Real-Time *in Situ* Monitoring of Lysozyme During Lyophilization Using Infrared Spectroscopy: Dehydration Stress in the Presence of Sucrose

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Purpose. First, to investigate the role of sucrose in stabilizing protein structure (as measured by changes in the amide I band of lysozyme) caused by dehydration encountered during lyophilization. Second, to demonstrate the utility of internal reflection spectroscopy as a tool for conducting controlled lyophilization experiments.

Methods. A custom-built internal reflection FTIR accessory was used to follow the entire freeze-drying process of solutions consisting of 49.4 mg/mL lysozyme in the presence and absence of 10% sucrose in real-time. Studies were carried out using D_2O as a transparent medium in the infrared region of the protein amide bands. Potential self-association of the protein in the presence of sucrose was investigated using dynamic light scattering. Hydration levels were determined using a multiple regression equation. Differential scanning calorimetry (DSC) permitted characterization of the final lyophilized product. Moisture content was determined using Karl Fischer titration.

Results. Throughout freezing and drying, minimal changes were observed both in frequency (1647 ± 1 cm^{-1}) and bandwidth (46 ± 1 cm^{-1}) of the amide I band in the presence of sucrose. In contrast, greater changes in frequency and band width were seen in the absence of sucrose. A successfully lyophilized cake was obtained which had properties of a glass as measured by DSC, with a Tg of 50°C. The lyophilized product containing sucrose had 4% moisture by weight. Three distinct rates of water desorption were discovered during drying under vacuum (50 mg/hr within the sample temperature range from −35° to −25°C; 30 mg/hr from −10° to 25°C; 1.2 mg/hr from 27° to 38°C).

Conclusions. The inclusion of sucrose served to minimize perturbations of protein structure caused by freezing and dehydration stresses encountered during lyophilization (compared to studies conducted in the absence of sucrose). The results support the water replacement hypothesis and underscore the role of the sugar in preserving a native structure in the dried state. This investigation demonstrates the usefulness of infrared spectroscopy in evaluating lyophilization process parameters and formulation design.

KEY WORDS: FTIR spectroscopy; attenuated total reflectance; internal reflection; lyophilization; lysozyme; sucrose.

INTRODUCTION

An understanding of the lyophilization process and the stresses involved is critical to resolving formulation development problems and attaining optimal product stability (i.e., long-term shelf-life). Some factors responsible for achieving a stable protein product include protein structure (1), residual moisture (2,3), choice of excipients (or solute stabilizers) (4,5) and process design (6). In order to preserve the native structure, the protein must survive cooling, freezing, and dehydration stresses. Often it is necessary to include stabilizing excipients in the formulation. However, knowledge of how such excipients stabilize proteins during lyophilization has remained incomplete due to a lack of techniques capable of studying the entire process at the macromolecular level. Effective strategies to preserve product viability must consider how different physical environments encountered during lyophilization impact the protein and affect the role of the stabilizing excipient. A new approach has been developed using internal reflection infrared spectroscopy to investigate the perturbing stresses on protein structure during lyophilization in real-time (7,8). In this study an empirical model is introduced to estimate hydration levels in the sample during primary and secondary drying. With this improvement, direct evidence for how water removal is associated with changes in protein structure in the presence and absence of sucrose can be studied.

MATERIALS AND METHODS

Lysozyme (3× crystallized) was purchased from Sigma (product L-2879) and used without any further purification. D_2O obtained from Sigma (product D-4501) was 99.9% deuterium enriched and used in the preparation of a 49.4 mg/ml protein solution. These experiments were conducted in D_2O to avoid potential difficulties associated with water subtraction (necessary when H_2O is the solvent) under freezing conditions. Using D_2O instead of H_2O permits a window of transparency in the region of the amide I band of proteins and allows the direct evaluation of this band during freeze-drying (7). J.T. Baker analyzed reagent grade sucrose (product 4072-01) was used as a solute stabilizer (10% w/v).

A custom-built single-reflection, horizontal, attenuated total reflection accessory in combination with a Bio-Rad FTS-7 infrared spectrophotometer (equipped with a Hg/Cd/Te [MCT] detector) was used to acquire the spectra in real-time. Spectra were collected at 4 cm^{-1} resolution and 1024 scans were signal averaged for each spectrum (constituting a time interval of 15 min per spectrum). Modifications were made on the apparatus to permit measurement of temperature and vacuum in the sample compartment, which were not available in earlier investigations (7,8). A schematic layout of the sample compartment is described in Figure 1. Two accessible openings to the sample compartment, one connected to the lyophilizer, the other to a pressure sensor for vacuum measurement, permitted a closed environment to conduct freeze-drying. A Labconco lyophilizer provided the condenser and vacuum necessary to carry out the experiment. The temperature and pressure of the condenser chamber in the lyophilizer were also monitored during the drying interval of the process. The dewar positioned above the sample, allowed cooling of the sample compartment by thermal conduction through a stainless steel holder that contained the germanium crystal (internal reflection element [IRE]). Temperature was controlled by adjusting the amount of liquid nitrogen

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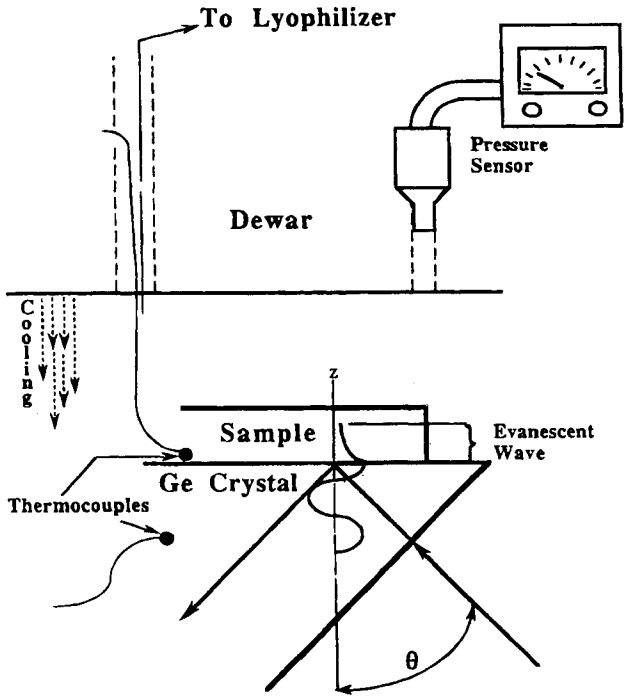

Fig. 1. A schematic representation of the single-reflection, horizontal, attenuated total reflection sample chamber. An opening into the chamber permitted accessibility to a pressure sensor used to monitor vacuum during the experiment. A second opening allowed connection to the lyophilizer. A dewar positioned on top of a stainless steel IRE holder was used to provide cooling to the Ge crystal. Thermocouples were positioned in contact with the Ge crystal and sample. Incident light is internally reflected at $\theta = 45°$. The theory concerning the evanescent wave has been described in referance 8.

in the dewar and compensating with heating elements embedded within the crystal holder.

Before a given sample was subjected to lyophilization, equilibrium involving hydrogen-deuterium exchange was established based upon no observable alterations in the amide I and II bands over an extended period of time (beyond several days in a refrigerator). A typical experiment involved cooling the sample far below the glass transition temperature of the amorphous phase. Tracking the cooling process over time permitted the determination of cooling rates of the sample and germanium crystal. Once the desired subzero temperature was attained, drying under vacuum commenced.

Differential scanning calorimetry (DSC) experiments were carried out on a Perkin-Elmer DSC-7. The instrument was calibrated with indium (melting transition, 156.1°C) and methylene chloride (melting transition, −95.6°C) prior to examination of the lyophilized product. About 5 mg of lyophilized product was weighed in a tared aluminum pan. The sample was cooled to 20°C in the DSC and data were collected during warming at 10°C/min.

Residual moisture of the final lyophilized product was measured by Karl Fischer titration (9) and found to be reliable within ±0.28%. Enzyme concentration and assay were determined spectrophotometrically as described earlier (10).

Dynamic light scattering experiments were conducted using a Malvern System 4700c sub-micron particle analyzer connected to a Langley-Ford autocorrelator (model 1096). A typical experiment was carried out using the 514.5 nm excitation line of an argon-ion laser and analyzing the samples under identical conditions of illumination intensity. The scattered light was collected at 90° with a 9 microsecond sample time. Samples consisted of 5 mg/ml lysozyme in water with and without 10% sucrose. Additionally, samples were passed through a 0.22 micron filter into a clean sterilized test tube prior to the light scattering measurement. Using a cumulant algorithm, the average protein diffusion coefficient was determined from the autocorrelation function. Once the diffusion coefficient was known, the Stokes-Einstein equation could be used to compute the effective diameter (2 × hydrodynamic radius) of the protein. Light scattering measurements were corrected for refractive index and viscosity differences associated with 10% sucrose solutions.

Hydration Determinations

Reference standards of known composition were prepared by weighing the desired amounts of sucrose and protein into a clean test tube and subsequently adding a specified volume of D_2O with a calibrated pipet. To assess the contribution of D_2O to the total weight of the solution, the density of D_2O at the temperature pipetted was multiplied by the volume added in each case. The resulting solution mixture was further checked by measuring the protein content at 280 nm and in all cases considered, agreement between weighed and spectrophotometrically measured fractions was achieved to within 1%. All liquid solutions were evaluated in this way, whereas water contents of the solid lyophilized products were directly measured by Karl Fischer titration. The weight fraction of D_2O in each solution multiplied by 100 produced the experimental %h values (represents the percent hydration of D_2O on a w/w basis) reported in Table I. The experimental values were also used to generate the regression formula coefficients (equation 1) using JMP (statistical software from SAS Institute Inc.).

In order to account for changes in hydration during lyophilization, an empirical approach was applied. This model assumes that Beer's Law is obeyed throughout a given experiment. Infrared spectra of each reference sample were taken. Since the O-D stretch region was targeted to assess hydration within a D_2O matrix, its absorption band (A_{OD}) was integrated

Table I. Comparison of Calculated (Equation 1) to Experimental Hydration

Experimental	Calculated	Residual
100%	95.6%	4.4%
95.7	98.7	−3.0
88.1	89.5	−1.4
91.7	90.3	1.4
61.8	63.2	−1.4
60.0	62.3	−2.3
65.9	66.3	−0.4
49.6	52.6	−3.0
7.0	5.9	1.1
4.0	3.2	0.8
3.7	4.7	−1.0
3.4	4.8	−1.4

from 2075 to 2748 cm^{-1} (Figure 2). However straightforward this may seem, it is complicated by the fact that there are overlapping contributions from sugar and protein H/D exchanged hydroxyl groups in this region of the spectrum. Such contributions become more significant as a result of freeze-concentration of the sample (to be discussed further below). Therefore, to account for this complexity the amide I (A_{al}) integrated from 1591 to 1722 cm^{-1} and the sugar band (A_s) integrated from 912 to 1161 cm^{-1} were also evaluated (assuming that changes in the absorbance of these bands would be consistent with changes in their contribution to the O-D stretch region of the spectrum) using the multiple regression formula:

$$\%h = k_1(A_{OD}) + k_2(A_{al}) + k_3(A_s) + c \quad (1)$$

where k_1, k_2 and k_3 were empirically determined coefficients, and c is a constant. Using the actual hydration values of the reference standards shown in Table I and the measured areas of the O-D, amide I and sucrose bands in their infrared spectra, the following coefficients for the regression formula were generated: $k_1 = 2.86758$, $k_2 = -3.32909$, $k_3 = -3.24102$ and $c = 13.77861$. A comparison of the actual hydration values to the predicted values obtained for each reference sample computed from equation 1 and their respective residuals is displayed in Table I. The fit of the data is reasonable with an $r^2 = 0.995$. This formula was subsequently used to evaluate changes in hydration during the course of primary and secondary drying.

RESULTS AND DISCUSSION

Freezing

In the cooling profile shown in Figure 3A, the sample temperature exhibited is lower than the temperature of the germanium crystal. In the earlier studies using this technique (7,8), it was assumed that the temperature of the sample at the surface of the crystal was the same as that of the germanium crystal. These data show that this is not the case. Interestingly, the temperature differential between the crystal and the sample increases as the temperature decreases. The plateaus shown in the cooling profile represent the time intervals of spectral collection where temperature was held constant (±1°C, Ge reading). An inital spectrum was collected at 25°C followed by cooling to 0°C at a rate of 5°C/min where the second spectrum was collected, as shown in Figure 3B. Subsequent cooling rates were between 1 and 2°C/min. At a sample temperature of 0°C, the spectrum does not exhibit the characteristics of ice, suggesting that supercooling of the sample had occurred. However, at −7°C there is evidence for freezing as exhibited by a shift to low frequency in the O−H and O−D stretch bands. Additionally, there is a dramatic increase in the absorption of the solute bands (shown in the range from 900 to 1800 cm^{-1}). A plausible explanation for the signal increase in the solute bands is "freeze-concentration". Hence, one is looking at an enhancement of solute at the germanium crystal surface with which the progression to lower temperatures is related more to the composition of the amorphous phase (as depicted by the increasing absorbance of the solute bands at −50° and −70°C). Another interesting observation is that the amide I band at −70°C (1647 cm^{-1}) does not shift much in comparison to its position at 25°C

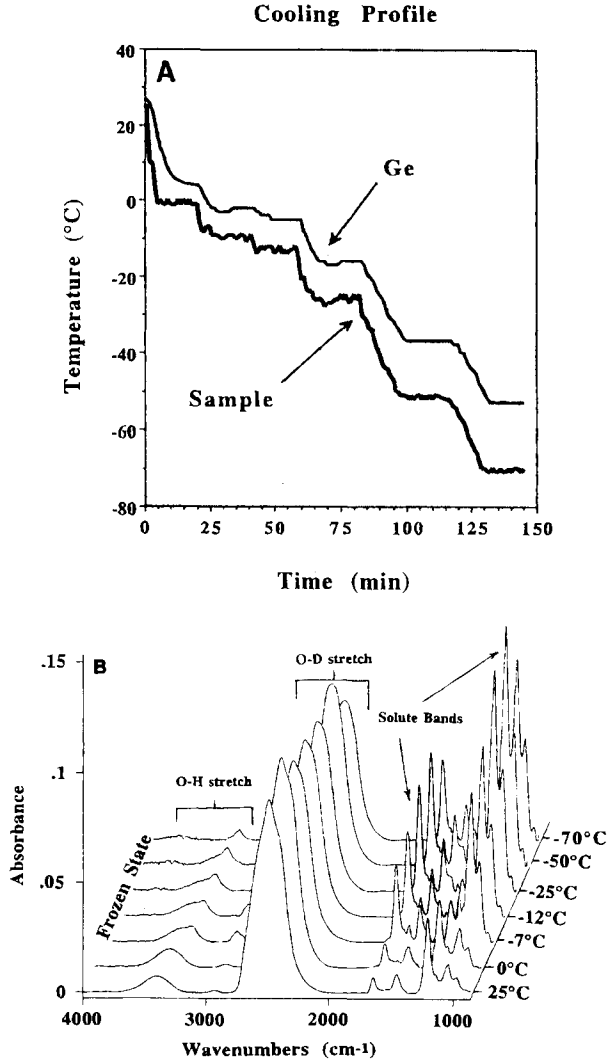

Fig. 3. The freezing process showing (A) the change in temperature of the sample in comparison to that of the germanium crystal (plateaus represent moments of infrared collection) and (B) the analogous infrared spectra displaying the temperature of the sample.

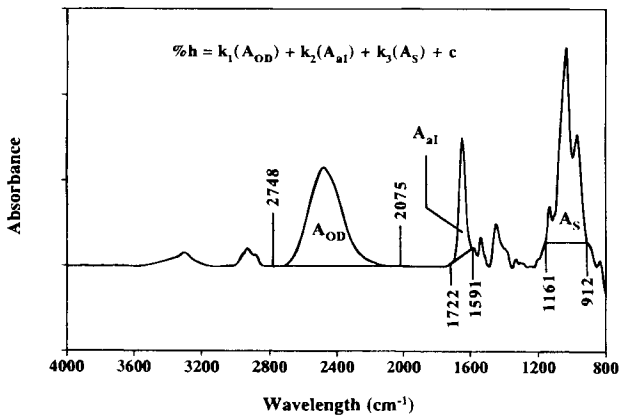

Fig. 2. The infrared spectrum of the final lyophilized product showing regions integrated which include the O−D stretch (A_{OD}; 2075–2748 cm^{-1}), amide I (A_{al}; 1591–1722 cm^{-1}) and the sucrose bands (A_s; 912–1161 cm^{-1}). Integration was evaluated above the line segments drawn between the defined wavenumber regions.

(1648 cm^{-1}). Experiments conducted in the absence of any stabilizer exhibited a shift in this band to 1640 cm^{-1} at −70°C (7), which indicated that there were perturbations of the native protein structure.

Drying

Upon warming under vacuum (Figure 4), the differential temperature between the sample and the germanium crystal was observed to narrow. Unexpectedly, after 11 hr of drying under vacuum there was an increase in the sample chamber pressure reaching a maximum of 1000 mtorr after approximately 13 hr (Figure 4). Furthermore, with this increase in pressure there was an increase in sample temperature which seemed to coincide with the completion of sample cooling caused by the sublimation of ice (primary drying). As discussed below, the completion of sublimation could also be detected in the infrared spectra of the sample. Another possibility for this behavior may have involved the presence of ice on the dewar. Although steps were taken to insulate the top surface of the dewar inside the sample compartment, cooling was mediated from the dewar and could have acted as a cold finger in the experiment. Therefore, moisture desorbed from the sample could have formed ice on the dewar and, upon warming (as the vapor pressure of the ice increased), may have contributed to the positive pressure observed in the sample compartment. Additionally, the magnitude of positive pressure seems to be linked to the amount of vacuum during drying. That is to say, when the vacuum is high this effect is substantially reduced.

The spectral data acquired during drying (Figure 5) were normalized to the amide I band and found to display a relative increase in the absorbance of the O−D stretch band from −45°C through −40°C. Although these changes were less dramatic than those observed in an earlier publication (8) where the sample was cooled at a much faster rate than these experiments, they suggest an increase in mobility at the surface of the Ge crystal (decrease in viscosity). These findings may indicate that the temperature was very close to the Tg′ of the amorphous phase where viscosity changes are known to occur (11).

Applying equation 1 to the spectral data permitted the determination of hydration levels throughout the experiment. These data are presented in Figure 6A as a function of time during drying. The changes in the O−D stretch region of Figure 5 in the time domain of increased mobility (between −45°C [0.42–0.67 hr] and −40°C [4.57–4.82 hr]) translate into an apparent increase in hydration as shown in Figure 6A. This apparent increase in hydration may be explained to result as a

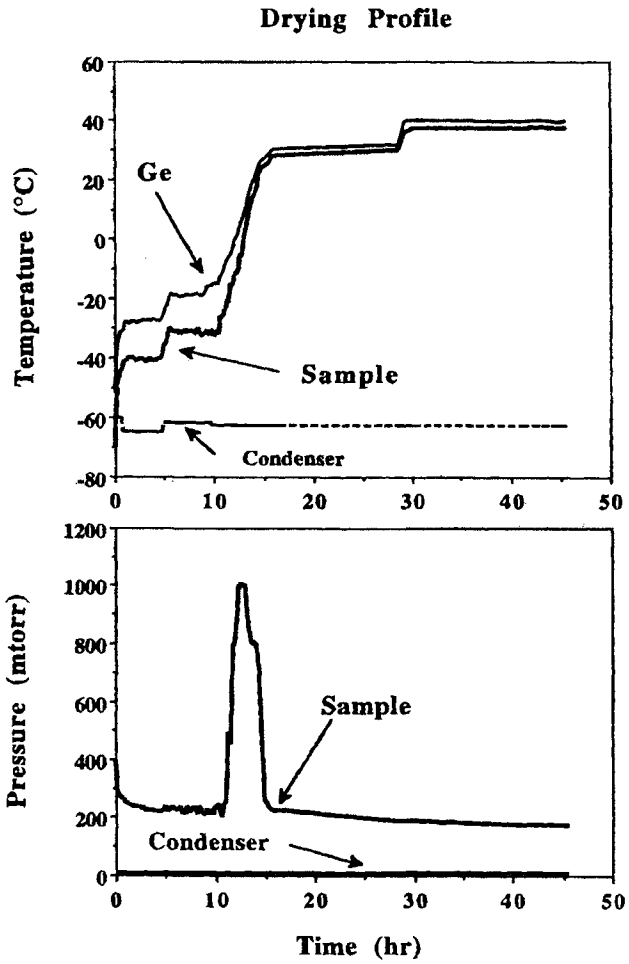

Fig. 4. The drying profile displaying the temperature of the sample, Ge crystal, and the condensor (top) in addition to the analogous pressure experienced in the sample and condensor chambers over the same time course (bottom).

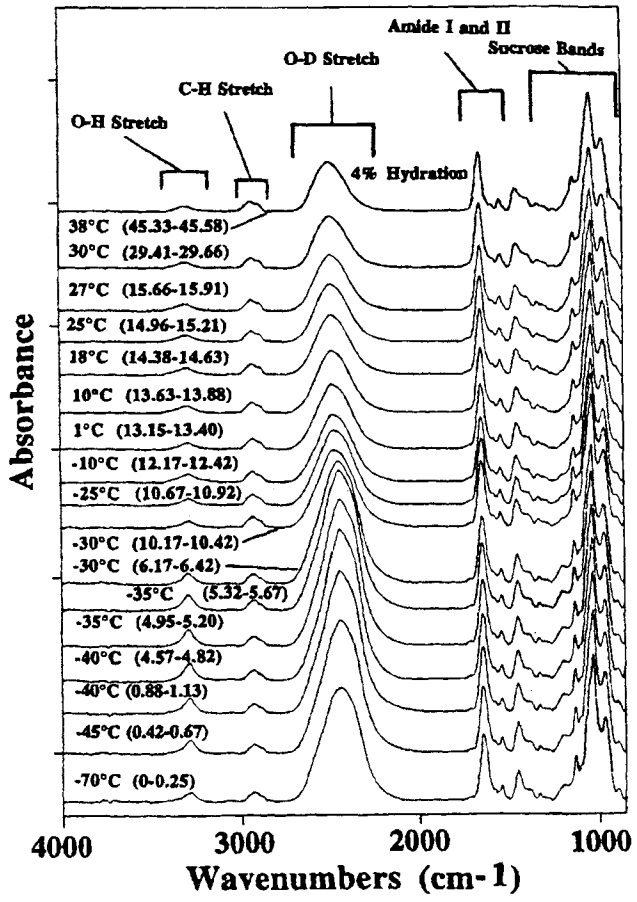

Fig. 5. Infrared spectra obtained throughout the entire drying experiment are accompanied with temperature and the time domain of each collected spectrum (times listed in parenthesis are in hours). Spectral regions are assigned.

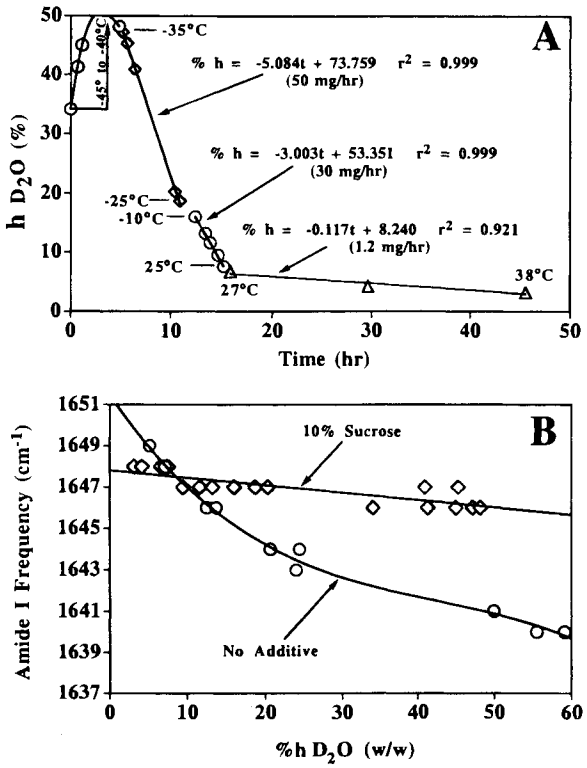

Fig. 6. Plots showing (A) the change in hydration (as determined from the infrared data using equation 1) with respect to time and (B) the behavior of the amide I frequency as a function of hydration level in the sample.

consequence of the increased molecular mobility (due to a reduction of viscosity in the vicinity of Tg′) that was sufficient to permit resumed crystal growth (8). Beyond this period in the drying process, a steady rate of dehydration was shown to occur until the temperature was allowed to increase from −25°C (10.67–10.92 hr) to −10°C (12.17–12.42 hr). At this point in the drying process there was a discontinuity observed and change in rate of dehydration experienced by the sample. Continued drying beyond this point resulted in a further change in rate of dehydration at 27°C (15.66–15.91 hr), which was considerably lower with progressive drying. By segmenting the three regions of different dehydration rates and fitting by linear least squares, three distinct linear rates of dehydration were obtained by the slope of the fitted lines (Figure 6A). The correlation coefficients of the fitted data (expressed as r^2) indicate a high quality of fit. A rate of 50 mg/hr was exhibited between −35°C and −25°C, followed by 30 mg/hr (intermediate dehydration rate) and 1.2 mg/hr (from 27°C to the conclusion of the experiment). It is worth mentioning that the identification of three distinct rates of water loss during drying has been observed before in a mannitol formulation of moxalactum di-sodium (12).

The discontinuity observed between −25°C and −10°C was interpreted as resulting from the end of primary drying and the beginning of secondary drying. It coincides with the abrupt increased chamber pressure noted earlier (Figure 4). Beyond this period in the drying process two distinctly sharp transition rates emerge. Both are ascribed to secondary drying, but between 25°C and 27°C the removal of water from the amorphous phase becomes much more difficult below a hydration level of 8%. At this moisture concentration, it is likely that the remaining water in the matrix is associated with protein backbone, sugar and charged groups. The most difficult water molecules to remove would be those associated with the charged groups of the protein (13). Specific interactions between sucrose and water have been implicated to account for the non-ideality in glass transition temperatures observed in the sucrose-water system (14). Moreover, in an amorphous phase predominantly comprised of sucrose and protein such interactions involving water, sucrose and charged groups are likely to account for the slowest rate of loss observed between 27°C and 38°C (the end of the experiment).

Structural Perturbations

Data describing the impact of dehydration on the shifting behavior of the amide I band (which is indicative of perturbations of the proteins secondary structure) in the presence and absence of 10% sucrose are shown in Figure 6B. The data exhibit much greater change in frequency in the absence of sucrose than with its inclusion. Hence, the presence of sucrose acts to minimize the perturbing effects of freezing and dehydration stresses on lysozyme structure.

In comparing the spectral changes in the amide I prior to freezing with and without sucrose, an interesting observation was made. The inclusion of 10% sucrose at 25°C reduced the band width of the amide I from 56 to 46 cm^{-1}. An understanding of such behavior could provide clues which would help in the interpretion of the spectral data prior to freezing (Figure 7). One possible explanation for this result could involve self-association of the protein in the presence of sucrose. To investigate this further, dynamic light scattering was used to measure the tendency of lysozyme to self-associate in the liquid in the presence and absence of sucrose. Evidence supporting self-association was obtained from these measurements. Comparing the effective diameter of the protein with 10% sucrose to that without sucrose, a ratio of 1.38 was measured suggesting a

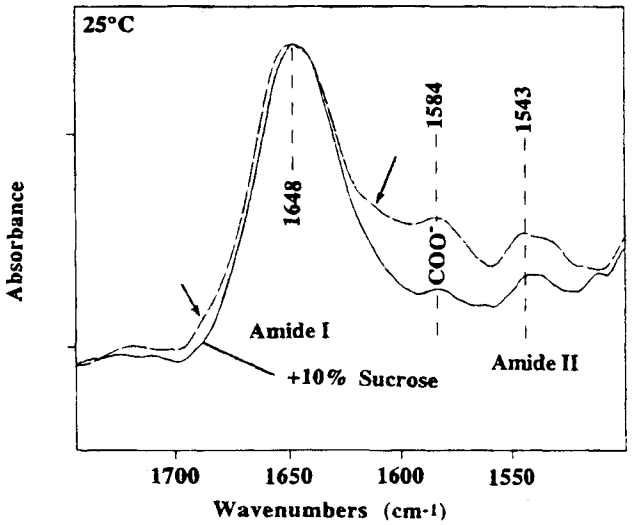

Fig. 7. Infrared spectra of 49.4 mg/ml lysozyme showing the region of the amide I for the liquid (prefrozen) state in the presence (solid contour) and absence (dashed contour) of 10% sucrose. Notice the narrowing of the amide I band (especially in the region shown by the arrows) in the presence of sucrose.

relative increase in the particle size of the protein in the presence of sucrose. This increase in average particle size with 10% sucrose provided evidence for the enhanced presence of self-associated protein forms prior to freezing. This may explain the narrowness observed in the spectral features when sucrose was added in the liquid state (Figure 7). In addition to self-association, a narrowing of the amide I band could also be due to the adoption of a more compact protein structure in the presence of sucrose, reducing surface exposure to the solvent.

Essentially no change in amide I band width was observed during freezing in the presence of sucrose (46 ± 1 cm^{-1}). Hence, the protein structure appears not to be altered during freezing in the presence of sucrose. This observation combined with minimal shifting of the amide I during freezing might be explained in terms of preferential exclusion (15–18). Some solutes by virtue of exclusion from the hydration envelope of proteins increase the chemical potential of the native state. However, because there is greater surface area exposed to the solvent for the denatured state in comparison to the native state, the extent of preferential exclusion is greater for the denatured state. Therefore, the increase in the chemical potential of the denatured state is even greater than that for the native state, making the compact "folded" protein more thermodynamically stable (19). Similarly, self-associated states of proteins are favored because there is a reduction in surface area exposed to the solvent in comparison to that for monomers. The stabilizing influences of sugars have been reported to operate in this way (19) and therefore, have been used as cryoprotectants (20). It has been hypothesized that solute exclusion is responsible for the continued stabilizing influence of cryoprotectants in the frozen state (16).

In contrast to the freezing behavior with sucrose, the band width of the amide I changed from 56 to 46 cm^{-1} at −12°C in the absence of sucrose (the same band width observed in the liquid state when sucrose was present but with an accompanying shift to lower frequency). This narrowing of the amide I after freezing in the absence of sucrose might again be explained in terms of self-association induced by freeze-concentration of the protein in the amorphous phase. It would seem likely that as water is removed during freezing, the remaining amorphous phase would become more protein enriched making it possible for protein-protein interactions to occur. However, this state is different from that with sucrose since a high degree of change in spectral features is exhibited with the progress of freezing and drying. Beyond −12°C during freezing, the amide I band broadened slightly to 49 cm^{-1} at −70°C prior to drying under vacuum. At the conclusion of drying, the amide I broadened to 52 cm^{-1}, which is probably due to protein unfolding. In contrast, the amide I band width of the sample dried in the presence of sucrose remained unchanged (46 ± 1 cm^{-1}) during the entire course of dehydration.

In addition to the observation that the amide I band width changed little during lyophilization in the presence of sucrose, the band width of the amide II remained approximately 27 ± 1 cm^{-1}. This was in striking contrast to broadening observed during drying in the absence of sucrose, where the band half width was approximately 36 cm^{-1} (7). It has been suggested that broadening of the amide II can arise from the denaturation of C=O ··· H−N peptidic hydrogen bonds (21). If this is the case, the presence of sucrose stabilizes these interactions during drying perhaps by maintaining a native compact structure.

Lyophilized Product Characterization

At the conclusion of the lyophilization experiment, the sample containing sucrose displayed features of a white lyophilized cake showing the presence of cracks arising from mechanical stress encountered in the drying process. In contrast, a product obtained in an earlier study where the Tg′ was exceeded on drying exhibited the physical features of a collapsed cake exhibiting a fractured transparent product (8). Collapse results in the disappearance of the freezing pattern with the passage of the sublimation front (22).

The lyophilized product containing sucrose was subsequently removed from the FTIR and examined by DSC. The thermal behavior of the dried material exhibited a Tg (glass transition characterized by an endothermic baseline shift) of 50°C. Moisture content of the sample was found to be 0.04g water/g dry weight. To further verify that this was a true glass transition, the sample after heating to 65°C was weighed gravimetrically so as to determine the loss of moisture on drying. The resultant moisture level was 0.02g water/g dry weight. A second DSC measurement was made and exhibited a Tg of 61°C. This was expected, since the removal of additional water should increase the glass transition temperature thus confirming that the final dried state of the product was indeed a glass.

Rehydration of the lyophilized cake yielded complete recovery of activity, consistent with results from earlier investigations (7,8). These results demonstrate that the perturbations observed in the amide I band are due to an average protein structure which is not irreversibly denatured.

Sugar-Protein Interactions During Drying

Based on the observation that drying in the absence of sucrose had a dramatic effect on band broadening for both the amide I and amide II (Figure 8A), this implies that the addition of sucrose served to stabilize the native-like structure under conditions of dehydration stress (minimize band broadening effects). A plausible explanation for this observation involves water replacement by sucrose at low hydration levels (23). At a hydration level of 4%h, if one assumes that all the water is uniformly distributed throughout the sample, there would be approximately 31 molecules of water per molecule of lysozyme. This amount of water is insufficient to completely hydrate the charged groups on the protein (13,24). It is manifested by a reduction in the absorbance of the carboxylate band near 1584 cm^{-1} in the infrared. Yet with this residual moisture content, the band is retained in the freeze-dried solid containing sucrose. Figure 8B illustrates the relative change in the carboxylate band area (taken as a ratio to the amide I [A_{aI}]) as a function of hydration (%h). The changes observed in Figure 8B with no sucrose match those determined by Careri *et al.* for lysozyme in D_2O (13), testifying to the validity of the hydration level estimates obtained from equation 1. Namely, at hydration levels below approximately 12%h (vertical dashed line) there is a consistent decrease in the carboxylate band area. In contrast, when studying the behavior in the presence of sucrose (described by the opened diamonds in Figure 8B), the reduction in the carboxylate band below a hydration of 12%h is not observed, suggesting sucrose is hydrogen bonding to the protein in place of desorbed D_2O. There were no spectral changes detected that would indicate at what point in the drying process sugars replaced desorbed D_2O. Rather, it appears that as mole-

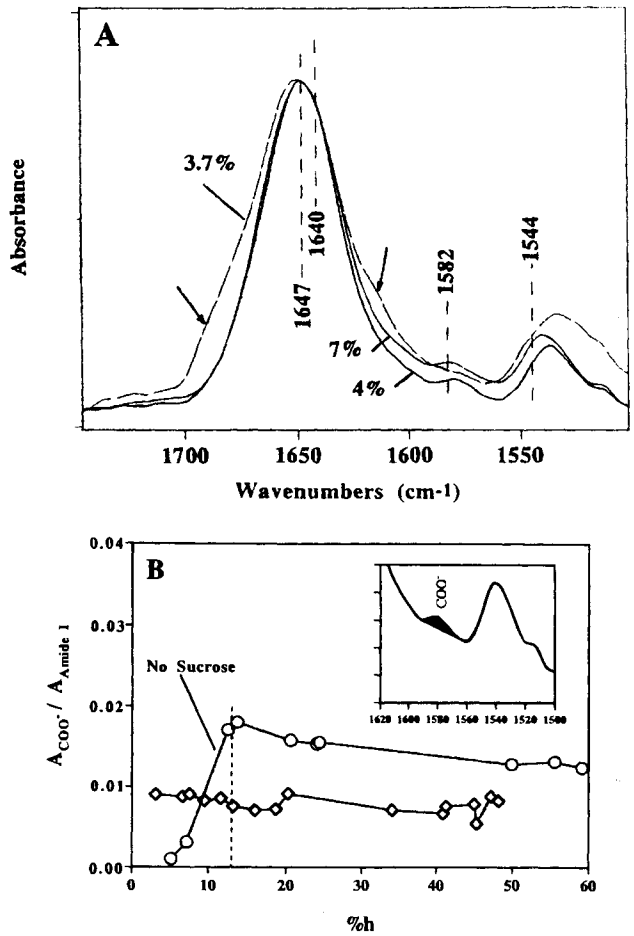

Fig. 8. Infrared data showing (A) spectra of the final lyophilized product depicting broad features in the amide I (described by arrows) and II bands obtained without sucrose (dashed contour) and data in the presence of sucrose (solid contours) at indicated moisture levels. In (B) are the relative changes in the carboxylate band area as a function of hydration level (%h) estimated from equation 1. The inlay shows the manner in which the carboxylate band area was determined (shaded region of the inlay spectrum). The open circles refer to the case without sucrose and the open diamonds represent the case in which sucrose was included. The verticle line represents the approximate hydration level reported by reference 13, where at lower hydration levels a decrease in the carboxylate band was observed.

cules in the protein hydration shell were removed, sucrose could take their place.

If one compares the A_{coo}-/A_{al} ratio prior to freezing (in the liquid state) to that observed during lyophilization, an interesting observation is made. Namely, the liquid solution without sucrose yielded a ratio of approximately 0.03, whereas that with sucrose was approximately 0.01. What one notices during lyophilization for the case without sucrose (Figure 8B) is that from a hydration level of 60% to approximately 12% there was a slight increase in the ratio approaching 0.02. However, in the presence of sucrose the relative intensity of the carboxylate band was essentially the same as that in the liquid state prior to freezing. This result supports the suggestion that the thermodynamic impact of solute exclusion in the liquid state is passed on into the frozen state. The spectroscopic consequence of this interaction is presumed to be present in the initial liquid and frozen states, where little change in protein bands was observed with sucrose acting as an excluded solute. Yet during the process of drying, solute exclusion was no longer operating (at relatively low levels of moisture) and water replacement by sucrose became the means by which the protein was stabilized.

Drawing from the evidence obtained from light scattering, even though an apparent reduction in hydration was observed from the carboxylate ratio in the presence of sucrose, as shown in Figure 7 (ascertained from 0.01 in the presence and 0.03 in the absence of sucrose in the liquid state), we acknowledge that this change may arise as a result of self-association and that the structural properties of the self-associated forms remain virtually unaffected throughout the course of the lyophilization experiment.

CONCLUSIONS

This investigation demonstrates the utility of infrared in monitoring the lyophilization process of protein formulations. Data depicting changes in spectral features due to freeze-concentration and dehydration have been described. The proposed linear regression formula applied to the spectroscopic data adequately accounted for sample moisture levels and augmented infrared monitoring of hydration levels during lyophilization. Moreover, these data provide compelling evidence in support of the water replacement hypothesis. Structural perturbations as described by the amide I and II (i.e., frequency shifts, band widths) have been shown to be minimized in the presence of sucrose (even at low hydration levels). Such behavior of a stabilizing excipient provides insight into the role it plays in maintaining a compact structure throughout the course of the lyophilization process.

The difference in magnitude in the carboxylate ratio from liquid to frozen state (from 0.03 to 0.014) for the case without sucrose suggests that dramatic changes in the overall hydration of the protein occurred during freezing and ultimately during dehydration. Furthermore, a different hydrogen-bonded state is attained in the presence of sucrose, where little change in protein structure was observed in moving from liquid to frozen to dehydrated solid states. Sucrose must satisfy this "hydrogen bonding need" by serving as a replacement for lost water in the dehydrated state.

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