of this change is found in the amide III region of the spectrum (Jakobsen et al., 1985) where increased absorption in the helix amide III band near 1300 cm^{-1} was the only significant change observed. The frequencies and assignments of the amide I bands of γ-globulin exposed to methanol are also listed in Table I.

Thus, the effect of methanol on either albumin or γ-globulin is very similar, i.e., increased infrared absorption near 1660 cm^{-1} and in the 1300 cm^{-1} region. These changes can only be interpreted that methanol increases the helix content of these two proteins. Methanol (Singer, 1962) has been found to induce helix formation in other proteins.

When γ-globulin is exposed to ethylene glycol instead of methanol, we also see the amide I (Figure 5) and amide III (Figure 5) changes that indicate helix formation. That is, the 1663 cm^{-1} peak (Figure 5A, exposed to saline) is replaced by a band at 1660 cm^{-1} (Figure 5B, exposed to ethylene glycol) in the amide I region. A peak in the helix amide III region at 1288 cm^{-1} also grows in intensity (Figure 6B) upon exposure to ethylene glycol. These amide I and amide III changes can only indicate helix formation. However, different from the methanol case, exposure to ethylene glycol produces changes in the amide I and amide III bands indicative of β-sheet structure (Figures 4 and 5). In the amide I region, ethylene glycol produces a shift from 1638 cm^{-1} Figure 5A) to 1629 cm^{-1} (Figure 5B), while in the amide III region there is a small shift from 1242 cm^{-1} (Figure 6A) to 1238 cm^{-1} (Figure 5B) and an increase in intensity at this frequency. All previous assignments for the amide I and amide III frequencies of helix structures, turns, or disordered conformations are at *higher* frequencies (Carey, 1982; Parker, 1983) than 1638 and 1239 cm^{-1}, respectively. Therefore, a shift to *lower* frequencies can only mean that the β-sheet conformation is retained, but the 9 cm^{-1} shift of the amide I band indicates that there must be significant structural differences between the β-sheet structure at the higher frequency and the β-sheet structure at the lower frequency. Thus, the effect of ethylene glycol upon γ-globulin is to produce both a new helix structure and a different type of β-sheet structure. A small amount of the old β-sheet structure is retained as indicated by the 1640 cm^{-1} shoulder seen in Figure 5B (after exposure to ethylene glycol). Table I lists the frequencies and assignments of the amide I bands of γ-globulin exposed to ethylene glycol.

Two other changes between γ-globulin exposed to saline (Figure 4A and 5A) and exposed to ethylene glycol (Figure 4B and 5B) should be noted. The first is the possible decrease in intensity of the 1260 cm^{-1} amide III band. This frequency strongly indicates a possible decrease in the disordered sections of γ-globulin and indicates that either the new helix structure or the different β-sheet structure could be forming from these disordered segments. The second change is the intensity reversal of the 1550/1535 cm^{-1} band ratio. Since this was not observed upon exposure of γ-globulin to methanol (Figure 4B), it is not likely to result from the production of new helix structures. Thus, the intensity ratio reversal is more likely associated with the formation of a different β-sheet structure.

When γ-globulin, dissolved in ethylene glycol, is flowed through the ATR cell, we can begin to get some idea of the mechanisms involved in the formation of a new form of β-sheet structure. After 10 min of adsorption, only a thin film has deposited on the ATR crystal (Figure 7A), and a band at 1629 cm^{-1} is evident, indicating formation of the new form of β-sheet structure. In fact, the intensity ratio of the 1629/1644 cm^{-1} bands or the new/normal ratio is such that much of the existing β-sheet structure at 10 min is of the new variety. The broad band at 1654 cm^{-1} has a 1644 cm^{-1} shoulder likely due to the original β-sheet structure while the 1654 cm^{-1} band is probably due on the high-frequency side to disordered structure (~1663 cm^{-1}) and at 1654 cm^{-1} to helix formation. However, at 10 min, the intensity of the 1629 cm^{-1} band is not as strong (relative to the other bands of the amide I vibration) as the 1638 cm^{-1} band intensity of adsorbed γ-globulin exposed to water (Figure 3A and 4A). This would suggest that both helix formation and new β-sheet structure result from the γ-globulin segments that were part of the original β-sheet structure.

After 180 min of adsorption, a thicker film has adsorbed (Figure 7B), and the intensity of the 1629 cm^{-1} band has increased relative to the other amide I bands, indicating formation of more of the new β-sheet structure. In fact, the intensity ratio of the 1629 cm^{-1} band relative to the other amide I frequencies is now much like the intensity ratio of the 1638 cm^{-1} band of adsorbed γ-globulin exposed to water. This would indicate the possibility not only that the new β-sheet structure is formed from the original β-sheet structure but also that some of it could result from the helix structure formed after 10 min of flow. This helix band, after 180 min of flow, has shifted to 1658 cm^{-1}, a frequency consistent with helix structure with little or no bound water (Jakobsen et al., 1986). Thus, the mechanism seems to indicate that γ-globulin is adsorbed onto the surface of the ATR crystal and rapidly both helix and new β-sheet structures are formed (if not already formed while in solution). With increasing flow time, more γ-globulin is absorbed on the ATR crystal (the film at 180 min is 4–6-fold thicker than the film at 10 min), and this γ-globulin film rearranges to form more of the new β-sheet structure. Some of this additional new β-sheet structure is formed from the helix structures as well as from the normal β-sheet structures. By 180 min, only a small amount of the normal or original β-sheet structure remains. The lack of resolution of a 1663 cm^{-1} frequency indicates that the disordered structure content has decreased both at 10 min and at 180 min of flow. Thus, since helix formation occurs rapidly (Ghellis & Yon, 1982), it is likely that some of the helix forms from the disordered segments of γ-globulin. Ethylene glycol has been reported (Singer, 1962) to first partially unfold proteins, and then some of the unfolded portions refold to form ordered structures. Such an unfolding of the original β-sheet structure could lead to a new β-sheet structure upon refolding.

Of interest is the 1550/1535 cm^{-1} intensity ratios of the amide II region of the spectra of Figure 7. After 10 min of flow time, this intensity ratio favors the 1550 cm^{-1} band even though a 1629 cm^{-1} amide I component is clearly visible. Only after the 1629 cm^{-1} band has grown in intensity (Figure 7B, 180 min of flow) does this intensity ratio in the amide II region reverse to the ratio seen in Figure 5B for adsorbed γ-globulin after exposure to ethylene glycol. This definitely confirms that the effects giving rise to an intense 1629 cm^{-1} band are also responsible for the observed 1550/1535 cm^{-1} ratio and that the 1535 cm^{-1} amide II band is also associated with the new β-sheet structure.

In order to shed more information on these structural changes and to compare our spectra–structure correlations with the structural information obtained by circular dichroism (Doi & Jirgenson, 1970), we also obtained spectra of dissolved γ-globulin (in saline) at pH 7.2 and at pH 2.2 (Figure 7 and 8). Here we also observed (Figure 8) a shift of the 1639 cm^{-1} band (pH 7.2) to 1630 cm^{-1} (pH 2.2) but no change in the 1650 cm^{-1} region which indicates no formation of helix structure (as is also indicated in Figure 9 by no gain in intensity in the 1300 cm^{-1} region). The 1639 cm^{-1} shift indicates formation of the new β-sheet structure. There was a small shift (1239 to 1240 cm^{-1}) and intensity increase in the amide III region of the spectrum (Figure 9) on changing from pH 7.2 to pH 2.2. The 1559/1543 cm^{-1} intensity ratio (Figure 8) also changed as the pH was lowered. Thus, the pH-induced changes involving the β-sheet structures were very similar to the changes which occurred when adsorbed γ-globulin was exposed to ethylene glycol (Figure 4 and 5). Contrary to the ethylene glycol case, pH changes did not induce any alteration in the helix structure.

Table II: Deconvoluted Frequencies (cm^{-1}) of Dissolved and Adsorbed γ-Globulin

assignment	dissolved in saline, pH 7.2	exposed to saline, pH 7.4
turns or β-sheet	1686	1690
β-sheet or turns	1675	1674
disordered	1651	1663
β-sheet	1639	1638

This interpretation of the pH-induced changes is supported by the circular dichroism results (Doi & Jirgenson, 1970) on solutions near the same pHs. Doi and Jirgenson (1970) report essentially no change in the total amount of β-sheet structure by CD at high and low pH but either formation of an overall more disordered structure or aggregation into a more disordered β-sheet structure at low pH. The infrared results (Figure 7 and 8) strongly support the latter explanation. Such aggregation into a more disordered β-sheet structure either could be intermolecular in nature or could involve intramolecular (possibly between chains) aggregation. Unfolding or loss of the original ordered β-sheet structure and re-formation into a new structure involving different parts of the molecule or different molecules could easily lead to a more disordered β-sheet structure.

Of particular interest is a comparison of the amide I frequencies of γ-globulin dissolved in saline at pH 7.2 and the frequencies of adsorbed (at pH 7.4) γ-globulin exposed to saline. These frequencies are listed for comparison in Table II. The frequencies assigned to β-sheet structures (which are mainly in the interior of the protein) are virtually identical. However, those assigned to disordered structures or turns (to a large extent on the exterior of the protein) show significant frequency differences between the dissolved and the absorbed protein. These differences are especially large for the disordered structures and could indicate that the point of attachment of the protein to the adsorbent surface (the ATR crystal) lies in the disordered segments of γ-globulin (or the side chains of the amino acids in the disordered segments). Conversely, these shifts and lack of shifts between dissolved and adsorbed γ-globulin can be used as evidence that the spectral secondary structure correlations or assignments are correct.

In an attempt to put all the observations together, a mechanism has been postulated for the adsorption behavior of γ-globulin dissolved in ethylene glycol. In aqueous solution or in the solid state, γ-globulin has mainly an ordered intramolecular β-sheet structure with some turns, some random or

disordered structure, and possibly some very short (single turn) helix structure (Amzel & Poljak, 1979). Due either to being dissolved in ethylene glycol or following a very short (10 min) protein adsorption period, some of the ordered intramolecular β-sheet structure has been lost, probably by unfolding since ethylene glycol is known (Singer, 1962) to partially unfold some proteins. This ordered β-sheet structure is replaced by both helix structures and a new form of β-sheet structure, although the helix structure is apparently formed from both the disordered segments and the original β-sheet segments. We believe that the new form of β-sheet structure is an intermolecular β-sheet formed from the unfolded portions of the original β-sheet which would explain a more disordered form of β-sheet structure. Although such a disordered β-sheet structure could be intramolecular in nature, we favor an intermolecular β-sheet based on the possibility of aggregation as reported in the CD results (Doi & Jirgenson, 1970), the low-frequency shift of the amide I band indicating stronger H bonds which could result from intermolecular bonding, and, mainly, that intermolecular β-sheet formation has been reported in the denaturation of other proteins (Clark et al., 1981; Painter & Koenig, 1976). After 180 min of protein adsorption, more of the disordered, intermolecular β-sheet has formed, while some of the helix structure and practically all of the ordered, intramolecular β-sheet structure have disappeared.

It has been previously discussed that either methanol or ethylene glycol narrows the amide I bandwidth of albumin as compared to the bandwidth of this vibration for adsorbed albumin exposed to saline. This bandwidth narrowing has been related to a decrease in the amount of water bound to the exterior of the albumin molecules (Jakobsen et al., 1986). When adsorbed γ-globulin is exposed to methanol, no such bandwidth narrowing can be detected. However, this lack of detection *may* be due to the formation of helix structure as shown by the more intense, high-frequency shoulder on the 1638 cm^{-1} β-sheet amide I band (Figure 4B) which tends to broaden this major amide I band at 1638 cm^{-1}. When adsorbed γ-globulin is exposed to saline, then to ethylene glycol, and then to saline again, the bandwidth of the major amide I band at 0.85 peak height goes from 24.0 to 15.0 cm^{-1} and then back to 23.6 cm^{-1}, respectively. This may reflect (as for albumin) a decrease in the amount of bound water, or it may be due to inherently different bandwidths due to the two forms of β-sheet structure; i.e., the ordered, intramolecular β-structure resulting from exposure to water may have a different amide I bandwidth than the disordered, intermolecular β-sheet structure resulting from exposure to ethylene glycol. Either explanation has interesting connotations for future infrared studies and indicates that such infrared studies can provide significant structural information.

In summary, the observed changes for each of the following systems are the following.

Adsorbed Albumin. The spectral changes in adsorbed albumin caused by exposure of the albumin to either methanol or ethylene glycol (Jakobsen et al., 1986) are identical. These changes can be related to an increase in the helix secondary structure, a decrease in the amount of bound water, and an increase in helix order.

Adsorbed and Dissolved γ-Globulin. The spectral changes (1) caused by exposure of adsorbed γ-globulin to nonaqueous solvents, (2) caused by changing the pH of aqueous solutions of γ-globulin, or (3) caused by adsorbing γ-globulin dissolved in ethylene glycol can be related to changes in secondary structure where one of the following occurs. (1) Exposure to methanol produces helix structure but does not affect the β-sheet structure. (2) Exposure to ethylene glycol both produces helix structure and changes the ordered, intramolecular β-sheet structure to a more disordered, probably intermolecular β-sheet structure. (3) Changes in the pH of aqueous solutions of γ-globulin do not produce helix structure but do change the β-sheet structure from the ordered, intramolecular form to the less ordered, intermolecular β-structure. (4) A qualitative mechanism for the adsorption of γ-globulin from ethylene glycol solutions has been proposed. This mechanism involves rapid formation of both helix structure and the less ordered, intermolecular β-sheet structure from both the disordered segments and the ordered, intramolecular β-sheet segments of γ-globulin. With increasing time of solution flow, more γ-globulin is adsorbed, and the adsorbed protein rearranges or converts to the less ordered, intermolecular β-structure at the expense of both helix segments and ordered, intramolecular β-sheet structure. The resulting adsorbed γ-globulin only has a trace of the ordered, intermolecular β-structure left at the end of the adsorption period.

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