Research Paper

The Effect of Stabilizers and Denaturants on the Cold Denaturation Temperatures of Proteins and Implications for Freeze-Drying

Xiaolin (Charlie) Tang[1,2] and Michael J. Pikal[1,3]

Purpose. The aim of the study is to investigate the effects of stabilizers and denaturants on the thermal and cold denaturation temperatures of selected proteins in systems of interest to freeze-drying.

Methods. β-Lactoglobulin and phosphoglycerate kinase (PGK) were chosen as model proteins. Protein thermal and cold denaturation temperatures were determined by both conventional and modulated differential scanning calorimetry and verified by tryptophan emission spectroscopy in selected systems.

Results. The cold denaturation of β-lactoglobulin was reversible, whereas the thermal denaturation was only reversible at high scanning rate (10°C/min). The cold denaturation temperatures of β-lactoglobulin decreased with an increase in protein concentration (self-stabilization). The cold denaturation temperature increased with increases in pH (from pH 2 to 7) with about 4.6°C increase per unit pH change. All stabilizers studied (i.e., sucrose, trehalose and glycerol) increased the thermal denaturation temperature of the proteins studied and decreased the cold denaturation temperature. The effect of sucrose in decreasing the PGK cold denaturation temperature [40°C per molar concentration increase (40°C/M)] was of the same magnitude as for β-lactoglobulin (36°C/M). The effect of stabilizers on cold denaturation temperatures is much greater than the effect on thermal denaturation temperatures. With sucrose, the β-lactoglobulin thermal denaturation temperature increases only about 5°C from 0 to 2.7 M, whereas the decrease in cold denaturation temperature was more than 35°C even at sucrose concentrations as low as 0.9 M. Denaturants (urea and guanidine hydrochloride) increased the cold denaturation temperatures of proteins and thereby destabilized protein; the magnitudes were 9°C/M (urea on T_{cd} of β-lactoglobulin) and 65°C/M (guanidine hydrochloride on PGK) compared with literature data of 16°C/M (guanidine hydrochloride on β-lactoglobulin). The cold denaturation temperatures of β-lactoglobulin and PGK extrapolated to zero concentration of denaturants were −14 and −26°C, respectively.

Conclusions. The protein cold denaturation temperature was pH-, protein concentration-, and additive-dependent. Stabilizers, such as sugars and/or polyols, can stabilize both protein thermal and cold denaturation, whereas the denaturants destabilize protein cold denaturation. The stabilization effect on protein cold denaturation is much larger than on thermal denaturation, a result of great importance in protein freeze-drying.

KEY WORDS: β-lactoglobulin; cold denaturation; phosphoglycerate kinase (PGK); protein unfolding; stabilizers; thermal denaturation; tryptophan fluorescence emission spectroscopy.

INTRODUCTION

Therapeutic proteins are frequently not sufficiently stable to be used as liquid formulations, and therefore, protein formulations are commonly freeze-dried to improve their storage stability. However, it is common knowledge that proteins are often structurally altered and/or degraded during the process, and even if the protein survives the process without damage, storage stability is not always sufficient, particularly at room temperature. A number of possible

"stresses" may develop during the freeze-drying process that can directly produce degradation during the process and/or decrease storage stability of the dried product. One such stress is the spontaneous unfolding of a protein at low temperature, known as "cold denaturation". Proteins are thermodynamically stable only in a defined temperature interval. Thermal unfolding at high temperature, or thermal denaturation, is well known and frequently studied, but protein cold denaturation, while accepted as a general phenomena (1–4), is much less studied and has been virtually ignored in systematic studies of instability during freezing drying. However, because freeze-drying is conducted at low temperatures (−20 to −50°C), cold denaturation may well play a role in freeze-drying. If cold denaturation does occur, and if spontaneous refolding upon reconstition of the freeze-dried protein is not complete, in-process degradation is the result. Moreover, because denatured or unfolded proteins are

[1] School of Pharmacy, U-2092, University of Connecticut, 372 Fairfield Road, Storrs, Connecticut 06269-2092, USA.

[2] Present address: Formulation Development, Centocor, Inc., 145 King of Prussia Rd., MS R-1-2, Radnor, Pennsylvania 19087, USA.

[3] To whom correspondence should be addressed. (e-mail: pikal@uconnvm.uconn.edu)

normally less stable during storage (5,6), cold denaturation may also impact storage stability.

Of course, cold denaturation is not the only thermodynamic stress operating, and all stresses would contribute to the destabilization of the native conformation. In a frozen system, enormous interfacial area between ice and aqueous protein forms, which may contribute to thermodynamic destabilization and unfolding at the ice surface, and selective crystallization of a buffer species may dramatically shift pH, which might also facilitate unfolding (7–9). Typically, stabilizers, such as sucrose or trehalose, are used in freeze-drying to improve both in-process and storage stability. In such formulations, freeze concentration produces a system of very high stabilizer concentration and high protein concentration, and we might expect the cold denaturation temperature to be impacted. Thus, investigation of the effects of high concentration of stabilizers on the cold denaturation phenomena has potentially important applications to protein stabilization by freeze-drying.

Investigation of protein cold denaturation is often complicated by ice formation above the cold denaturation temperature (1,2). Many proteins were investigated at destabilizing pH values or in the presence of denaturants such that the cold denaturation temperatures are above the freezing point of the protein solutions (10–12). Although many polyol stabilizers, such as sucrose and trehalose, are widely used in protein formulation and freeze-drying, the literature is nearly silent on the effect of stabilizers on cold denaturation. In one study, it was shown that methanol destabilized both the thermal and cold denaturation of lactate dehydrogenase in a linear manner, and it was mentioned that glycerol, a polyol, stabilized thermal and cold denaturation of lactate dehydrogenase (no data were shown for the glycerol systems) (11).

Our goal is to investigate the effects of stabilizers and denaturants on the thermal and cold denaturation temperatures of selected proteins in systems of interest to freeze-drying. One objective is to determine the conditions under which cold denaturation is a significant thermodynamic stress in practical freeze-drying. In a future study, we also intend to study the kinetics of protein unfolding in highly viscous systems using protein cold denaturation as an unfolding model and sugars as viscosity enhancers. However, sugars are protein stabilizers and might dramatically decrease the protein cold denaturation temperatures and complicate analysis of kinetic experiments. To maintain constant cold denaturation temperature in a study, denaturants may have to be added to counteract the stabilization effect of sugars. Therefore, a second objective of the present study is to serve as a necessary prelude to the future kinetics study (13).

The first requirement of a good model protein system is that it undergoes cold denaturation in a mostly reversible fashion. Irreversible processes greatly complicate the thermodynamic analysis of denaturation and would also complicate the kinetic analysis of unfolding we plan as a future study. Literature data suggest that phosphoglycerate kinase (PGK) would serve as a good model protein for cold denaturation studies because cold denaturation is reversible, and the cold denaturation temperature is above the ice formation temperature in the presence of a small amount of guanidine hydrochloride (14,15). Further, a number of

spectroscopic methods have been employed to study both the thermodynamics and kinetics of its unfolding (in dilute aqueous solution) (16). Finally, some thermodynamic data exist from which we can evaluate the effect of denaturants on the cold denaturation temperature to compare with our data (15–18). β-Lactoglobulin is another well-studied model protein that undergoes reversible cold denaturation, although the data availability is a bit more limited than with PGK (10,19). In this research, different polyols such as sucrose and trehalose (disaccharides), which are extensively used as stabilizers for freeze-drying and glycerol, will be investigated as stabilizers. Different denaturants (guanidine hydrochloride and urea) will be investigated for their ability to increase the cold denaturation temperature.

MATERIALS AND METHODS

PGK precipitated and suspended in 1.4 M sodium sulfate (Sigma, St. Louis, MO) was dialyzed (overnight at 5°C with gentle stirring) in 2 mM sodium phosphate buffer at pH 6.5 twice to remove the excipients from the suspension, followed by freeze-drying below the collapse temperature of about −10°C. The vials used for freeze-drying are 5-ml serum tubing vials with 20-mm finish from Fisher, and stoppers are Daikyo (20 mm) from West Pharmaceutical Services, Lionville, PA.

β-Lactoglobulin (freeze-dried powder), sucrose, trehalose, and glycerol were purchased from Sigma and used without further purification. All the reagents are of analytical grade.

Buffer Solutions

Sodium phosphate buffers (20 mM) were used for both β-lactoglobulin and PGK. The pHs of the formulations ranged from 2 to 7 for β-lactoglobulin and was 6.5 for PGK. In PGK formulations, 1 mM of dithiothreitol (from SigmaUltra, St. Louis, MO) was added as an antioxidant (14,18).

Protein Thermal and Cold Denaturation Temperature by Modulated Differential Scanning Calorimetry

The protein cold denaturation temperature was evaluated using modulated differential scanning calorimetry (MDSC model 2920, TA Instruments, Newcastle, DE) with relatively high concentrations (>1% w/v) of protein. The DSC was calibrated using standard indium (TA Instruments) and deionized water (electric resistance >18 MΩ) at the same heating rate as used in the protein experiments. The samples (18 μl) were sealed in 20-μl hermetic aluminum pans (TA Instruments pans). Buffer solution was used in the reference pan. For modulated DSC experiments, the typical scan rate (heating or cooling) was 1°C/min, with modulation amplitude of ±0.5°C and a period of 80 s. The scan rate was 10°C/min for conventional DSC experiments. The cold and thermal denaturation events (peak and onset temperatures) and the heat capacity change upon denaturation were analyzed by Universal Analysis 95 (TA Instruments). The error bars in figures are standard deviations of three replicates.

Protein Cold Denaturation Detected by Tryptophan Fluorescence Emission Spectroscopy

The protein cold and thermal denaturation temperatures were also studied by temperature-controlled fluorescence spectroscopy (LS 50, Perkin Elmer, Norwalk, CT). The excitation wavelength was 295 nm, and fluorescence spectra were collected between 300 and 400 nm. The scan rate of the spectrum collection was 100 nm/min. About 150-μl protein solution was filled into the quartz microfluorescence cell (capacity 200 μl). The concentration of protein was in the range from 0.3 to 5%.

Linear Curve Fitting

The least-squares fitting method was used for the linear curve fitting. R^2 was presented as an indicator of the goodness-of-fit statistics. Note that in several cases, only three data points were available, so it is obvious that in these cases, insufficient data exist for a rigorous test of linearity. Nevertheless, while we are often unable to conclude that exact linearity exists, the trends in the data are obvious regardless of this ambiguity.

RESULTS AND DISCUSSION

DSC Studies of Protein Thermal and Cold Denaturation

For both β-lactoglobulin and PGK, the heat flow data showed clear transitions at both high and low temperatures. β-Lactoglobulin (5% in 4 M urea at pH 2) was cooled from room temperature (25°C) to −12°C to show cold denaturation and then heated from −12°C to room temperature to show protein renaturation or refolding (Fig. 1). The process of cooling and heating was repeated three times to determine if the protein cold denaturation process is reversible. Finally, the sample was heated past the thermal denaturation temperature to 80°C and then cooled back to −12°C to determine if the thermal denaturation was reversible. The DSC thermogram is presented in Fig. 1a. The DSC results showed repeatable peaks at 0°C (cold denaturation during cooling) and 18°C (refolding). The peaks were endothermic in heating and exothermic in cooling. Similar peaks were observed in previous works (20). Figure 1 shows that the cold denaturation (and renaturation) of β-lactoglobulin is reversible. The temperature difference of about 18°C between peak position of unfolding and refolding (at the scan rate of 1°C/min) is likely a result of unfolding and refolding kinetic effects. That is, the unfolding kinetics and scan rate are on roughly the same time scale, so the time required for conformational change drives the observed cold denaturation temperature down and the observed renaturation temperature up. One would expect the true thermodynamic transition to be roughly the average of the observed cold denaturation and renaturation temperatures. In support of this interpretation, note that the cold denaturation temperature (peak position) of β-lactoglobulin changed from 0 to −10°C when the scan rate increased from 1 to 10°C/min, although the onset temperature changed about only 2°C (Fig. 1b). However, when the same

sample was heated at 1°C/min to above the thermal denaturation temperature (70°C) (curve A) and then cooled (curve B), the cold denaturation peak did not appear (Fig. 1a), which means that thermal denaturation irreversibly damaged the protein conformation. That is, thermal denaturation is not reversible at a scan rate of 1°C/min. Similar data were obtained by conventional DSC using a scan rate of 10°C/min (Fig. 1b). However, in contrast to the modulated DSC data obtained at 1°C/min, the cold denaturation peak (and even the thermal renaturation peak) did reappear after the sample was heated past the thermal denaturation temperature and then cooled, which means that thermal denaturation did not completely and irreversibly damage the protein conformation at the high heating and cooling rates used in this experiment (10°C/min). This observation suggests that the nonreversible "thermal denaturation" includes an irreversible kinetic event (such as protein aggregation), which needs sufficient time to complete. Thus, the nonreversible "thermal denaturation" is likely a rapid reversible unfolding step, followed by slower irreversible aggregation.

The reversibility of protein cold denaturation (1,2) is important because many "bulk drug" proteins are stored at very low temperatures (−70 or −80°C) in frozen solutions, where the storage temperature is likely below the protein cold denaturation temperature, which means the proteins are potentially cold-denatured during storage. However, if cold denaturation is reversible, the proteins will refold when thawed, although the material would remain denatured during frozen storage.

To illustrate the denaturation of PGK, the sample was held at −10°C for 1 h and then heated at 1°C/min past the thermal denaturation temperature. A typical DSC thermogram is shown in Fig. 1c. There were three endothermic peaks at 3, 10, and 40°C. The peaks below room temperature were PGK refolding or "renaturation" transitions corresponding to the two structure domains, and the peak at highest temperature was the thermal denaturation peak or the "melting peak" of PGK. These results are consistent with previous reports on PGK (2,14).

The reversing heat flow from MDSC measures the heat capacity of samples. Heat capacity changes corresponding to refolding and denaturation were observed directly from DSC results for both proteins (refer to the ΔC_p in Fig. 1a and c). As expected, the heat capacity increased during protein denaturation and decreased during protein refolding. Although the heat capacity changes are not large (about 3–4 kJ/mol protein/K, a typical value for protein denaturation) (2), the shifts are experimentally significant. The direct determination of protein heat capacity change during cold denaturation has not previously been reported.

Protein cold denaturation was studied at different scan rates to investigate the impact of kinetics on the cold denaturation temperature. It is expected that if the protein unfolding or refolding kinetics cannot follow the DSC scan rate, the DSC results will not exactly represent the thermodynamic transitions, and the value of the cold denaturation temperature will depend on scan rate. The DSC experiment was performed for 5% β-lactoglobulin (in 20 mM phosphate buffer, pH 2.0, with 4 M urea) at 10°C/min, and the results were compared with those at 1°C/min scan rate. The onset of cold denaturation temperature did change but only slightly (change from 1.2°C at a scan rate of 1°C/min to −2°C at a scan rate of 10°C/min).
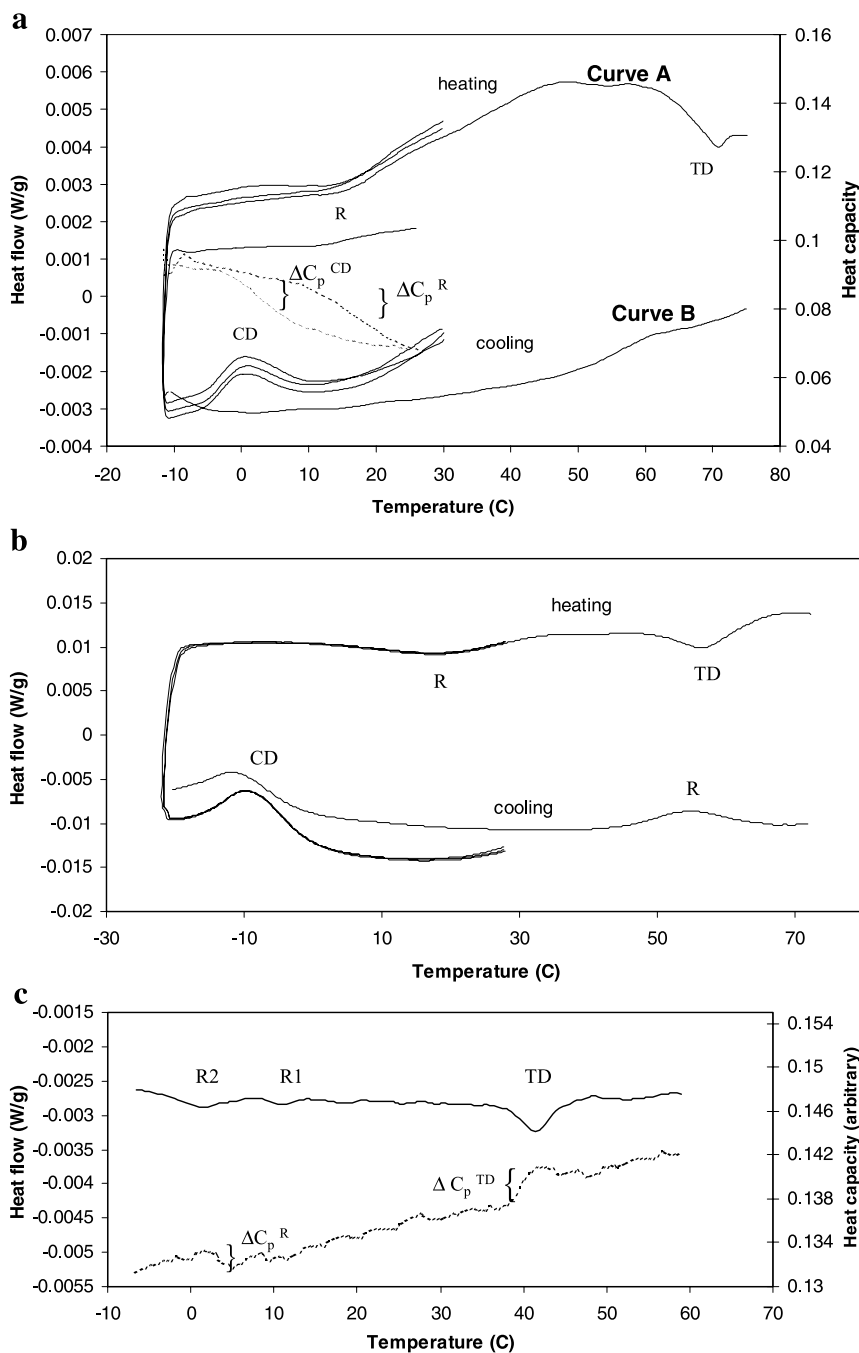
Fig. 1. Protein denaturation and renaturation detected by differential scanning calorimetry (DSC). CD = cold denaturation; TD = thermal denaturation; R = refolding. (a) The thermogram (scanning rate of 1°C/min) of 5% β-lactoglobulin (in 20 mM phosphate buffer, pH 2.0, with 4 M urea) showed reversible cold denaturation/renaturation and nonreversible thermal denaturation. The dashed lines represent heat capacity change during cold denaturation and renaturation. (b) The conventional DSC thermogram (scanning rate of 10°C/min) of 5% β-lactoglobulin (in 20 mM phosphate buffer, pH 2.0, with 4 M urea) showed both reversible cold denaturation/renaturation and reversible thermal denaturation. (c) The modulated DSC thermogram (1°C/min) of 5% PGK (in 20 mM phosphate buffer, pH 6.5, with 0.7 M guanidine hydrochloride) showed renaturation (R2 at 3°C and R1 at 11°C) and thermal denaturation peaks (TD at 41°C). The solid line represents total heat flow. The dashed line represents the heat capacity. A rough measure of the heat capacity changes at R2 and TD is indicated by the brackets.

Therefore, the protein cold denaturation temperature determined by DSC is partially determined by kinetics, but the impact of kinetics appears to be moderate.

Tryptophan Fluorescence Emission Spectroscopy Studies of Protein Thermal and Cold Denaturation

Tryptophan fluorescence emission spectroscopy was also used to detect protein denaturation at both high and low temperatures. Fluorescence emission spectroscopy data were collected for 5% β-lactoglobulin in 20 mM phosphate buffer (pH 7.0) and 3 M urea at both room temperature (25°C native conformation) and −10°C (cold-denatured) (Fig. 2). The fluorescence emission peak position was around 340 nm at 25°C, representing the "native" structure, where the tryptophan residue is buried inside the compact native protein structure (15). As expected, the peak position shifted to higher wavelength (around 350 nm and increased in intensity) for the cold-denatured state at low temperature (−10°C).

A temperature scan fluorescence experiment was performed for the same protein in 10% (∼0.3 M) sucrose and phosphate buffer (20 mM, pH 7.0, and 4 M urea). In this experiment, the sample temperature was changed from room temperature (25°C) to 0°C and then heated up from 0 to 75°C. The spectra were collected at different temperatures (data not shown). The peak positions shifted to higher wavelength when the sample temperature was cooled from room temperature to 0°C, indicating cold denaturation, and shifted back when the sample was heated from 0°C to room temperature, indicating refolding. Further heating of the sample from room temperature to high temperature shifted the peak to high wavelength again, indicating thermal denaturation. The cold and the thermal denaturation temperatures determined by tryptophan fluorescence emission spectroscopy were around 15 and 60°C, respectively. These results are fully consistent with the DSC results.

β-Lactoglobulin has two tryptophan residues in the molecule (W19 and W61), with W19 being buried inside the molecule and the other (W61) being close to the protein surface in the native structure. Typically, tryptophan emission fluorescence shows a peak at about 330 nm when it is inside the protein in a hydrophobic environment and at about 350 nm when it is exposed to the aqueous solution (21,22). Consistent with these expectations, our results show a broad peak at 340 nm at room temperature, which shifted to 353 nm at −10°C. The tryptophan emission fluorescence peak of β-lactoglobulin is a combination of both tryptophan residuals. At room temperature, β-lactoglobulin is in native conformation (23), and the fluorescence peak position (340 nm) should include contributions from both the inside residue W19 (330 nm) and the edge residual W61 (between 330 and 350 nm). The peak position at −10°C (350 nm) indicates that both tryptophan residuals were exposed to the buffer solution, which means the protein was unfolded. Therefore, the fluorescence spectroscopy results are fully consistent with our interpretation of the events at low temperature (−10°C) being cold denaturation/renaturation and the events at high temperature (>50°C) presenting thermal denaturation.

The Effect of Protein Concentration and pH on Protein Cold Denaturation Temperatures

Cold denaturation temperatures of β-lactoglobulin at different concentrations (from 1 to 10% at pH 2 in 4 M urea) were investigated by MDSC. The onset temperatures of protein cold denaturation (T_{cd}) are plotted against the protein concentrations in Fig. 3. The T_{cd} of β-lactoglobulin decreases significantly as protein concentration increases. Because the lower the cold denaturation temperature of the protein the more stable the protein, β-lactoglobulin is thermodynamically more stable at high concentration. While it has been reported that β-lactoglobulin forms dimers or even trimers at neutral pH, this protein exists only as monomers at low pHs (pH < 2.5) (24). Thus, stabilization by forming multimers does not appear to be the origin of the effect shown in Fig. 3. It is well known that many proteins are
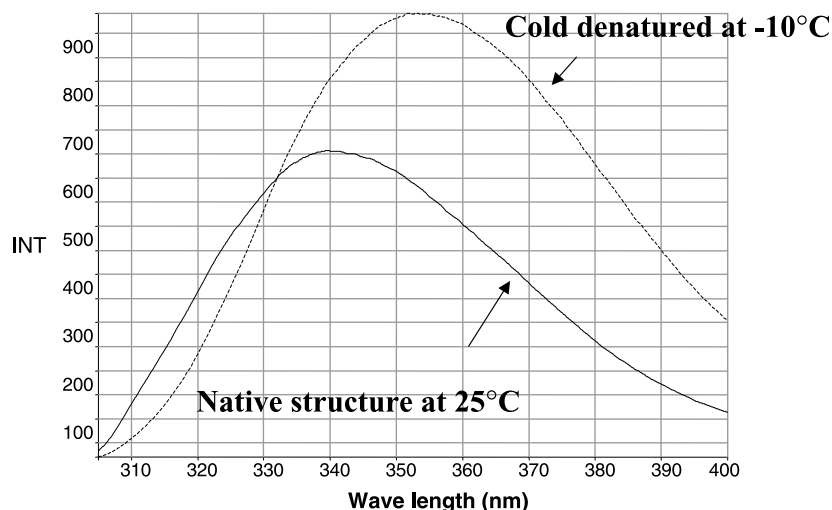

Fig. 2. Protein cold denaturation detected by tryptophan fluorescence emission spectroscopy. β-Lactoglobulin in 20 mM phosphate buffer, pH 7.0, with 3 M urea. The peak position was 340 nm at room temperature (native structure), which shifted to 352 nm at −10°C (cold denatured).
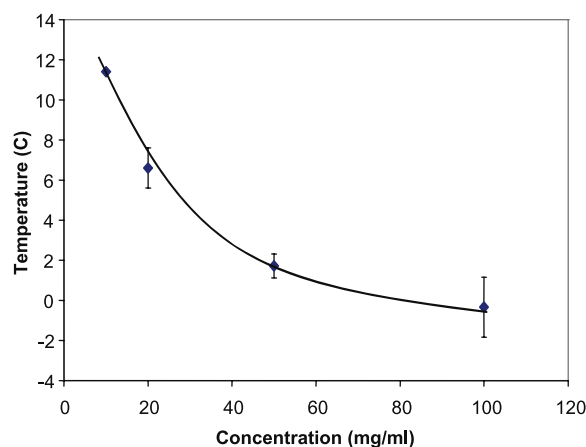
Fig. 3. The effect of protein concentration on β-lactoglobulin cold denaturation temperatures as determined by DSC. β-Lactoglobulin was in 20 mM phosphate buffer, pH 2.0, with 4 M urea. Error bars are not given for the 10 mg/mL system because here, there was no replication.

range. The results imply some kind of saturation effect because the self-stabilization effect levels out as protein concentration increases.

The effect of pH on cold denaturation temperature of β-lactoglobulin (from pH 2.0 to 7.0) was also determined by MDSC (Fig. 4). The protein formulations were the same as before, i.e., 5% protein in 20 mM phosphate buffer (pH from 2 to 7) with 4 M urea. The T_{cd} showed a linear correlation with the pH values by the least-squares fitting method (based on three data points, $R^2 = 1.00$), with higher denaturation temperatures (T_{cd}) at higher pH, as shown in Eq. (1).

$$T_{cd} = 4.6\,pH - 8.16 \qquad (1)$$

Thus, β-lactoglobulin is more stable at low pH. While proteins are generally unstable at very low and very high pH, this protein is very stable at very low pH (pH 2) and less stable at neutral pH (pH 7).

The effect of pH on protein cold denaturation has implications for freeze-drying. It is not unusual for protein formulations to suffer pH changes during freezing, particularly when a buffer component crystallizes (29). Dramatic pH shifts were reported when high concentrations of sodium phosphate were used (7). Thus, the pH shift during freezing could induce protein cold denaturation during freeze-drying if the pH shift significantly increases the protein cold denaturation temperature.

After ice formation, the protein is in a freeze concentrate, which may have high concentration of solutes, including high protein concentration, which means the protein self-stabilization effect could play a major role in the freeze-drying of pure protein. However, note that the self-stabilization seems to approach a "saturation" limit when the concentration is higher than about 5% (Fig. 3).

stabilized by increasing the protein concentration (25,26). The mechanisms are assumed to be either formation of more stable multimers or minimization of surface effects in multiphase systems. However, our system is a single phase, and, as indicated above, multimers apparently do not form below pH 2.5. It appears that β-lactoglobulin self-stabilizes by volume exclusion effect or the macromolecular-crowding effect arising from nonspecific steric repulsion between molecules (27,28). The macromolecular-crowding effect simply favors and shifts the equilibrium toward formation of compact product, i.e., from unfolding state to native conformation. We observed that the self-stabilization effect is more significant at low concentration. Moreover, the self-stabilization effect was undetectable whenever the stabilizer (sucrose) was present because the sucrose-excluded volume effect dominated. The tryptophan emission spectroscopy did not change in the protein concentration range from 0.1 to 5% at room temperature (data not shown), suggesting no significant change in protein tertiary structure over this concentration
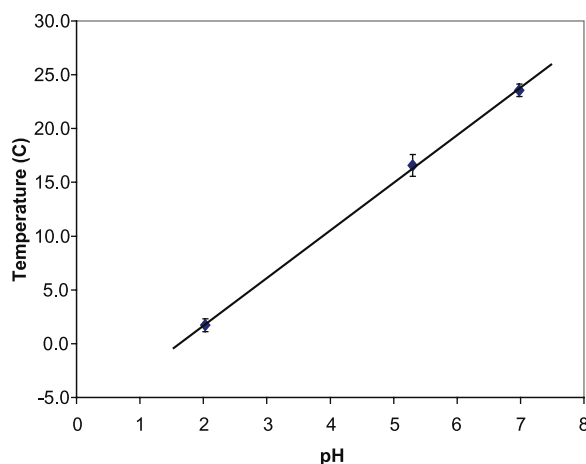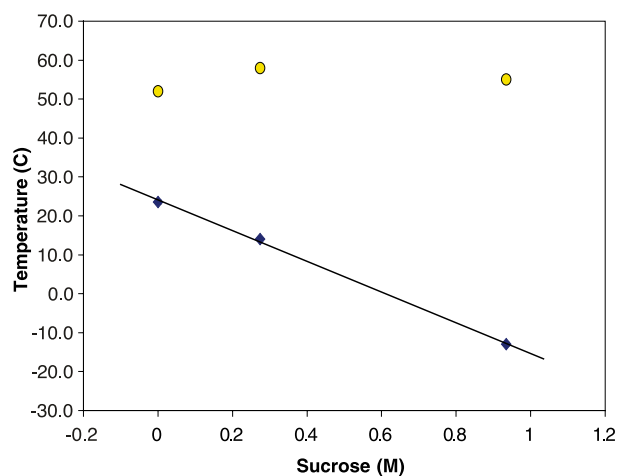

Fig. 4. The effect of pH on β-lactoglobulin cold denaturation temperatures as determined by DSC. β-Lactoglobulin (5%) was in 20 mM phosphate buffer with 4 M urea.

Fig. 5. The effect of stabilizers on protein cold and thermal denaturation temperatures. β-Lactoglobulin (in 20 mM phosphate buffer, pH 7.0, with 4 M urea) cold denaturation temperature as a function of sucrose concentration. The slope of the linear correlation between T_{cd} and sucrose concentrations was −40°C/M. Diamonds represent cold denaturation temperature, and circles represent thermal denaturation temperature.

The Effects of Stabilizers and Denaturants on Cold Denaturation and Thermal Denaturation

Thermal denaturation temperatures were determined by MDSC for β-lactoglobulin (20 mM phosphate buffer, pH 7.0) with different concentrations of sucrose (from 0 to 2.6 M). As expected (30,31), sucrose increased the thermal denaturation temperature of β-lactoglobulin. The stabilization effect plateaus at high concentrations (after 1.0 M), with the maximum effect being only about 5°C (from 60 to 65°C). The thermal denaturation temperatures were also determined for the same system with 4 M urea present (Fig. 5). The results were similar to the previous system without urea, i.e., the increase in thermal denaturation temperature of β-lactoglobulin caused by sucrose is very limited (about 5°C). While the thermal denaturation temperatures were depressed about 10°C by denaturant (4 M urea), the effect of sucrose on the thermal denaturation temperature was of the same magnitude with or without denaturant.

Cold denaturation temperatures (T_{cd}) for β-lactoglobulin (in 20 mM phosphate buffer at pH 7.0 with 4 M urea) in different concentrations of sucrose were determined by MDSC. The T_{cd} decreased linearly and sharply with increasing sucrose concentration with a slope of −40°C/M (based on three data points, $R^2 = 1.00$), demonstrating the enormous stabilization effect of sucrose (Fig. 5). It should be noted that the cold denaturation temperature of β-lactoglobulin extrapolates to about −100°C in a system of 3.3 M sucrose, a concentration roughly equal to that in a maximally freeze-concentrated sucrose system. The stabilization effect of sucrose is pH-dependent. While a linear correlation was also observed between sucrose concentration and T_{cd} at pH 2, the stabilization effect of sucrose at pH 2 is much less than that at pH 7. The slope is only −23°C/M at pH 2 (i.e., vs. −40°C/M at pH 7).

The effects of sucrose on thermal and cold denaturation in β-lactoglobulin systems are significantly different in magnitude. If the effect of sucrose on free energy of the native structure (state) is similar for both cold and thermal denaturation, the different stabilization results must be caused by the different effects of sucrose on cold and thermal unfolded states, which likely means the unfolded states from cold denaturation and thermal denaturation are different. The difference in nature of the unfolded states may also be a factor in the irreversibility of thermal denaturation, as contrasted with the reversibility of cold denaturation. The stabilization effect of stabilizer (sucrose) is less effective when the protein is in the more stable state (at pH 2).

The effects of different stabilizers (sucrose, trehalose, and glycerol) on protein cold denaturation (5% β-lactoglobulin in 20 mM phosphate buffer, pH 7.0, 4 M urea) were studied by MDSC. We find that all stabilizers decrease the cold denaturation temperature of β-lactoglobulin, thus stabilizing the protein. The effects of sucrose and trehalose were similar and were greater than that of glycerol on a molar basis. All data seemed to form a universal curve when plotted against the molar concentration of hydroxyl groups (Fig. 6). This result strongly suggests that the sugar stabilization effect involves hydroxyl groups, perhaps involving hydrogen-bonding with water. This "universal stabilization effect" of polyols on protein cold denaturation has not previously been reported.

The effect of stabilizer (sucrose) on the thermal and cold denaturation temperature of PGK was also investigated by MDSC. Protein samples in 20 mM phosphate buffer (pH 6.5) with 0.7 M guanidine hydrochloride and different concentrations of sucrose were scanned by MDSC. As expected, sucrose increased the thermal denaturation temperature and decreased the cold denaturation temperature of PGK (Fig. 7). The effect of sucrose on PGK cold denaturation was much greater than the effect on thermal denaturation, a result essentially the same as that found for thermal and cold denaturation of β-lactoglobulin. The T_{cd} vs. sucrose concentration plot shows a linear correlation with a slope of about −36°C/M, which is almost exactly the same value as found for β-lactoglobulin at pH 7. This result suggests that the stabilization effects of sugars on cold denaturation of the two proteins investigated are relatively nonspecific.

Fig. 7. The effect of sucrose concentration on the cold and thermal denaturation temperature of PGK (in 20 mM phosphate buffer, pH 6.5, with 0.7 M guanidine hydrochloride). The linear correlation between the cold denaturation temperature (T_{cd}) and sucrose concentration (C_s) is $T_{cd} = -36.1C_s + 18.9$ ($R^2 = 0.99$).
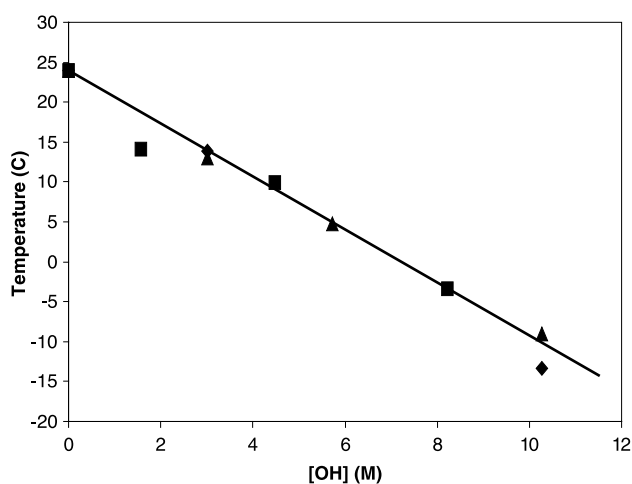

Fig. 6. The cold denaturation temperature of β-lactoglobulin (in 20 mM phosphate buffer, pH 7.0, with 4 M urea) vs. hydroxyl group concentration for different stabilizers. Diamonds represent sucrose, squares represent glycerin, and triangles represent trehalose. The molar concentration of hydroxyl groups was calculated by multiplying the molar concentration of the sugar by the number of hydroxyl groups in the sugar molecule.
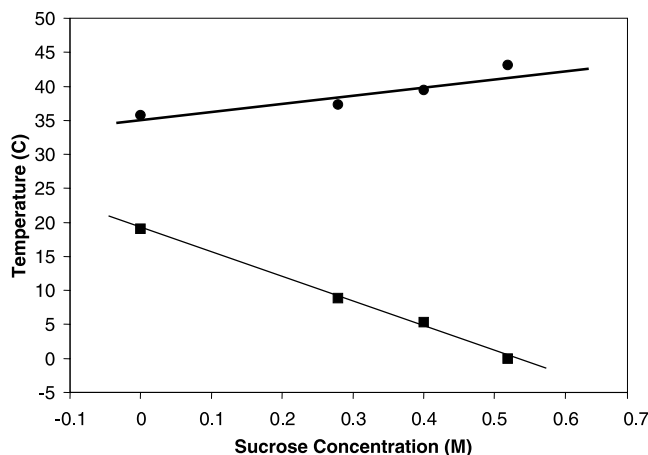
The stabilization effects of polyols are very important for freeze-drying of protein formulations. Sucrose and trehalose are the most commonly used stabilizers, and their empirical effects on protein stability are well known. However, there is little literature regarding their effect on protein cold denaturation. Our results showed that polyols are very efficient stabilizers for protein cold denaturation. The slopes of the linear correlation between T_{cd} and stabilizer concentration are high for both proteins and nearly the same magnitude [40°C/M for β-lactoglobulin and 36°C/M ($R^2 = 0.99$) for PGK], implying that the stabilization mechanisms of polyols are the same for both protein systems. Because freeze-drying is usually conducted at very low temperature, it is obvious that protein cold denaturation could cause stability problems. However, because of freeze concentration during the last stages of freezing when the temperatures are low, the polyol concentration is high, which means the stabilization effect of the polyols (i.e., depression of cold denaturation temperature) will be very great. Thus, depression of cold denaturation temperature should play an important role in protein stabilization during freeze-drying.
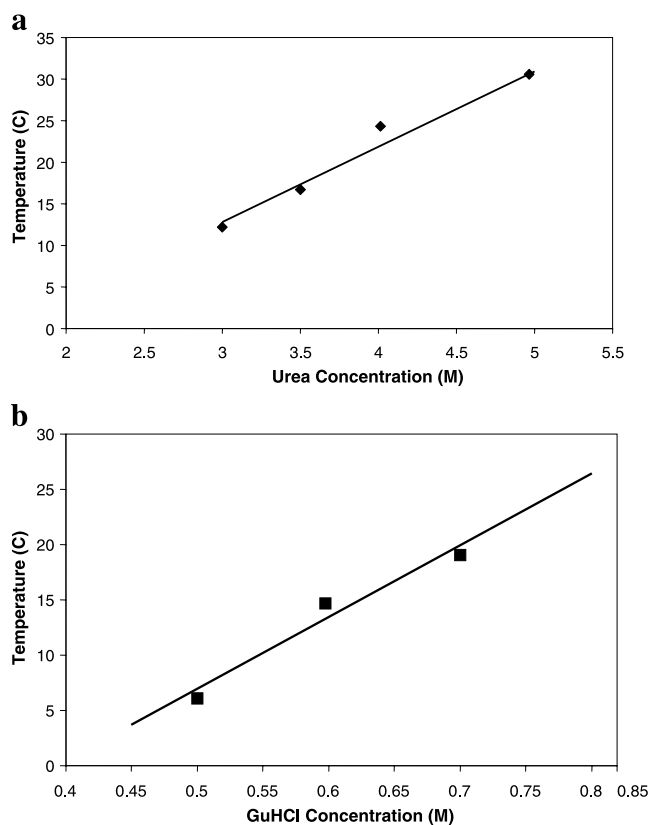
Most importantly, we find that the stabilizers are far more effective in stabilization toward cold denaturation than in protein thermal denaturation. Obviously, because cold denaturation is of greater relevance to freeze-drying than thermal denaturation, stabilization-screening studies need to be focused on cold denaturation rather than thermal denaturation when freeze-drying applications are intended. Finally, as noted earlier, even if the protein is thermo-dynamically destabilized during the process, for structural damage to result, the rate of unfolding needs to be fast enough so that significant structural change may result on the time frame of the process. This is the topic of the next paper in this series.

Denaturants, such as urea and guanidine hydrochloride, are well known for their effect on protein thermal denaturation. In this investigation, we studied the effect of denaturants on protein cold denaturation using DSC. The cold denaturation temperature of 5% β-lactoglobulin in 20 mM phosphate buffer (pH 7.0) with different concentrations of urea from 3 to 5 M was determined by MDSC. The thermograms were analyzed, and the cold denaturation temperatures for β-lactoglobulin were plotted against urea concentrations in Fig. 8a. The T_{cd} of β-lactoglobulin showed a good linear correlation with urea concentrations. The T_{cd} of β-lactoglobulin in pure buffer solution at pH 7.0 was obtained by extrapolating T_{cd} to zero urea concentration. The extrapolated cold denaturation temperature (T_{cd}) was −14°C, which is far under the freezing temperature of the solution (about −1°C) but still in the temperature range relevant to freezing and freeze-drying.

The effect of denaturant on the cold denaturation temperature of PGK is illustrated in Fig. 8b. Extrapolation of the linear regression line (based on three data points, $R^2 = 0.95$) yielded a cold denaturation temperature of PGK (−25.6°C) in 20 mM phosphate buffer at pH 6.5. Thus, the T_{cd} of PGK is even lower than that of β-lactoglobulin but still in a range relevant to stability during freezing.

The slope of the linear correlation of β-lactoglobulin T_{cd} with urea concentration was about 9°C/M ($R^2 = 0.97$), and the slope of PGK T_{cd} vs. guanidine hydrochloride concentrations was about 65°C/M. Literature data describing the effect of guanidine hydrochloride on the T_{cd} of β-lactoglobulin yield a slope value of 16°C/M (32). Thus, the effects of denaturants on protein cold denaturation temperatures seem to be quite specific to the denaturant and to the proteins, supporting the concept of preferential denaturant binding to the unfolded state vs. binding to the folded state.

Fig. 8. The effect of denaturants on protein cold denaturation temperature. (a) The effect of urea on cold denaturation temperature of β-lactoglobulin (in 20 mM phosphate buffer, pH 7.0). The linear correlation between the cold denaturation temperature (T_{cd}) and urea concentration (C_u) is $T_{cd} = 9.1C_u − 14.4$ ($R^2 = 0.97$). Thus, the cold denaturation temperature of the "pure" protein is −14.4°C. (b) The effect of guanidine hydrochloride on the cold denaturation temperature of PGK (in 20 mM phosphate buffer, pH 6.5). The linear correlation between the cold denaturation temperature (T_{cd}) and urea concentration (C_u) is $T_{cd} = 65.0C_u − 25.6$ ($R^2 = 0.95$). The cold denaturation temperature of the "pure" protein is −25.6°C.

CONCLUSIONS

Protein cold and thermal denaturation can be detected by MDSC, and MDSC was able to directly detect the specific heat capacity change during protein cold denaturation. The protein cold denaturations are typically reversible, whereas the thermal denaturations are nonreversible if the scan rate is slow (1°C/min), but partially reversible at high scan rate (10°C/min). Most importantly, stabilizers such as polyols can stabilize both protein thermal and cold denaturations, but the stabilization effect on protein cold denaturation is much larger than the corresponding effect on thermal denaturation,

which has important consequences for freeze-drying of proteins. Denaturants (urea and guanidine hydrochloride) significantly increase protein T_{cd}, and the effects are protein- and denaturant-specific.

ACKNOWLEDGMENTS

REFERENCES

1. F. Franks, R. H. M. Hatley, and H. L. Friedman. The thermodynamics of protein stability. Cold destabilization as a general phenomenon. *Biophys. Chemist.* **31**:307–315 (1988).
2. P. L. Privalov. Cold denaturation of proteins. *Crit. Rev. Biochem. Mol. Biol.* **25**:281–305 (1990).
3. M. I. Marques, J. M. Borreguero, H. E. Stanley, and N. V. Dokholyan. Possible mechanism for cold denaturation of proteins at high pressure. *Phys. Rev. Lett.* **91**:138103–138104 (2003).
4. J. L. Neira and J. Gomez. The conformational stability of the *Streptomyces coelicolor* histidine-phosphocarrier protein. Characterization of cold denaturation and urea–protein interactions. *Eur. J. Biochem.* **271**:2165–2181 (2004).
5. J. F. Carpenter, L. Kreilgaard, S. D. Allison, and T. W. Randolph. Roles of protein conformation and glassy state in the storage stability of dried protein formulations. *Pharm. Formul. Dev. Pept. Proteins* 178–188.
6. M. J. Pikal. Book of Abstracts, 211th ACS National Meeting, New Orleans, LA, March 24–28, 1996, p. BIOT-135.
7. B. A. Szkudlarek, T. J. Anchordoquy, G. A. Garcia, M. J. Pikal, J. F. Carpenter, and N. Rodriguez-Hornedo. Book of Abstracts, 211th ACS National Meeting, New Orleans, LA, March 24–28, 1996, p. BIOT-138.
8. E. Shalaev, T. Johnson-Elton, L. Change, and M. J. Pikal. Thermophysical properties of pharmaceutically compatible buffers at sub-zero temperatures: implications for freeze drying. *Pharm. Res.* **19**:195–211 (2002).
9. M. J. Pikal. "Lyophilization". In *Encyclopedia of Pharmaceutical Technology*, Marcel Dekker Inc., New York, 2001.
10. Y. V. Griko and P. L. Privalov. Calorimetric study of the heat and cold denaturation of .beta.-lactoglobulin. *Biochemistry* **31**:8810–8815 (1992).
11. F. Franks and R. H. M. Hatley. Stability of proteins at subzero temperatures: thermodynamics and some ecological consequences. *Pure Appl. Chem.* **63**:1367–1380 (1991).
12. N. Poklar, G. Vesnaver, and S. Lapanje. Comparison of the results of thermal denaturation of .beta.-lactoglobulin obtained by DSC and UV-spectroscopy. *Calorim. Anal. Therm.* **24**:363–366 (1993).
13. X. C. Tang and M. J. Pikal. Measurement of the kinetics of protein unfolding in viscous systems and implications for protein stability in freeze drying. *Pharm. Res.* **22**:1187–1196 (2005).
14. Y. V. Griko, S. Y. Venyaminov, and P. L. Privalov. Heat and cold denaturation of phosphoglycerate kinase (interaction of domains). *FEBS Lett.* **244**:276–278 (1989).
15. G. Damaschun, H. Damaschun, K. Gast, R. Misselwitz, J. J. Mueller, W. Pfeil, and D. Zirwer. Cold denaturation-induced conformational changes in phosphoglycerate kinase from yeast. *Biochemistry* **32**:7739–7746 (1993).
16. L. S. Taylor, P. York, A. C. Williams, H. G. M. Edwards, V. Mehta, G. S. Jackson, I. G. Badcoe, and A. R. Clarke. Sucrose reduces the efficiency of protein denaturation by a chaotropic agent. *Biochim. Biophys. Acta* **1253**:39–46 (1995).
17. G. V. Semisotnov, M. Vas, V. V. Chemeris, N. Y. Kashparova, N. V. Kotova, O. I. Razgulyaev, and M. A. Sinev. Refolding kinetics of pig muscle and yeast 3-phosphoglycerate kinases and of their proteolytic fragments. *Eur. J. Biochem.* **202**:1083–1089 (1991).
18. K. Gast, G. Damaschun, H. Damaschun, R. Misselwitz, and D. Zirwer. Cold denaturation of yeast phosphoglycerate kinase: kinetics of changes in secondary structure and compactness on unfolding and refolding. *Biochemistry* **32**:7747–7752 (1993).
19. B. Wang and F. Tan. DSC study of cold and heat denaturation of .beta.-lactoglobulin A with urea. *Chin. Sci. Bull.* **42**:123–127 (1997).
20. B. Wang and F. Tan. DSC study of denaturation of .beta.-lactoglobulin B. *Sci. China, Ser. B* **38**:138–144 (1995).
21. C. Bhattacharjee and K. P. Das. Thermal unfolding and refolding of beta-lactoglobulin. An intrinsic andextrinsic fluorescence study. *Eur. J. Biochem.* **267**:3957–3960 (2000).
22. B. Nolting. Temperature-jump induced fast refolding of cold-unfolded protein. *Biochem. Biophys. Res. Commun.* **227**:903–908 (1996).
23. Y. V. Griko and P. L. Privalov. Calorimetric study of .beta.-lactoglobulin heat denaturation in urea and phosphate containing solutions. *Mol. Biol. (Mosc.)* **26**:150–157 (1992).
24. R. K. Owusu Apenten and D. Galani. Thermodynamic parameters for beta-lactoglobulin dissociation over a broad temperature range at pH 2.6 and 7.0. *Thermochim. Acta* **359**:181–188 (2000).
25. N. B. Bam, J. L. Cleland, J. Yang, M. C. Manning, J. F. Carpenter, R. F. Kelley, and T. W. Randolph. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. *J. Pharm. Sci.* **87**:1554–1559 (1998).
26. L. S. Jones, N. B. Bam, and T. W. Randolph. Surfactant-stabilized protein formulations—A review of protein–surfactants interactions and novel analytical methodologies. *ACS Symp. Ser.* **675**:206–222 (1997).
27. A. P. Minton. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J. Biol. Chem.* **279**:10577–10580 (2001).
28. R. J. Ellis. Macromolecular crowding: obvious but underappreciated. *Trends Biochem. Sci.* **26**:597–604 (2001).
29. K. A. Pikal-Cleland, N. Rodriguez-Hornedo, G. L. Amidon, and J. F. Carpenter. Protein denaturation during freezing and thawing in phosphate buffer systems: monomeric and tetrameric .beta.-galactosidase. *Arch. Biochem. Biophys.* **384**:398–406.
30. J. C. Lee and S. N. Timasheff. The stabilization of proteins by sucrose. *J. Biol. Chem.* **256**:7193–7201 (1981).
31. T. M. Foster, J. J. Dormish, U. Narahari, J. D. Meyer, M. Vrkljan, J. Henkin, W. R. Porter, H. Staack, and J. F. Carpenter *et al.* Thermal stability of low molecular weight urokinase during heat treatment. III. Effect of salts, sugars and Tween 80. *Int. J. Pharm.* **134**:193–201 (1996).
32. A. I. Azuaga, M. L. Galisteo, O. L. Mayorga, M. Cortijo, and P. L. Mateo. Heat and cold denaturation of .beta.-lactoglobulin B. *FEBS Lett.* **309**:258–260 (1992).