Dehydration-induced Conformational Transitions in Proteins and Their Inhibition by Stabilizers

Steven J. Prestrelski,* Nicole Tedeschi,* Tsutomu Arakawa,* and John F. Carpenter‡
*Department of Protein Chemistry, Amgen, Inc., Thousand Oaks, California 91320; ‡School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado 80262, USA

ABSTRACT Dehydration of proteins results in significant, measurable conformational changes as observed using Fourier-transform infrared spectroscopy and resolution-enhancement techniques. For several proteins these conformational changes are at least partially irreversible, since, upon rehydration, denaturation and aggregation are observed. The presence of certain stabilizers inhibited these dehydration-induced transitions; the native structure was preserved in the dried state and upon reconstitution. Conformational transitions were also observed in a model polypeptide, poly-L-lysine, after lyophilization and were inhibited with the addition of stabilizing cosolutes. The ability of a particular additive to preserve the aqueous structure of dehydrated proteins and poly-L-lysine upon dehydration correlates directly with its ability to preserve the activity of lactate dehydrogenase, a labile enzyme, during drying.

INTRODUCTION

Water is essential for the formation of native protein structures (1, 2). However, the effects of dehydration upon protein conformation have long been a point of controversy. Kuntz and Kauzmann (3) have demonstrated from theoretical considerations that dehydration should result in significant conformational changes. Several sorption isotherm studies of progressive rehydration of dried proteins employing vibrational spectroscopy, nuclear magnetic resonance, and hydrogen exchange techniques have indicated hydration-related changes in conformation and flexibility (4–6). In particular, differences in the rehydration behavior between α-lactalbumin and lysozyme were attributed to conformational differences in the dried state. Dehydration-induced changes in conformation-sensitive modes observed in a freeze-dried protein with Raman spectroscopy studies have also supported this claim (7); however, effects due solely to removal of water could not be ruled out. Furthermore, it is well known that dehydration can completely and irreversibly inactivate some enzymes (8). This inactivation occurs presumably through loss of structure. In fact, Hanafusa (9) has observed such dehydration-induced changes in tertiary and quaternary structure upon rehydration of dried myosin and catalase, respectively.

In contrast, other investigators, also using vibrational spectroscopy to follow protein changes upon progressive rehydration, have developed a hydration model for lysozyme that does not involve significant changes in conformation (10–12). Spectral changes observed in conformation-sensitive modes were ascribed solely to changes in hydration. The strongest evidence provided for the absence of a conformational change during dehydration comes from an electron spin resonance (ESR) study. In the ESR study, carried out at −160°C, two spin probes placed on lysozyme were found not to shift more than 1.0 Å as a function of hydration levels (11).

This controversy has not been reconciled, in part, because of the lack of methodology for detailed examination of protein structure in the both the aqueous and dehydrated states. For example, previous studies have been inconclusive because spectral differences observed upon dehydration were difficult to interpret directly in terms of conformation (6, 10). Furthermore, the studies of Rupley and Careri (see Ref. 11 for a review) have focused upon a single and unusually stable protein, lysozyme, for which all dehydration-induced spectral shifts are fully reversible. The more recent development of combining Fourier-transform infrared (FTIR) spectroscopy with mathematical band narrowing techniques has provided the capacity for detailed conformational studies of proteins in aqueous solution (13, 14). Application of these techniques to dehydrated proteins allows more rigorous and detailed interpretation of the drying-induced spectral changes than was previously possible. In this report, an extensive study of the effects of dehydration on the structure of several proteins with diverse physicochemical properties is described. Supporting experiments using poly-L-lysine as a model polypeptide system are also reported. Additionally, the effects of various stabilizing and destabilizing additives on protein conformation during dehydration are examined.

MATERIALS AND METHODS

Materials

Basic fibroblast growth factor (bFGF), γ-interferon (γ-IFN), and granulocyte-colony-stimulating factor (G-CSF) were human sequences produced recombinantly at Amgen (Thousand Oaks, CA). α-Lactalbumin (bovine), α-casein (bovine), lactate dehydrogenase (LDH; from rabbit muscle) and poly-L-lysine (M_r = 57,400 by low-angle laser light scattering) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. These proteins are widely different in conformation, molecular weight, isoelectric point, and quaternary structure. All other com-

Received for publication 10 July 1992 and in final form 16 March 1993.
Address reprint requests to Dr. Steven J. Prestrelski, Amgen, Inc., Amgen Center, 1840 DeHavilland Drive, Thousand Oaks, CA 91320.

0006-3495/93/08/661/11 $2.00

pounds were acquired from commercial sources. The polyethylene glycol (PEG) used had a M_r of 8000.

Spectroscopy

All infrared spectra were recorded on a Nicolet (Madison, WI) 800 FTIR system. Two-hundred fifty-six double-sided interferograms were averaged and Fourier-transformed after application of a Happ-Genzel apodization function. The spectral resolution was 2 cm^{-1}. Second-derivative spectra were calculated as described by Susi and Byler (15) using Nicolet software, and smoothed with a 13-point smoothing function (9 points for the spectra of LDH). Spectra of aqueous protein solutions were recorded at a concentration of 50 mg/ml in 10 mM Tris-Cl, 0.1 M NaCl (pH = 7.5) using infrared cells with CaF_2 windows and 6-μm tin spacers.

Lyophilization

All proteins were dialyzed against 10 mM Tris-Cl, 0.1 M NaCl (pH = 7.5). The proteins were lyophilized from ~20–25 mg/ml solutions in the Tris-HCl buffer using a Virtis (Gardiner, NY) lyophilizer. For purposes of conducting parallel activity studies, LDH was lyophilized at a concentration of 1 mg/ml. Solutions were frozen on the lyophilizer shelf at −45°C. Primary drying was achieved by lyophilizing for 24 h at a shelf temperature of −45°C. Secondary drying was achieved at shelf temperatures of −10°C (24 h) and +20°C (24 h). The condenser temperature was −60°C; the pressure was ~30 mTorr.

The dried protein samples containing 0.5 to 1.0 mg of protein were mixed and ground with KBr and placed into a die. The die was then placed under vacuum for at least 16 h, and afterward the sample was pressed into a pellet under vacuum. The pellets were transferred to IR cells in a nitrogen-purged dry box. No absorbance due to water was observable in the spectra of blank KBr pellets prepared in this manner. As a control for examining the effects of KBr pellet preparation on protein conformation the spectra of several dried proteins were collected using an attenuated total reflectance sampling device that did not require mixing and pressing with KBr. These spectra were essentially identical to those for samples prepared as KBr pellets, although the required sample size was much greater. Additional control experiments were performed using IR cards (3M Company, St. Paul, MN). IR cards consist of a thin polyethylene sheet onto which a sample can be placed. Samples of dried powders were placed between two cards, and the infrared spectrum was collected. Again, no sample processing was necessary. Furthermore, this is a transmission experiment, and thus, none of the problems associated with reflectance measurements are present. No significant differences were noted between identical samples prepared by the three techniques, indicating that making a KBr pellet does not affect the protein conformation. The KBr pellet technique provides the highest signal and the lowest potential for artifacts (i.e., scattering, reflectance artifacts) and is useful for all situations.

The dried proteins were reconstituted by dissolving the powders in H_2O to a concentration of 50 mg/ml, a concentration identical to that needed to acquire the spectrum of the prelyophilized aqueous protein. In cases where rehydration resulted in aggregation and precipitation, the dried protein samples were rehydrated directly onto a CaF_2 infrared crystal and sandwiched with a second crystal and a 6-μm spacer.

LDH activity assays

LDH enzymatic activity was measured at 25°C as follows. The 1.0-ml reaction mixture contained 25 mM Tris-HCl, 0.1 M KCl (pH = 7.5) containing 2 mM pyruvate and 0.215 mM reduced nicotinamide adenine dinucleotide. The dried LDH samples were rehydrated to a concentration of 1 mg/ml. The reaction was initiated by the addition of 5 μl of an LDH solution that had been prediluted to 25 μg/ml. Activity was measured by the decrease in the absorbance at 340 nm using a Beckman DU 650 UV spectrophotometer with programmable temperature control. Assays were performed immediately before freezing and after rehydration. The activity is expressed as the percentage of initial activity.

Quantitative analysis of spectra

To quantify the overall similarity between two second-derivative spectra, the simple correlation coefficient, r, was calculated using the following equation:

$$r = \frac{\sum^N x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}}$$

where x_i and y_i represent the spectral absorbance values of the reference and sample spectra at the ith frequency position. Here, x and y are treated as two independent variables. The r value is used here to compare the spectrum of a given lyophilized protein to that for the aqueous protein. All spectral absorbance values of the smoothed second-derivative spectra in the region between 1720 and 1610 cm^{-1}, which corresponds to the amide I region, were used for the calculation. For spectra collected at 2 cm^{-1} resolution, this results in absorbance values every 1 cm^{-1}. For identical spectra, a value of 1.0 will be returned. Spectra that have differences will show lower values. Additionally, spectra do not have to be normalized. Thus, calculation of the correlation coefficient, r, is a suitable method for comparing spectra of proteins in different environments.

Residual water measurements

The method used for measurement of residual water is similar to that of Crowe et al. (16). To examine the effect of stabilizers on residual hydration, 10 mg of sucrose and increasing amounts of protein or, conversely, 10 mg of protein and increasing amounts of sucrose were added to 1 ml of water. To each mixture, 1 μl of ^3H_2O was added. Three 1-μl aliquots used as standards were extracted and placed in 1 ml of H_2O and mixed with 15 ml of scintillation cocktail. Three 300-μl aliquots were transferred to vials and lyophilized using the protocol described above. The lyophilized samples were reconstituted in 1 ml of H_2O and mixed with 15 ml of scintillation cocktail. Radioactivity in the samples was measured using a Beckman LS1800 liquid scintillation counter.

RESULTS AND DISCUSSION

Lyophilization of proteins

Fig. 1 shows the second-derivative IR spectra in the amide I region for several proteins in the aqueous and dehydrated (lyophilized) states. The hydration level of the proteins after lyophilizing using this procedure was 0.04 g/g or less in the absence of stabilizers. (The uncertainty in this value arises because the amount of tritium that exchanges with the protein is unknown.) Proteins of all conformational classes are represented including α (γ-IFN, G-CSF), α/β (α-lactalbumin), β (bFGF), and disordered (α-casein), and all, except γ-IFN (a dimer), are monomeric. The IR spectrum of each protein is significantly altered upon dehydration, although the degree of the spectral change varies. In the spectrum of dehydrated bFGF (Fig. 1*A*) a peak observed in the aqueous spectrum at 1657 cm^{-1} is no longer resolved. Furthermore, the relative intensity of the peak at 1622 cm^{-1} in the aqueous spectrum is significantly reduced, and a new band at 1614 cm^{-1} is resolved. The remaining peaks shift in frequency from 2 to 5 cm^{-1} upon dehydration. In general, all of the resolved peaks are broader. Similarly, in the spectrum of dehydrated γ-IFN (Fig. 1*B*) extensive band broadening and peak frequency shifts are observed. Comparison of the spectrum of dehydrated α-lactalbumin (Fig. 1*C*) to that of the aqueous protein indicates that the six peaks resolvable in the aqueous protein

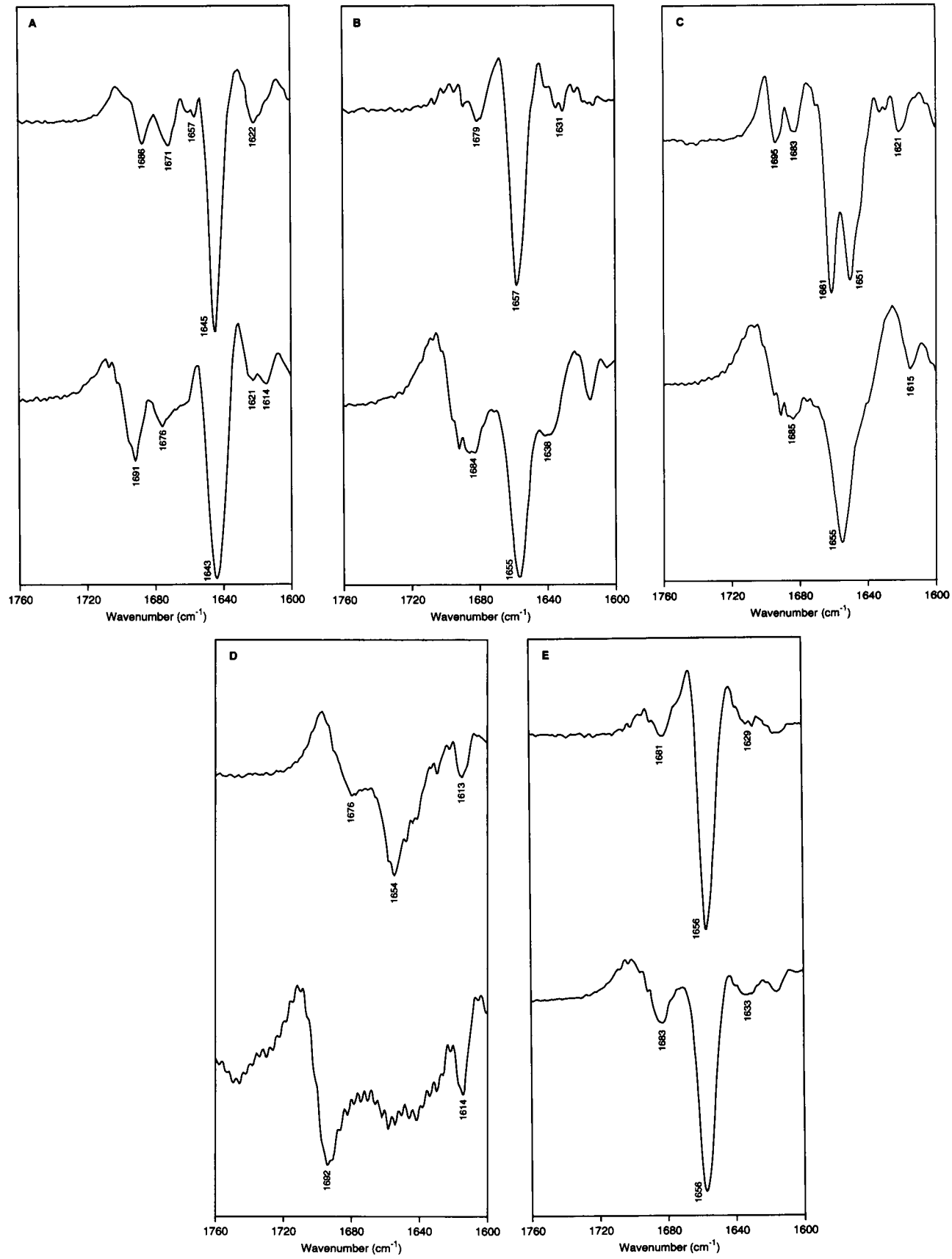

FIGURE 1 Second-derivative infrared spectra in the amide I region of several proteins in the aqueous (*upper curves*) and dehydrated (*lower curves*) state. (*A*) bFGF; (*B*) γ-IFN; (*C*) α-lactalbumin; (*D*) α-casein; (*E*) G-CSF.

are reduced to two at 1655 and 1685 cm^{-1} in the dried state. Furthermore, a new peak is observed at 1615 cm^{-1}. The loss of resolution may be due to overall band broadening or a loss of certain spectral features, or some combination of the two. It is not possible to distinguish between these alternatives with the available information. The spectrum of aqueous casein (Fig. 1*D*) has the characteristics of an unordered protein, in agreement with previous studies (13). However, the spectrum is greatly altered after dehydration and shows a large fraction of β-sheet as evidenced by predominant bands near 1615 and 1690 cm^{-1}. (This is similar to the behavior of random coil poly-L-lysine, described below.) In contrast to the other proteins, the spectrum of dehydrated G-CSF (Fig. 1*E*) appears little changed from the aqueous spectrum. A relatively small degree of broadening is observed along with small band frequency shifts.

For all of the proteins studied the dominant spectral change observed upon dehydration is broadening of the individual amide I components, indicative of a general disordering of the protein backbone. This disordering is likely to be static (multiple stable conformations) rather than dynamic because the protein's mobility is greatly decreased upon dehydration. In addition, several protein spectra show relative peak intensity differences as well as loss of spectral feature(s) upon dehydration. Shifts in peak positions are also observed. From a knowledge of the relationship between amide I components and protein structure (13, 14), these spectral changes are indicative of conformational changes.

This interpretation of dehydration-induced spectroscopic changes is in conflict with that of Rupley et al. (12), who report an absence of conformational change in lysozyme with dehydration. While Rupley et al. have observed spectral differences in the amide I band upon dehydration, they attribute these changes to removal of water in the absence of a conformational change. This interpretation was based on an ESR study that did not indicate a conformational change. Several possibilities exist for this discrepancy. First, it is possible that conformational changes on the order observed here are not observable with the ESR technique, which examines the distance between spin probes at a temperature of −160°C as a function of hydration level. Second, it is possible that the spectral differences observed upon dehydration may be due to the removal of water, per se, and not conformational differences, as interpreted by Rupley et al. Removal of the hydration shell may alter the environment such that the vibrational properties are altered. Such changes could, in theory, shift amide I frequencies and intensities. If this were true, however, similar changes in the spectra of each protein upon dehydration would be expected. However, quite large differences in the nature of the spectral changes are observed upon dehydration of the different proteins studied. Compare, for example, the changes in the G-CSF spectrum to those of α-casein (Fig. 1, *D* and *E*). Another possibility for the discrepancy is that lysozyme retains the native conformation upon dehydration, whereas the proteins examined here do not. To compare our results more directly with those of Rupley et al., we have examined the behavior of lysozyme (data not shown) under the conditions used here and found that spectrum in the dried state is also indicative of drying-induced conformational changes. Upon rehydration, the protein returns to the prelyophilized structure, indicating that the unfolding is reversible. Based on the results presented here for a wide variety of proteins it appears that the conclusions reached by Rupley and Careri (11) may have been biased by basing their studies on a single protein.

Quantitative analysis

Quantitative analysis of the spectra presented in this report is a difficult task. First, from a structural perspective, no information is available on the structure of dehydrated proteins that is analogous to crystallographic structures of proteins in the aqueous state. While it is clear that conformational changes occur, this lack of information makes it impossible to make any concrete quantitative statements concerning the structure of dehydrated proteins. From a spectroscopic perspective, quantitative analysis also poses problems. Fourier self-deconvolution (17) is the most widely used analytical tool for quantitation of the amide I components because it does not affect relative band areas (13, 14). We have also attempted to apply this tool to the spectra of dehydrated proteins. However, in order to make meaningful comparisons between two spectra, one must use the same deconvolution parameters (i.e., an estimate of the half-width and a resolution enhancement factor) for both spectra. It is apparent from the second-derivative spectra presented that dehydration induces significant changes in the component bandwidths. Thus, in our experience, it has not been possible to deconvolute spectra of aqueous and dehydrated proteins in a manner that allows meaningful comparisons to be made between them for an array of different proteins.

However, an important attribute of the spectrum of a dried protein is its degree of similarity to the spectrum of the aqueous protein. We have used the spectral correlation coefficient, as described in Materials and Methods, as a quantitative measure of the similarity of the spectra and, therefore, by inference, of the structure of proteins in the dehydrated and aqueous states. The results of this analysis are listed in Table 1. As is observed visually, dehydration results in varied changes in the amide I infrared spectra of the different proteins examined. The correlation coefficients for proteins lyophilized in the absence of stabilizers ranges from 0.696 for casein, which undergoes a large transition, to more than 0.9 for G-CSF, which is little affected by dehydration. To examine variability, the spectra of several identical samples of proteins were collected in duplicate under identical conditions. The mean difference in the computed correlation coefficients for spectra of identical samples relative to the same aqueous protein spectrum was 0.011, $\sigma = 0.0085$ ($n = 4$).

Rehydration

The spectral correlation coefficients for the rehydrated proteins relative to the initial aqueous spectra are listed in Table

TABLE 1 Correlation coefficients for second derivative spectra of dehydrated and rehydrated proteins

Additive	bFGF	γ-IFN	α-Lactal-bumin	Casein	G-CSF
Dehydrated					
Buffer only	0.892	0.743	0.774	0.696	0.907
Sucrose	0.972	0.929	0.898	0.843	0.923
Lactose	0.954	0.858	0.843		0.927
Mannose	0.951	0.849	0.876		0.933
Glucose	0.965	0.914	0.893		0.917
Galactose	0.952	0.762	0.783		0.841
Mannitol	0.954	0.719	0.805	0.833	0.850
Myoinositol	0.932	0.551	0.801		0.795
PEG	0.932	0.858	0.739		0.902
Rehydrated					
Buffer only	0.909	0.795	0.984	0.741	0.991
Sucrose	0.992	0.992	0.989	0.952	0.995

1. The amide I IR spectra of bFGF, γ-IFN, and casein after rehydration were still altered relative to the prelyophilized aqueous spectra as evidenced by r values significantly less than 1.0. This indicates that at least partial, irreversible conformational changes occurred upon lyophilization and rehydration. γ-IFN and casein also exhibited extensive aggregation and precipitation upon rehydration; the rehydrated casein was essentially insoluble. The dehydration-induced changes appeared reversible, however, for α-lactalbumin and G-CSF, which give r values near 0.99 relative to the initial aqueous spectra. These results indicate that while the observed dehydration-induced conformational changes are sometimes irreversible, some proteins appear to be inherently stable under the stresses introduced during freeze-drying.

The irreversible spectral changes observed for several of the proteins provide further support for the contention that the spectroscopic differences observed upon dehydration result from conformational changes. If drying-induced changes were due solely to H_2O removal they should be fully reversible upon rehydration. Thus, it appears that the results of Rupley and Careri (11) may have been biased by examination of only a single protein and, further, by the choice of a model protein that is inherently stable under dehydration. In the present study, it is apparent from the results of several different proteins that the conformational behavior upon dehydration is protein dependent. Three types of behavior are observed for proteins upon dehydration and rehydration. First, a protein can be resistant to conformational change during drying and therefore retain the native conformation upon rehydration such as G-CSF. Second, a protein may unfold during dehydration but refold to the native state upon rehydration, as is observed for α-lactalbumin and lysozyme. Third, a protein may unfold during dehydration and not regain the native conformation, resulting in irreversible conformational changes and denaturation. Thus, the inherent stability of a protein to survive dehydration and subsequent rehydration must be related to its capacity to resist conformational changes during dehydration or to refold into the native structure upon rehydration. With the present information it is not possible to relate the observed stabilities to a type or degree of conformational change observed upon dehydration.

Correlation of dehydration-induced structural and functional alterations

An apparent manifestation of the observed loss of structure is the observation that upon dehydration, several labile enzymes completely lose their biological activity (18). We have examined this proposal in detail using the enzyme lactate dehydrogenase as a model. Table 2 lists the spectroscopic and enzymatic activity results for lactate dehydrogenase upon lyophilization and rehydration. When freeze-dried only from the buffer solution the spectrum of LDH indicates a high degree of unfolding, indicated by a correlation coefficient essentially equal to zero. Thus, dehydration induces a large-scale unfolding of LDH. This high degree of unfolding correlates with an essentially complete loss of activity upon rehydration. Thus, the unfolding of the native conformation of LDH upon dehydration is apparently irreversible and leads to loss of biological activity. For technical reasons it is not possible to collect the spectra of the rehydrated LDH at 1 mg/ml with sufficient signal-to-noise ratios to examine the second-derivative spectra. However, it is reasonable to regard the level of enzymatic activity as a functionally relevant indicator of the presence of native structure.

Lyophilization of poly-L-lysine

To understand more fully the nature of the dehydration-induced spectral changes, we have examined the behavior of poly-L-lysine during lyophilization. In solution poly-L-lysine adopts an α-helical, β-sheet or unordered conformation, depending on the pH and temperature (19–21). These solution conformations have been extensively characterized by FTIR spectroscopy (22). Fig. 2 shows the second-derivative amide I spectra of aqueous and lyophilized poly-L-lysine under several initial conditions of pH and temperature. At neutral pH, poly-L-lysine exists as an unordered polypeptide, as indicated by the strong peak near 1649 cm^{-1}. Freeze-drying induces a transition from an unordered polypeptide to a highly ordered β-sheet (Fig. 2*A*). Lyophilization from a pH 11.2 solution,

TABLE 2 Spectral correlation coefficients and activity retention values for lyophilized and rehydrated lactate dehydrogenase

Additive*	r value	Activity retention
Buffer only	−0.003	11.6 ± 0.04
Sucrose	0.832	118.6 ± 1.5
Lactose	0.805	105.5 ± 1.5
Mannose	0.812	103.0 ± 8.8
Glucose	0.781	95.4 ± 2.2
Galactose	0.870	103.0 ± 1.3
Mannitol	0.334	40.6 ± 0.3
Myoinositol	0.762	92.9 ± 2.5
Polyethylene glycol	0.788	41.2 ± 2.7

* The concentration of the additives for this study was 25 mg/ml.

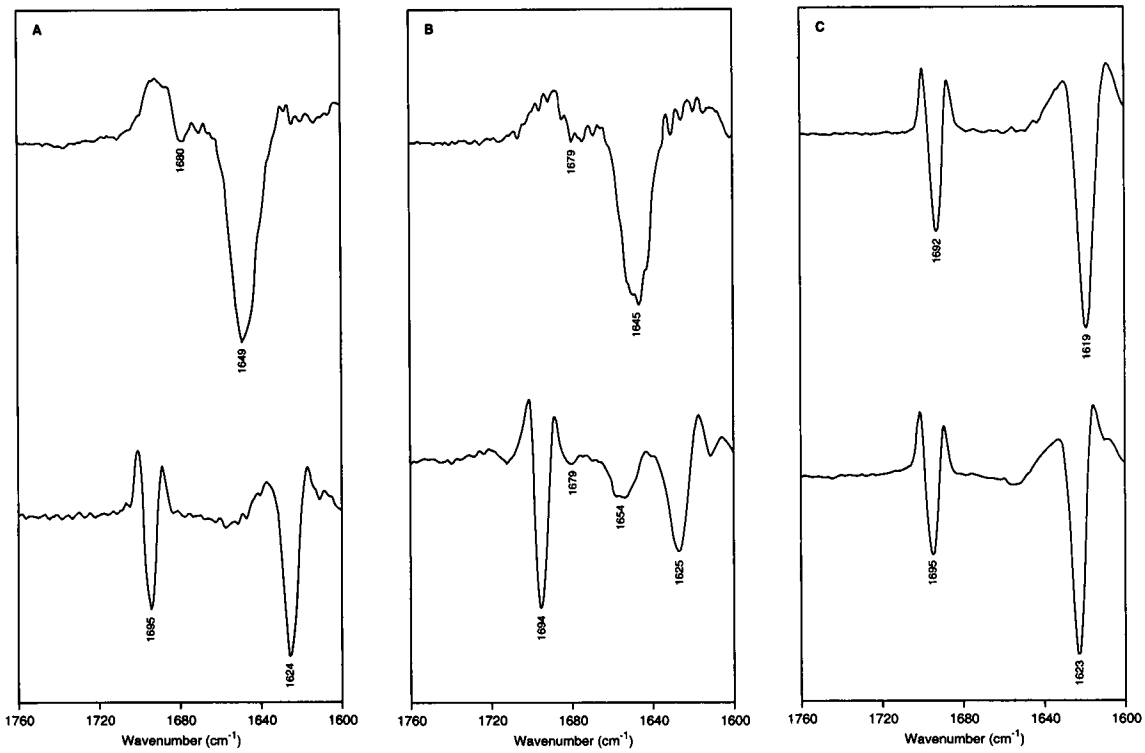

FIGURE 2 Second-derivative infrared spectra of poly-L-lysine in aqueous solutions (*upper curves*) and after lyophilization (*lower curves*) from the following solutions: (*A*) Tris buffer (pH 7.5); (*B*) NaOH solution (pH = 11.2); (*C*) NaOH solution (pH 12.0) heated at 75°C for 30 min. The heated solution was frozen quickly in a dry ice/acetone bath so that reversion to the α-helical state was inhibited.

where the polypeptide adopts an α-helical conformation, also induces a transition to a β-sheet, although a small amount of α-helix is apparently still observable, as indicated by the peak near 1654 cm^{-1} (Fig. 2*B*).

Heating a pH 12.0 solution of poly-L-lysine results in formation of a β-sheet. In a sample lyophilized after this treatment, the β-sheet is preserved upon dehydration (Fig. 2*C*). Apparently the preferred conformation in the dried state is β-sheet regardless of the initial conformation in aqueous solution. The changes in this spectrum upon dehydration are very small, limited to small frequency shifts (3–4 cm^{-1}) for each of the two peaks indicative of the β-sheet conformation. No changes in band shape, width, or relative peak intensities are apparent upon dehydration. Thus, from this result it is apparent that the spectroscopic changes due solely to removal of water from the polypeptide, independent of a conformational change, are very small. This observation agrees with results indicating that local association effects have the greatest influence and that solvent factors have little effect on carbonyl stretching vibrations (23, 24). The results of the poly-L-lysine studies provide strong evidence that the spectral changes in the amide I region observed upon dehydration of proteins are predominantly related to conformational changes and that the effect of water removal independent of a conformational change is minimal.

The dehydration-induced conformational transitions observed for poly-L-lysine appear to be due to compensation for the loss of hydrogen bonding interactions with H_2O during dehydration. In solution, a random coil has its peptide hydrogen bonds satisfied by water molecules. Upon dehydration, these hydrogen bonds are lost. To compensate for this loss the polypeptides form intermolecular hydrogen bonds, resulting in the observed β-sheet conformation of the dried polypeptide. Furthermore, in the absence of water, the partial charges of hydrogen bonding groups are less screened due to the lower dielectric environment, increasing the electrostatic attraction between dehydrated peptides. Thus, in the dried state, the hydrogen bonding interaction energy between amide groups should be stronger than in aqueous solution. The conformational transition observed for α-helical poly-L-lysine is also consistent with this mechanism. At low hydration levels, the β-sheet conformation is energetically more favorable than the α-helix because the β-sheet requires a lower degree of solvation (25). The β-sheet structure has a higher degree of intermolecular hydrogen bonding. Thus, as the hydration shell is removed from the helical polypeptide, a transition to β-sheet is induced.

These proposed mechanisms for dehydration-induced conformational transitions can be extrapolated to proteins. During dehydration, the protein rearranges its conformation to maximize intra- and interchain hydrogen bonding to replace lost hydrogen bonds to water. In addition, several different types of interactions are present in proteins that are not in the poly-L-lysine model, including hydrophobic interac-

tions. Thus, the effects of dehydration on different proteins are more complex than the polylysine model. It is clear that significant conformational rigidity exists in some proteins because only a small conformational change is observed after dehydration (e.g., G-CSF, Fig. 1*E*). In contrast, many proteins are unstable during lyophilization and after reconstitution lose all or part of their structure. Thus, while it is reasonable to focus upon the hydrogen bonding interactions in explaining the dehydration-induced conformational changes, it is clear that it is more complicated and more studies are necessary to completely account for all interactions in the aqueous and dehydrated states.

Effects of additives on the structure of dried proteins

Studies of labile enzymes have shown that certain additives can stabilize proteins during lyophilization and rehydration (18). Carbohydrates, disaccharides in particular, appear to be the most effective stabilizers. Based on the observation that lyophilization induces (sometimes irreversible) conformational transitions in proteins, it is reasonable to speculate that the stabilizing effect of these solutes may involve the protein's conformation. Fig. 3 shows the infrared spectra of several proteins lyophilized with sucrose, an additive that has been shown to be particularly effective at protecting labile enzymes during dehydration (18). In all cases, the dried state spectra of proteins lyophilized in the presence of sucrose resemble closely the respective spectra of the same proteins in aqueous solution (compare with Fig. 1). This is also apparent from the spectral correlation values listed in Table 1. Lyophilization in the presence of sucrose results in an r value much closer to 1.0 than the value in the absence of any additives. Thus, the stabilizer preserves the native or aqueous conformation of the protein during dehydration.

In addition to sucrose, we have examined the effects of numerous disaccharides, monosaccharides, and polyhydric alcohols on protein spectra in the dried state. The data listed in Table 1 indicate that the effects of the additives examined fall into three classes. First, several additives, disaccharides in particular, result in spectra that are very similar to the aqueous spectra. The r values for the spectra of proteins lyophilized with disaccharides range from ~0.85 to 0.97, significantly higher than r values for unprotected proteins. Second, certain additives have an intermediate or no significant effects upon the spectra of the dried proteins. For example, PEG has a minimal effect on the spectra of dried G-CSF. Finally, certain additives further denature the proteins upon dehydration, as evidenced by a decreased correlation coefficient relative to the protein dried from buffer alone. For example, γ-IFN lyophilized from buffer has a correlation coefficient of 0.743 relative to the aqueous protein spectra, but when lyophilized from myoinositol it has a correlation coefficient of 0.551. Finally, the effect of a given additive varies with the protein examined, indicating that the protein itself plays an important role in determining the protein-additive interaction.

Numerous studies have emphasized the importance of the physical state of buffer and stabilizer components in stabilizing proteins during freeze-drying (26–28). While these reports clearly indicate that the amorphous or crystalline nature of additives is important in achieving optimal protein stability, the results presented here indicate that effects of the physical characteristics of an additive cannot be extrapolated to all proteins. In other words, one cannot assume that the observed effects of a given additive in a given protein system are universally applicable. The results for γ-IFN are an illuminating example. Mannitol is often used as a bulking agent in preparing lyophilized proteins with the assumption that because it crystallizes upon freezing it is therefore inert with respect to the protein. However, the spectra of γ-IFN indicate that mannitol, and other crystallizing components such as myoinositol, are destabilizing in this case and induce further unfolding during dehydration. Thus, focusing upon the nonprotein components, while clearly necessary, provides a limited view of the effects of the lyophilization process on the protein of interest.

The effects of the various additives on dehydration-induced conformational transitions of poly-L-lysine have also been examined. Several additives (sucrose, lactose, and maltose) inhibited the random coil to β-sheet transition (spectra not shown). In contrast, several other additives that do not protect protein structure (e.g., mannitol, myoinositol, PEG) did not prevent conformational transitions of poly-L-lysine. Galactose had an intermediate effect. A mixture of random coil and β-sheet is observed after dehydration. Additionally, lyophilization with sucrose also resulted in the retention of α-helical poly-L-lysine during dehydration when this is the initial solution conformation. These results support the conclusion that the mechanism of protein stabilization by additives during lyophilization is through maintenance of the native conformation in the dehydrated state.

In addition to the effects that the presence of carbohydrates has on the spectra of dried proteins, the proteins also produce similar changes in the spectra of the carbohydrates, as demonstrated by Carpenter and Crowe (29). Sugars lyophilized in the presence of proteins give infrared spectra very similar to those of sugars in aqueous solution. These results were interpreted as an indication that proteins are hydrogen bonding to the carbohydrates in the dried state. Combined with the present results, the results of Carpenter and Crowe (29) provide a picture of a protein hydrogen bonded to sugars in the dried state, the sugars substituting for water during dehydration.

Effects of stabilizers upon rehydration

Rehydration of the dehydrated sucrose/protein mixtures resulted in spectra essentially identical to those before lyophilization, and in contrast to results for proteins dried without stabilizers, no aggregate formation was apparent. The r

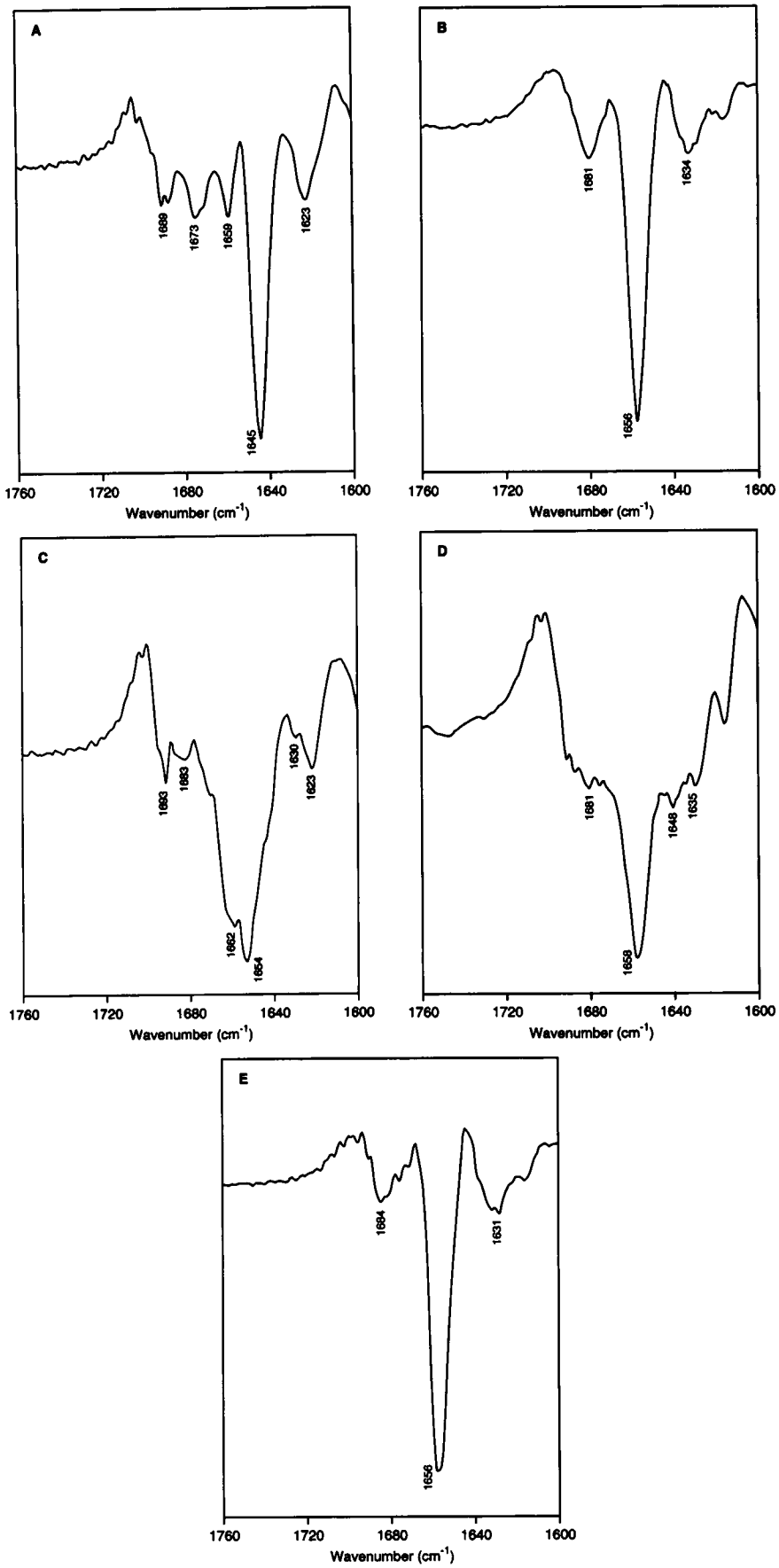

FIGURE 3 Second-derivative infrared spectra of several proteins lyophilized in the presence of 200 mg/ml sucrose. (*A*) bFGF; (*B*) γ-IFN; (*C*) α-lactalbumin; (*D*) α-casein; (*E*) G-CSF.

values for proteins rehydrated from sucrose are all close to 0.99 (Table 1), indicating that they are essentially identical to the prelyophilized conformation. Thus, in addition to preserving the native conformation effectively upon dehydration, the stabilizer fosters the retention of the native structure after rehydration.

Effects of stabilizers on LDH structure and activity recovery

We have also examined the effect of various stabilizers for their capacity to preserve the structure and activity of LDH. Table 2 lists the percentage activity recovered after lyophilization and reconstitution of LDH in the presence of various additives. Also listed are the spectral correlation coefficients for the dried-state spectra of LDH lyophilized under identical conditions. A strong correlation is apparent between the preservation of the native structure during dehydration and retention of enzymatic activity upon reconstitution. Lyophilization of LDH in the absence of stabilizers results in a loss of native structure and enzymatic activity upon rehydration. In contrast, lyophilization in the presence of stabilizers results in the preservation of the native structure, as indicated by increasing r values, and enhanced recovery of enzymatic activity after reconstitution. Spectral correlation coefficients near 0.8 result in essentially complete recovery of activity, and lower values result in intermediate levels of activity recovery. The results for polyethylene glycol are the only exception. However, cloudiness was observed upon rehydration of the PEG-containing sample, indicating precipitation. It is possible that the high concentration of PEG, a strong protein precipitant (30), induced precipitation of the protein during rehydration, resulting in the lower activity value. Linear regression of a plot of the percentage of enzymatic activity recovered versus the spectral correlation coefficient for LDH dried in the presence of various stabilizers (excepting that for PEG) gave a coefficient of determination, r^2, of 0.97. This indicates that, for LDH under these conditions, an essentially linear correlation exists between the preservation of the native structure, as indicated by the spectral correlation coefficient, and the recovery of enzymatic activity after rehydration. We conclude from these results that (*a*) the mechanism by which dehydration induces loss of biological activity in proteins is unfolding of the native protein structure and (*b*) the protective effect observed for stabilizers takes place through preservation of the native structure during dehydration.

Effects of destabilizing additives

If stabilizing agents preserve the native structure of proteins upon dehydration, then destabilizing compounds should further perturb the conformation. Fig. 4 shows the spectra of bFGF and γ-IFN freeze-dried in the presence of 0.3 M sodium isothiocyanate, a potent protein conformational destabilizer (31, 32). This concentration of the denaturant did not alter the solution structure of the proteins. Comparison of these results with those in Figs. 1 and 3 (*A* and *B*) shows that the addition of destabilizing solutes results in a further loss of native structure relative to lyophilization in the absence of cosolutes. The r values for bFGF and γ-IFN relative to the aqueous spectra are 0.538 and 0.140, respectively. Again, these results are consistent with the interpretation that the spectral differences observed upon dehydration are due to conformational changes.

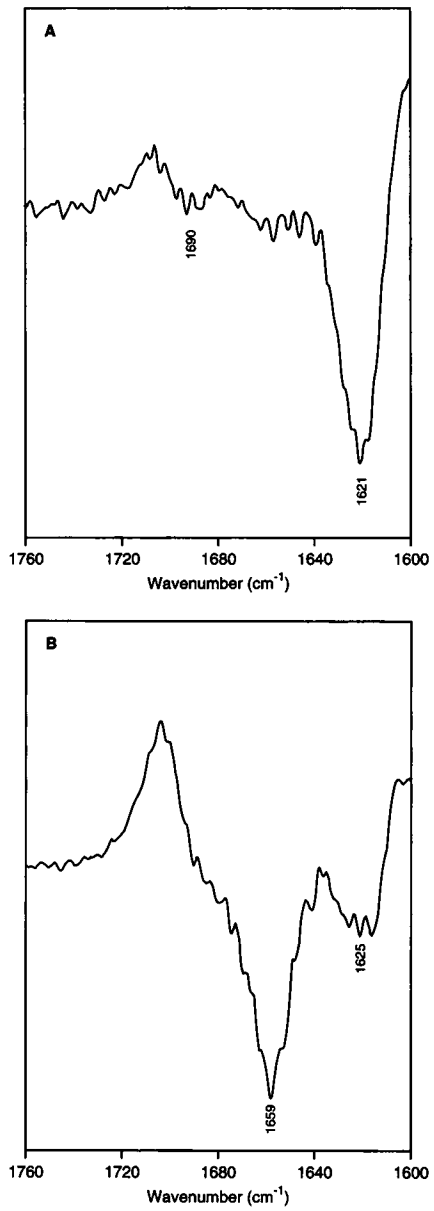

FIGURE 4 Spectra of bFGF (*A*) and γ-IFN (*B*) after lyophilizing in the presence of sodium isothiocyanate. The concentration of NaSCN is low enough (0.3 M) such that perturbation of the structure in the aqueous state is not observed.

Interactions between stabilizers and proteins in the dehydrated state

At least two mechanisms have been suggested for the preservation of biological activity of labile proteins by sugars

during dehydration. Carpenter and Crowe (29) have proposed that the hydroxyl groups of the sugar molecules act as water replacements in the dried state. Alternatively, it has been suggested that sugars increase the amount of residual water present, thus increasing protein hydration and avoiding dehydration-induced damage. Experiments with tritiated water indicate that the hydration level of the proteins after lyophilizing using the procedure employed here was 0.04 g/g or less in the absence of stabilizers. Lyophilization of sucrose in the absence of protein indicated that the amount of residual water is rather small, ~2 mol/mol of sucrose. In addition, as demonstrated in Fig. 5, titration of sucrose with increasing amounts of protein results in less residual water after lyophilization. At low initial concentrations, the addition of protein results in a decrease in the level of hydration of sucrose, indicating a direct interaction. At higher concentrations of protein, the effect is lessened as the sucrose becomes saturated with protein. The reverse experiment gave similar results. Titrating a protein with increasing amounts of sucrose results in a lower level of residual water than when increasing sugar concentration in the absence of protein (data not shown).

The most reasonable explanation for these results is that residual water is displaced from the dried protein by direct interactions between the protein and the sugars. That is, these results support the contention that interaction of the carbohydrate hydroxyl groups with polar and charged groups on the protein are responsible for the observed preservation of the aqueous structure upon dehydration. Furthermore, the carboxylate bands at 1600–1580 cm^{-1} can be used to monitor hydrogen bonding independent of a conformational change in the protein (5, 6). Examination of the carboxylate bands in the spectrum of α-lactalbumin (Fig. 6) indicates that the addition of carbohydrate maintains these bands in the hydrogen-bonded or "hydrated" form after dehydration, as had been observed previously (29). The contention that carbohydrates serve as a water substitute is also supported by work that shows perturbations of the carbohydrate hydroxyl vibrations in the presence of added protein (29) and by studies indicating that the molar ratio of various additives to protein, and not bulk concentration, correlates with protein stabilization (33). This "water replacement" effect of carbohydrates is well established for the stabilization of dried membranes (34).

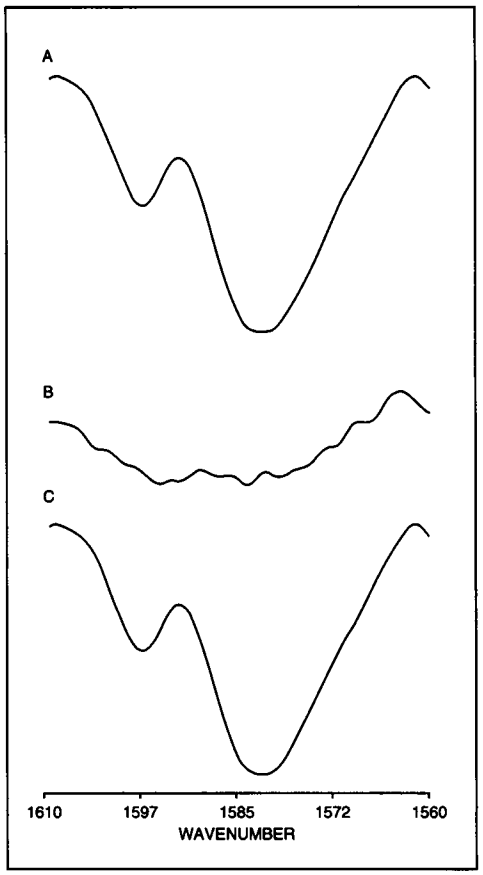

FIGURE 6 Carboxylate bands of α-lactalbumin. Second-derivative spectra of aqueous (*A*) and lyophilized (*B*) α-lactalbumin and of α-lactalbumin lyophilized in the presence of 200 mg/ml sucrose (*C*).

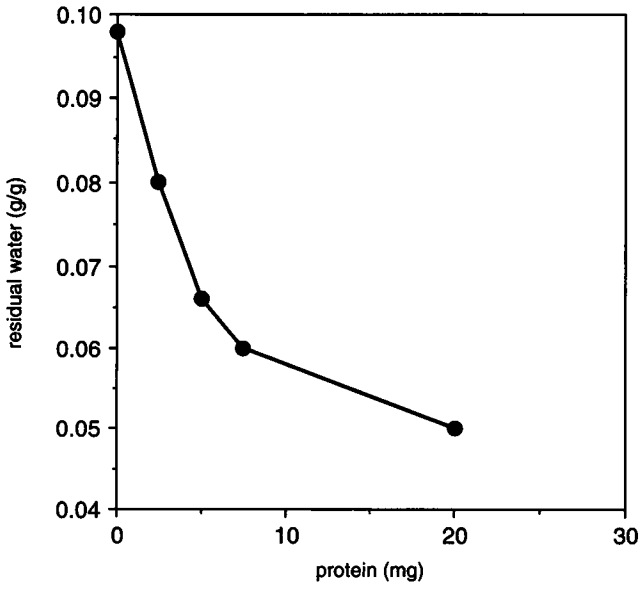

FIGURE 5 Effect of protein on the residual water after lyophilization. The amount of residual water bound to sucrose is plotted versus protein concentration. b-FGF was used as the protein component.

The results for poly-L-lysine dried in the presence of protein stabilizers also support the contention that carbohydrates serve as water substitutes. Additives that preserved the native structure of proteins during dehydration also inhibited the conformational transitions observed for poly-L-lysine and resulted in retention of the solution structure in the dried state as described above. Thus, in the less complex model system, where the interactions are better understood, it is clear that compounds which can hydrogen bond to the dry peptide also preserve the aqueous structure during dehydration.

CONCLUSION

We have demonstrated conclusively that dehydration induces significant, measurable conformational changes in proteins.

Several pieces of evidence support this conclusion: (*a*) vastly different spectroscopic results are observed for different proteins upon lyophilization; (*b*) in several cases, spectral changes induced upon dehydration are irreversible; (*c*) results from studies of a model polypeptide show clear, complete conformational transitions upon dehydration; (*d*) additional results from the model polypeptide experiments indicate that removal of water in the absence of a conformational change has no significant effects on the amide I vibrational mode. It has been further demonstrated that these dehydration-induced transitions can be inhibited with the addition of stabilizers and that the mechanism of these stabilizers derives from maintenance of the native structure in the dried state. Additionally, a direct correlation is observed between the degree of preservation of the native structure in the dried state and recovery of biological activity upon reconstitution of a dried enzyme. Finally, these results demonstrate that infrared spectroscopy is a useful tool for studying protein conformation upon dehydration. In particular, the IR methodology, based on its ability to examine structure in the dehydrated state, appears to have strong potential as a predictive tool in developing effective formulations for stabilizing proteins during lyophilization.

REFERENCES

1. Kauzmann, W. 1951. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* 14:1–63.
2. Edsall, J. T., and H. A. McKenzie. 1983. Water and proteins. II. The location and dynamics of water in protein systems and its relation to their stability and properties. *Adv. Biophys.* 16:53–183.
3. Kuntz, I. D., and W. Kauzmann. 1974. Hydration of proteins and polypeptides. *Adv. Protein Chem.* 28:239–345.
4. Poole, P. L., and J. L. Finney. 1983. Hydration-induced conformational and flexibility changes in lysozyme at low water content. *Int. J. Biol. Macromol.* 5:308–310.
5. Poole, P. L., and J. L. Finney. 1983. Sequential hydration of a dry globular protein. *Biopolymers.* 22:255–260.
6. Poole, P. L., and J. L. Finney. 1984. Sequential hydration of dry proteins: a direct difference IR investigation of sequence homologs lysozyme and α-lactalbumin. *Biopolymers.* 23:1647–1666.
7. Yu, N-T., and B. H. Jo. 1973. Comparison of protein structure in crystals and in solution by laser raman scattering. I. Lysozyme. *Arch. Biochem. Biophys.* 156:469–474.
8. Carpenter, J. F., J. H. Crowe, and T. Arakawa. 1990. Comparison of solute-induced protein stabilization in aqueous solution and in frozen and dried states. *J. Dairy Sci.* 73:3627–3636.
9. Hanafusa, N. 1969. Denaturation of enzyme protein by freeze-thawing and freeze-drying. *In* Freezing and Drying of Microorganisms. T. Nei, editor. University of Tokyo Press, Tokyo. 117–129.
10. Careri, G., E. Gratton, P-H. Yang, and J. A. Rupley. 1980. Correlation of IR spectroscopic heat capacity, diamagnetic susceptibility and enzymatic measurements of lysozyme powder. *Nature (Lond.).* 284:572–573.
11. Rupley, J. A., and G. Careri. 1991. Protein hydration and function. *Adv. Protein Chem.* 41:37–172.
12. Rupley, J. A., P-H. Yang, and G. Tollin. 1980. Thermodynamic and related studies of water interacting with proteins. *In* Water in Polymers. S. P. Rowland, editor. ACS Symp. Ser. 127:111–132.
13. Byler, D. M., and H. Susi. 1986. Examination of the secondary structure of proteins by deconvoluted FTIR spectra. *Biopolymers.* 25:469–487.
14. Surewicz, W. K., and H. H. Mantsch. 1988. New insight into protein conformation from infrared spectroscopy. *Biochim. Biophys. Acta.* 952: 115–130.
15. Susi, H., and D. M. Byler. 1983. Protein structure by Fourier transform infrared spectroscopy: second derivative spectra. *Biochem. Biophys. Res. Commun.* 115:391–397.
16. Crowe, J. H., B. J. Spargo, and L. M. Crowe. 1987. Preservation of dry liposomes does not require retention of residual water. *Proc. Natl. Acad. Sci. USA.* 84:1537–1540.
17. Kaupinnen, J. K, D. J. Moffatt, H. H. Mantsch, and C. C. Cameron. 1981. Fourier self-deconvolution: a method for resolving intrinsically overlapped bands. *Appl. Spectrosc.* 35:271–276.
18. Carpenter, J. F., L. H. Crowe, and J. H. Crowe. 1987. Stabilization of phosphofructokinase with sugars during freeze-drying: characterization of enhanced protection in the presence of divalent cations. *Biochim. Biophys. Acta.* 923:109–116.
19. Townend, R., T. F. Kumosinski, S. N. Timasheff, G. D. Fasman, and B. Davidson. 1966. The circular dichroism of the β structure of poly-L-lysine. *Biochem. Biophys. Res. Commun.* 23:163–169.
20. Tiffany, M. L., and S. Krimm. 1969. Circular dichroism of the random polypeptide chain. *Biopolymers.* 8:347–359.
21. Epand, R. M., G. E. Wheeler, and R. M. Moscarello. 1974. Circular dichroism and proton magnetic resonance of random chain poly-L-lysine. *Biopolymers.* 13:359–369.
22. Jackson, M., P. I. Haris, and D. Chapman. 1989. Conformational transitions in poly-L-lysine: studies using Fourier transform infrared spectroscopy. *Biochim. Biophys. Acta.* 998:75–79.
23. Bellamy, L. J., and R. L. Williams. 1959. Infrared spectra and solvent effects. *Faraday Soc. Trans.* 55:14–18.
24. Cutmore, E. A., and H. E. Hallam. 1969. Molecular configuration and interactions of the amide group. I. Solvent effects on vibrational frequencies. *Spectrochim. Acta.* 25A:1767–1784.
25. Barlow, D. J., and P. L. Poole. 1987. The hydration of protein secondary structures. *FEBS Lett.* 213:423–427.
26. Franks, F. 1989. Improved freeze-drying: an analysis of the basic scientific pronciples. *Process Biochem.* 24:iii–vii.
27. Hatley, R. H. M., and F. Franks. 1991. Aplications of DSC in development of improved freeze-drying processes for labile biologicals. *J. Therm. Anal.* 37:1905–1914.
28. Chang, B. S., and C. S. Randall. 1992. Use of subambient thermal analysis to optimize protein lyophilization. *Cryobiology.* 29:632–656.
29. Carpenter, J. F., and J. H. Crowe. 1989. An infrared spectroscopic study of the interaction of carbohydrates with dried proteins. *Biochemistry.* 28:3916–3922.
30. Arakawa, T., and S. N. Timasheff. 1985. Mechanism of poly(ethylene glycol) interaction with proteins. *Biochemistry.* 24:6756–6762
31. Von Hippel, P. H., and K-Y. Wang. 1965. On the conformational stability of globular proteins. *J. Biol. Chem.* 240:3909–3923.
32. Busby, T. F., D. H. Atha, and K. C. Ingham. 1981. Thermal denaturation of antithrombin. III. Stabilization by heparin and lyotropic anions. *J. Biol. Chem.* 256:12140–12147.
33. Tanaka, R., T. Takeda, and K. Miyajima. 1991. Cryoprotective effect of saccharides on denaturation of catalase by freeze-drying. *Chem. Pharm. Bull. (Tokyo).* 39:1091–1094.
34. Crowe, J. H., F. A. Hoekstra, and L. M. Crowe. 1992. Anhydrobiosis. *Annu. Rev. Physiol.* 54:579–599.