Pharmaceutical Research, Vol. 22, No. 7, July 2005 (© 2005)
DOI: 10.1007/s11095-005-6036-3

Research Paper

Measurement of the Kinetics of Protein Unfolding in Viscous Systems and Implications for Protein Stability in Freeze-Drying

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

Received September 21, 2004; accepted April 12, 2005

Purpose. The aim of the study is to determine the degree of coupling between protein unfolding rate and system viscosity at low temperatures in systems relevant to freeze-drying.
Methods. The cold denaturation of both phosphoglycerate kinase (PGK) and β-lactoglobulin were chosen as models for the protein unfolding kinetics study. The system viscosity was enhanced by adding stabilizers (such as sucrose), and denaturant (guanidine hydrochloride or urea) was added to balance the stabilizing effect of sucrose to maintain the cold denaturation temperature roughly constant. The protein unfolding kinetics were studied by both temperature-controlled tryptophan emission fluorescence spectroscopy and isothermal high-sensitivity modulated differential scanning calorimetry (MDSC) (T_{zero}). Viscometers were used to determine the system viscosity. To verify the predictions of structure based on protein unfolding dynamics, protein formulations were freeze-dried above the glass transition temperatures, and the protein structures in dry products were determined by fluorescence spectroscopy of reconstituted solids by extrapolation of the solution data to the time of reconstitution.
Results. Empirical equations describing the effect of sucrose and denaturant (urea and guanidine hydrochloride) on protein cold denaturation were developed based on DSC observations [X. C. Tang and M. J. Pikal. The Effects of Stabilizers and Denaturants on the Cold Denaturation Temperature of Proteins and Implications for Freeze-Drying. *Pharm. Res.* Submitted (2004)]. It was found that protein cold denaturation temperature can be maintained constant in system of increasing sucrose concentration by simultaneous addition of denaturants (urea and guanidine hydrochloride) using the empirical equations as a guide. System viscosities were found to increase dramatically with increasing sucrose concentration and decreasing temperature. The rate constants of protein unfolding (or the half-life of unfolding) below the cold denaturation temperature were determined by fitting the time dependence of either fluorescence spectroscopy peak position shift or DSC heat capacity increase to a first-order reversible kinetic model. The half-life of unfolding did slow considerably as system viscosity increased. The half-life of PGK unfolding, which was only 3.5 min in dilute buffer solution at −10°C, was found to be about 200 min in 37% sucrose at the same temperature. Kinetics of protein unfolding are identical as measured by tryptophan fluorescence emission spectroscopy and by high-sensitivity modulated DSC. The coupling between protein unfolding kinetics and system viscosity for both proteins was significant with a stronger coupling with PGK than with β-lactoglobulin. The half-lives of PGK and β-lactoglobulin unfolding are estimated to be 5.5×10^{11} and 2.2 years, respectively, even when they are freeze-dried in sucrose formulations 20°C above T'_g. Thus, freeze-drying below T'_g should not be necessary to preserve the native conformation. In support of this conclusion, native PGK was obtained after the freeze-drying of PGK at a temperature more than 60°C above the system T'_g in a thermodynamically unstable system during freeze-drying.
Conclusions. Protein unfolding kinetics is highly coupled with system viscosity in high viscosity systems, and the coupling coefficients are protein dependent. Protein unfolding is very slow on the time scale of freeze-drying, even when the system is freeze-dried well above T'_g. Thus, it is not always necessary to freeze-dry protein formulations at temperature below T'_g to avoid protein unfolding. That is, protein formulations could be freeze-dried at product temperature far above the T'_g, thereby allowing much shorter freeze-drying cycle times, with dry cake structure being maintained by the simultaneous use of a bulking agent and a disaccharide stabilizer.

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

[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., R-1-2, Radnor, Pennsylvania 19087, USA.
[3] To whom correspondence should be addressed. (e-mail: pikal@uconnvm.uconn.edu)

INTRODUCTION

Optimization of the target product temperature is perhaps the most important step in freeze-drying process development. It is well known that the higher the product temperature, the faster the freeze-drying. Therefore, a higher product temperature is preferred if the protein integrity is not compromised (2,3). Generally, an optimized freeze-drying process chooses the target product temperature during primary drying to be several degrees below the T_g' or collapse temperature (T_c). However, many protein formulations have very low T_g' or T_c because of electrolytes and stabilizers (such as sucrose or trehalose). In some cases, the T_g' or T_c of protein formulations could be below −40°C, which makes freeze-drying very difficult.

As we know, protein formulations can be freeze-dried at a temperature far higher than its T_g' without collapse by adding a crystalline bulking agent. However, stability is always a concern for proteins. The well-accepted rule for freeze-drying protein formulations is that the product temperature should be below the T_g' to avoid protein unfolding during freeze-drying. It is assumed that unfolding will be slow on the time scale of freeze-drying below the glass transition temperature, T_g', but rapid at temperatures significantly above T_g'. However, there are no experimental data to support this assumption.

In many protein formulations, sugars are added as stabilizers, which will yield high viscosity in the freeze concentrate. The high viscosity might be expected to greatly slow protein unfolding such that even if the protein is freeze-dried at a product temperature above the T_g', the protein still cannot unfold on the time scale of freeze-drying. Calculations on protein unfolding half-lives, assuming coupling between unfolding kinetics and system viscosity, suggest that freeze-drying the protein above the T_g' would generally not unfold the protein (4). The question becomes, "how high can the product temperature be during freeze-drying without unfolding (and decomposing) the protein?" To answer this question, more information is needed about protein thermodynamic stability and unfolding kinetics at low temperature and high viscosity in systems relevant to freeze-drying conditions.

Proteins are temperature sensitive and are thermodynamically stable only in a defined temperature interval. At high temperatures, they spontaneously unfold or denature (thermal denaturation). At low temperature, they experience cold denaturation (5–7). Cold denaturation is a process whereby the protein loses the native conformation at low temperature. In this study, we used protein cold denaturation as a model to investigate protein unfolding kinetics. The cold denaturation model is useful because this phenomenon provides an opportunity to study the unfolding kinetics under conditions most like the conditions that prevail in freeze-drying without interference from irreversible process that would dominate at high temperature. In addition, the combination of low temperature and high solute concentration means high viscosity systems are easily formed.

The potential correlation between chemical reaction rate and the system viscosity was first proposed by Kramers (8). It has also been reported in a review that the kinetics of many protein folding reactions do not correlate with folded state stability or with the size of the folding unit, but exhibit "surprisingly strong" dependence on solvent viscosity (9). The rates of both folding and unfolding kinetics of GCN4-p2′, a simple α-helix protein, are correlated with system viscosity (10). However, sometimes, refolding kinetics are decoupled from solvent viscosity, at least in relatively low viscosity systems (11).

The cold denaturation of β-lactoglobulin, myoglobin, RNase, and especially phosphoglycerate kinase (PGK) have been investigated for many years using well-established methodology (5,6,12,13). PGK appear to be a particularly good model protein for our study. At pH 6.5 and with 0.7 M GuHCl, PGK undergoes reversible cold denaturation between 20 and 0°C, with a clear indication of denaturation with differential scanning calorimetry (DSC) and both fluorescence and ultraviolet spectroscopy. The half-life for cold denaturation is several minutes in dilute buffer (0.7 M guanidine hydrochloride and 20 mM phosphate buffer) (6,14,15). Furthermore, extensive data describing the effects of both sucrose and GuHCl on the thermodynamics of PGK denaturation are available (16). Literature studies suggest that β-lactoglobulin might also serve as a satisfactory model, although the data available are much more limited than with PGK (13). It has been reported that β-lactoglobulin has a cold denaturation temperature above 0°C in the presence of denaturants, and the cold denaturation event was detectable using DSC (17–19).

Our goal is to study the protein unfolding kinetics in a system representative of a real pharmaceutical formulation for freeze-drying or at least close to it and to determine the correlation between viscosity and unfolding rate close to T_g'. This information could also be used to predict protein unfolding during secondary drying if knowledge of the moisture content allows estimation of viscosity. Our hypothesis is that with high viscosity (high concentration of sugars), the protein-unfolding rate will be slow on the time scale of freeze-drying, such that a protein may be freeze-dried above the system glass transition temperature without unfolding. If this hypothesis is valid, the freeze-drying process could be much faster. Literature data and also work in our laboratory suggest that at least some proteins can be freeze-dried at a temperature higher than T_g' without compromising either in-process stability or storage stability (20,21) (Tchessalov *et al.* 2002, unpublished observations).

For proper data interpretation, it is essential that we are able to maintain roughly constant cold denaturation temperature by keeping the temperature of cold denaturation constant as we add components that increase viscosity. That is, as we shift the temperature lower to initiate cold denaturation, we must ensure that this temperature remains well below the cold denaturation temperature as we change composition in a series of experiments. Different polyols such as sucrose and trehalose (disaccharides), which are extensively used as stabilizers for freeze-drying, will be used as viscosity enhancers. However, these stabilizers decrease the cold denaturation temperature. Denaturants [guanidine hydrochloride (GuHCl) and urea] will therefore be used to counteract the stabilizing effects of sucrose and thereby allow the cold denaturation temperature to remain constant.

MATERIALS AND METHODS

Phosphoglycerate kinase (PGK) precipitated and suspended in 1.4 M sodium sulfate (Sigma, St. Louis, MO) was dialyzed in 2 mM sodium phosphate buffer at pH 6.5 twice and freeze-dried below the collapse temperature (T_c, about −10°C).

β-Lactoglobulin (freeze-dried powder), sucrose, trehalose, and glycerol were purchased from Sigma and used without further purification. All the reagents were of analytical grade. All the vials used for freeze-drying were 5-ml serum tubing vials from Fisher.

Buffer Solutions

Sodium phosphate buffers (20 mM) were used for both β-lactoglobulin and phosphoglycerate kinase (PGK). The pHs of the formulations were from 2 to 7 for β-lactoglobulin as required and 6.5 for PGK. In PGK formulations, 1 mM of dithiothreitol (from SigmaUltra, St. Louis, MO) was used as an antioxidant (6,15).

System Viscosity Measurement

To investigate the coupling between protein unfolding kinetics and the system viscosity, cold denaturation was studied in systems of varying viscosities, obtained by addition of sucrose and denaturants. System viscosity was measured using two types of viscometers, the Advanced Rheometric Expansion System (Rheometer Scientific, Piscataway, NJ), with a liquid nitrogen cooling system, or the Universal Dynamic Spectrometer (Physica UDS 200, Paar Physica, Glen Allen, VA), with a refrigeration cooling system. The viscosities of buffer solutions with different concentrations of sucrose were determined at different temperatures and shear rate to investigate the correlation between system viscosity and temperature and to examine the correlation between system viscosity and shear rate. The viscosities below 50 cP were determined using the Physica UDS 200.

Protein Unfolding Kinetics by High-Sensitive Modulated Differential Scanning Calorimetry (T_{zero})

The protein cold denaturation temperature was measured at a linear scan rate of 1°C/min using modulated DSC (MDSC 2920, TA Instruments, Newcastle, DE) (1). Denaturation kinetics were monitored by measuring heat capacity change during protein cold denaturation using high-sensitive modulated differential scanning calorimeter (MDSC; T_{zero}, TA Instruments) in isothermal mode. Relatively high concentrations (about 5% w/v) of proteins were used to obtain measurable heat capacity change (ΔC_p) during unfolding. The samples (18 or 60 μl) were sealed in 20 or 80 μl hermetic aluminum pans. Buffer solution (the same components except for proteins) of the same volume was used as reference. The DSC was precooled to the final experimental temperature with the reference pan, which is much lower than protein cold denaturation temperature. Next, the sample pan was loaded into the precooled DSC cell, and the DSC was operated isothermally with modulation amplitude of ±0.5°C and period 80 s for 18-μl samples and modulation amplitude of ±0.5°C and period 120 s for 60-μl samples. Extreme care must be taken to ensure no ice forms during precooling or sample loading, and the pans should have flat bottoms for good thermal conductivity.

Protein Unfolding Kinetics by Tryptophan Fluorescence Emission Spectroscopy

The protein cold denaturation rate was determined by temperature-controlled fluorescence spectroscopy (LS 50, Perkin Elmer, Norwalk, CT). The excitation wavelength light is 295 nm. The emission fluorescence spectra were collected between 300 and 400 nm with a spectrum scan rate of 100 nm/min. The peak positions were estimated by drawing a horizontal line across the peak at half-height and calculating the average of both curve wavelengths at the intersections between the peak and the horizontal line deducted by an offset value (6.5 nm). The offset value was obtained by fitting the peak positions of multiple scan emission data (10 scans). Although the multiple scan data have less noise and easier to recognize peak positions, we need to use single scan peak data because multiple scanning takes too long for use in kinetics measurement.

About 150 μl protein solution (from 3 to 50 mg/ml protein) was filled into the quartz microfluorescence cuvette (capacity 200 μl). The fluorescence cell was precooled to the final experimental temperature for the kinetic study, and the sample was loaded into the holder. The spectra were then collected as a function of time. Data were collected for at least four times the protein unfolding half-life ($t_{1/2}$).

Tryptophan fluorescence emission spectroscopy was used to detect protein denaturation at both high and low temperatures. For low-temperature studies, the protein samples were quenched in a precooled cuvette well below the cold denaturation temperatures, and the tryptophan fluorescence emission spectra were collected as a function of time. The temperature history during quench was evaluated using a thermocouple placed in the sample. It was found that about 20 s was required for the sample to change from room temperature to as low as −20°C.

Maintaining the Cold Denaturation Temperature Constant as Sucrose is Added

Experimentally, the protein cold denaturation temperature should be kept at a temperature high enough so the solution will not freeze. Moreover, while the viscosity will be enhanced and the freezing point depressed by adding stabilizers, such as sucrose, such addition will also depress the cold denaturation temperature of the protein. To keep the cold denaturation temperature constant, i.e., the unfolding free energy constant, the addition of denaturants, such as GuHCl, is necessary. The effect of denaturant on the free energy of denaturation may often be written in a linear form as follows (22):

$$\Delta G_d = \Delta G_d^0 + \gamma \cdot [\text{GuHCl}] \quad (1)$$

where ΔG_d is the free energy of denaturation, ΔG_d^0 is the free energy of denaturation at zero concentration of de-

naturant, which is a function of polyol concentration and pH, and γ is a constant independent of polyol concentration. Stability is increased by the polyol according to the following equation:

$$\Delta G_d^0 = \Delta G_0^0 + m[\text{polyol}] \quad (2)$$

where ΔG_0^0 is the free energy at zero concentration of both denaturant and stabilizer and m is a constant relatively independent of protein (22). From Eqs. (1) and (2), it is obvious that as long as an increase in stabilizer concentration is properly balanced by an increase in denaturant concentration, m[polyol] = γ[GuHCl], the free energy of denaturation, ΔG_d, may be held constant.

Therefore, by the combined use of denaturants and polyols, the protein cold denaturation temperature can be controlled to allow us to investigate unfolding kinetics at constant thermodynamic driving force (i.e., constant ΔG_d) within a one-phase system without ice forming.

The stabilization effect of sucrose and the destabilization effect of urea in β-lactoglobulin in 20 mM phosphate buffer pH 7.0 were reported previously [1]. The results may be summarized in the equation below:

$$T_{cd} = 9.1 \cdot C_u - 40.2 \cdot C_s - 13.1 \quad (3)$$

where T_{cd} is the cold denaturation temperature of β-lactoglobulin (°C), C_s is the concentration of sucrose (M), and C_u is the concentration of urea (M). The effect of urea on β-lactoglobulin cold denaturation temperature at pH 2.0 was reported (13), and the slope of the linear correlation was 9.5°C/M, which is consistent with our results (slope 9.1°C/M) at pH 7.0, assuming that the effect of denaturants on the cold denaturation of β-lactoglobulin is roughly independent of pH. Thus, the cold denaturation temperature of β-lactoglobulin in the mixture of sucrose and urea can be estimated using Eq. (3). The effect of the guanidine hydrochloride (GuHCl) on the β-lactoglobulin cold denaturation temperature at pH 3 (23) may be summarized by the bilinear form,

$$T_{cd} = 15.6 \cdot C_{GuHCl} - 40.2 \cdot C_s - 13.1 \quad (4)$$

Additional cold denaturation experiments with different ratios of stabilizer and denaturants were carried out, and the results were found to be in agreement with Eqs. (4) and (5) (data not shown). These results show that there is no sucrose–urea or sucrose–GuHCl interaction term in Eqs. (3) or (4), which means that the effects of stabilizer (sucrose) and denaturant on β-lactoglobulin stability are independent.

For PGK, it is found that the stabilizer, sucrose, has an effect on the thermodynamics of unfolding which is dependent on the level of GuHCl. The literature data (16) were analyzed, and the empirical result is as follows:

$$\Delta G_d(\text{kcal/mol}) = 3.3 - (4.24 - 1.176 \cdot C_s)(C_{GuHCl} - 0.7) + 1.3 \cdot C_s \quad (5)$$

where C_s is the molar concentration of sucrose and C_{GuHCl} is the molar concentration of GuHCl. Considering the freeze-concentrated phase has a concentration of sucrose about 80% w/w, which is about 2.7 M, the calculated concentration of GuHCl required to keep the free energy of the system the same as in the absence of stabilizer is more than 10 M. Practically, it is impossible to prepare such a high concentration of stabilizers (80%) with denaturant (>10 M). Fortunately, we can easily prepare the mixtures of stabilizer (sucrose 60%) and calculated denaturant (GuHCl 4.9 M) with viscosity similar to a sucrose freeze-concentrate and study protein-unfolding kinetics in such systems. The PGK cold denaturation temperatures from our studies were in good agreement with Eq. (3) (data not shown). Equation (5) shows that the destabilization effect of guanidine hydrochloride is reduced by addition of sucrose.

RESULTS AND DISCUSSION

Protein Unfolding Kinetics Determined by Tryptophan Fluorescence Emission Spectroscopy

The protein formulations were designed so that the protein is thermodynamically stable at room temperature but unstable (cold denaturation) below room temperature by using Eqs. (3) and (4) for β-lactoglobulin in systems of urea and guanidine hydrochloride with sucrose, respectively, and Eq. (5) for PGK in systems of guanidine hydrochloride with sucrose. One example of the protein unfolding kinetic experiment using tryptophan fluorescence emission spectroscopy is presented in Fig. 1. PGK in 37% sucrose was quenched from room temperature to −10°C, and the tryptophan fluorescence emission spectra were collected at different time points. The peak position of the spectra shifted with time from low wavelength (about 338 nm) to high wavelength (about 348 nm). The experimental peak shifts were plotted *vs.* time in Fig. 1b and found to be in good agreement with the "best fit" values from the first-order reversible kinetic model (smooth line). The kinetic parameters, such as the rate constant (k) or the half-life of protein unfolding ($t_{1/2}$), are obtained from the regression results (Fig. 1b). The time for the peak position to reach halfway of the total wavelength change ("half-life, $t_{1/2}$") was 200 min (Fig. 1), which is significantly longer than the reported $t_{1/2}$ (~3 min) determined in dilute solution (16).

The method of determination of protein unfolding kinetics using tryptophan fluorescence emission spectroscopy is limited by the experimental time scale, although the temperature gradient inside the small sample (150 μL) is not a major concern. For the quench experiment, it took about 20 s for the sample in the precooled cuvette to go from room temperature to −20°C. Therefore, determination of any kinetic event shorter than 20 s is not practical. Furthermore, the scan time from 300 to 350 nm (the peak position ~340 nm) took 30 s and a whole spectrum scanning from 300 to 400 nm took 60 s. Therefore, this methodology cannot be used for very fast unfolding events (e.g., $t_{1/2} < 2$ min).

Protein Unfolding Kinetics Determined by High-Sensitivity DSC (T_{zero})

Protein formulations, which are thermodynamically stable at room temperature, but would cold denature below room temperature, were quenched in a precooled DSC cell. The sample temperature was stable at the final temperature after several seconds. The heat capacities of the samples were

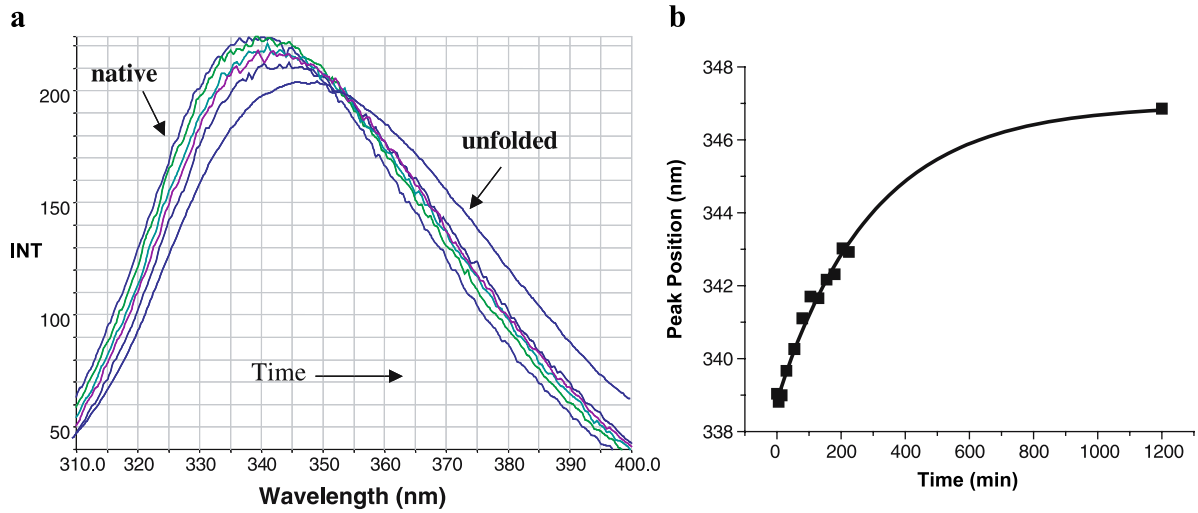

Fig. 1. Unfolding kinetics by tryptophan fluorescence emission spectroscopy. (a) Phosphoglycerate kinase (PGK) (in 20 mM phosphate buffer at pH 6.5 with 1.79 M guanidine chloride and 37% sucrose) tryptophan fluorescence emission spectroscopy peak positions at different times; curves left to right represent the times 1, 30, 105, 155, 225, and 1,200 min at −10°C. (b) The plot of peak position shifts *vs.* time. The symbols represent raw data, and the smooth line represents the "best fit" values from the first-order reversible kinetic model.

determined by isothermal modulated DSC. Two examples of PGK and β-lactoglobulin studies are presented in Fig. 2. The results show significant heat capacity increase when the PGK formulation was held at −10°C (Fig. 2a) and when the β-lactoglobulin formulation was held at −20°C (Fig. 2b). The heat capacity *vs.* time data are well fit by the first-order reversible kinetic model, yielding the unfolding kinetics rate constant (k) and half-life ($t_{1/2}$) (Fig. 2). The $t_{1/2}$ of PGK in 28.6% sucrose was about 42 min at −10°C, and the $t_{1/2}$ of β-lactoglobulin in 55.6% sucrose was about 159 min at −20°C.

The half-lives ($t_{1/2}$) for unfolding both proteins (PGK and β-lactoglobulin) determined by the DSC method are compared with corresponding data the fluorescence spectroscopy method in Fig. 3. The agreement between DSC and tryptophan fluorescence emission spectroscopy results is excellent for both PGK and β-lactoglobulin. The unfolding half-life ($t_{1/2}$) for both proteins increased significantly with increase in sucrose concentration (or system viscosity). The effect of sucrose concentration on the $t_{1/2}$ of PGK was even greater than for β-lactoglobulin.

This study represents the first attempt to quantify the kinetics of protein unfolding from isothermal DSC data, and at present, the method has some limitations. The heat capacity *vs.* time curves are extremely noisy both because of the small sample size (18 or 60 μl) and the small change in heat capacity upon protein cold denaturation (<0.02 J/K per gram of total sample) in the dilute solution (5% protein). Although the DSC measured heat capacity change of protein unfolding (β-lactoglobulin in 20 mM phosphate with 4 M urea, pH 2) is 0.38 ± 0.048 J/K per gram of protein (four replicates), the DSC signal change during protein cold

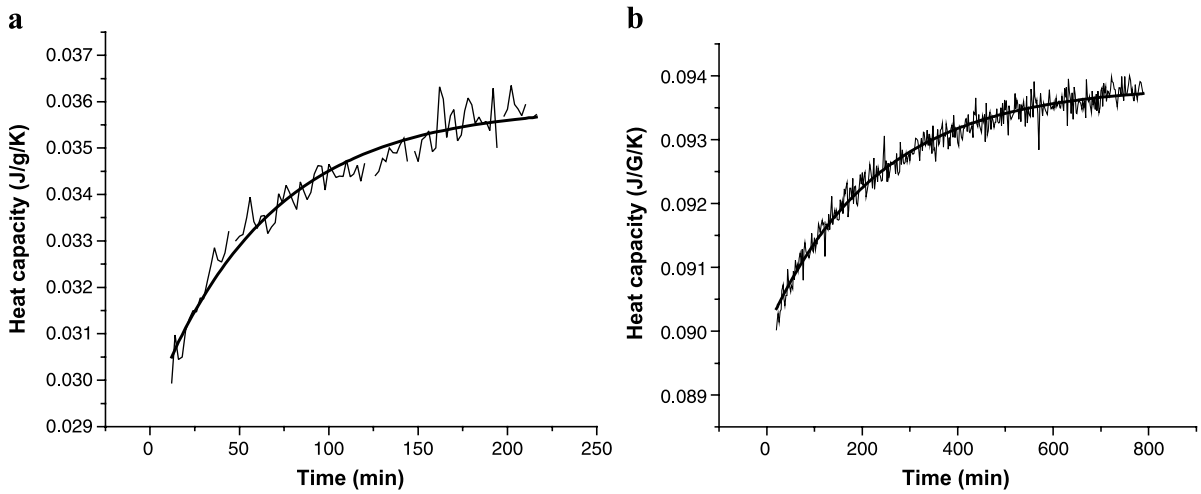

Fig. 2. Unfolding kinetics by high-sensitive differential scanning calorimetry (DSC) (T_{zero}). The heat capacity change during protein unfolding can be fitted to the first-order reversible kinetic model. (a) PGK (5%) in 1.36 M guanidine and 28.6% sucrose at −10°C. (b) β-Lactoglobulin (5%) in 7 M guanidine and 55.6% sucrose (pH 7) at −20°C.

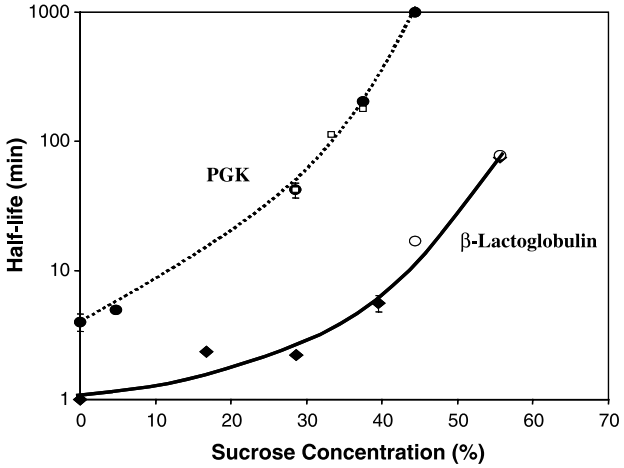

Fig. 3. Protein unfolding kinetics: fluorescence method compared with the DSC method. The results are consistent by two different methods. Filled circles = fluorescence results for PGK; filled squares = fluorescence results for **β**-lactoglobulin; open circles = DSC results for **β**-lactoglobulin; open squares = DSC results for PGK.

denaturation is only about 0.00008 W/K (or 0.00008 W/min) energy flow when the sample size is 18 μl of 5% protein. In addition, the DSC sample and the reference mass should be well matched. Too small a sample size relative to the reference size often causes a negative heat capacity response from the DSC, whereas a larger mass difference between sample and reference compromises the signal sensitivity. That is, the very small heat capacity change in protein unfolding, (<0.02 J/g/K) is lost in the huge heat capacity of water (about 4.2 J/g/K). Both the sample and reference pans must be well sealed because a trace of solvent evaporation will dominate the heat capacity change and ruin the experiment. The pans (samples and reference) should have good thermal conductivity and should not be moved during the experiment (by vibration). Thus, care should be taken to ensure the DSC cells are flat. Also, the MDSC experiment cannot determine fast unfolding kinetics ($t_{1/2}$ < 10 min) because it takes several minutes for the MDSC to equilibrate. The advantage of the MDSC method is that it yields kinetic results from one automated experiment.

Unfolding Kinetics of β-Lactoglobulin as a Function of Temperature

The glass transition temperatures (T_g) of the formulations were determined by DSC (MDSC 2920, TA Instruments) equipped with a liquid nitrogen cooling system. The T_g of the formulation of 5% β-lactoglobulin in 55.6% sucrose and 7 M guanidine hydrochloride (20 mM phosphate buffer, pH 7.0) is −75°C. The unfolding half-life ($t_{1/2}$) of β-lactoglobulin in this system was determined by both tryptophan fluorescence emission spectroscopy and the DSC method at various temperatures below the cold denaturation temperature. The half-lives are plotted against $T - T_g$ in Fig. 4. The $t_{1/2}$ increases slowly when the experimental temperature is far removed from the glass transition temperature of the sample (and the viscosity is modest). However, $t_{1/2}$ increases dramatically when the experimental temperature approaches within about 40°C of the glass transition temperature (Fig. 4). The unfolding $t_{1/2}$ was about 0.3 h at 85°C above T_g (experimental temperature 10°C) and increased to 5.3 h at 40°C above T_g (experimental temperature −35°C). If the $t_{1/2}$ *vs.* $T - T_g$ data are analyzed by Williams-Landel-Ferry (WLF) equation [Eq. (6)], to evaluate the constants, C_1 and C_2, $t_{1/2}$ of β-lactoglobulin unfolding at the glass transition temperature (T_g) is calculated as 322 days.

$$\log\left(\frac{t_{1/2}(T)}{t_{1/2}(T_g)}\right) = \frac{-C_1(T - T_g)}{C_2 + (T - T_g)} \tag{6}$$

In Eq. (6), $t_{1/2}(T)$ is the half-life at temperature T, $t_{1/2}(T_g)$ is the half-life at the reference temperature, i.e., glass transition temperature (T_g), and C_1 and C_2 are constants (determined as 5.7 and 32.6, respectively, from the fit). Assuming the WLF equation extrapolation gives a result of the correct order of magnitude, β-lactoglobulin would be kinetically stable even if it is freeze-dried well above T_g'.

System Viscosity at Different Sucrose Concentrations: Shear Thinning

The viscosities as a function of sucrose concentration and temperature were determined at various shearing rates. The shear rate dependence of viscosity is illustrated in Fig. 5a and b. The viscosity was much higher for the high concentration of sucrose (55.6%) than for the lower concentration of sucrose (28%), as expected. The log(η) *vs.* log(shearing rate) plot showed a linear relationship for both formulations at high and low concentrations of sucrose. The slope for the low concentration sucrose (28%) was only about −0.22 (Fig. 6a), but the slope for high concentration of sucrose (55.6%) was much larger in magnitude, i.e., −1.44 (Fig. 6b). The slopes of the linear correlation of the log(η) *vs.* log(shearing rate) for the formulation containing high concentrations of sucrose (55.6 and 60%) were plotted against experimental temperatures (Fig. 6). Note that the slopes were significantly larger in magnitude at low temperature than at high temperature (from −1.9 at −20°C to −0.23 at 25°C). The system viscosities decreased with temperature increase, fol-

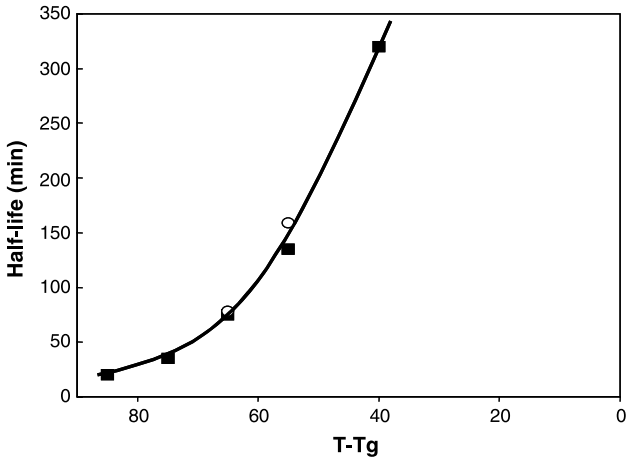

Fig. 4. Unfolding kinetics as a function of temperature ($T - T_g$). β-Lactoglobulin in sucrose (55.6% w/w) and GuHCl (7 M) at pH 6.5, T_g = −75°C. By WLF equation analysis with C_1 = 5.7 and C_2 = 32.6: half-life > 100 days at $T = T_g$. Squares = fluorescence; circles = DSC.

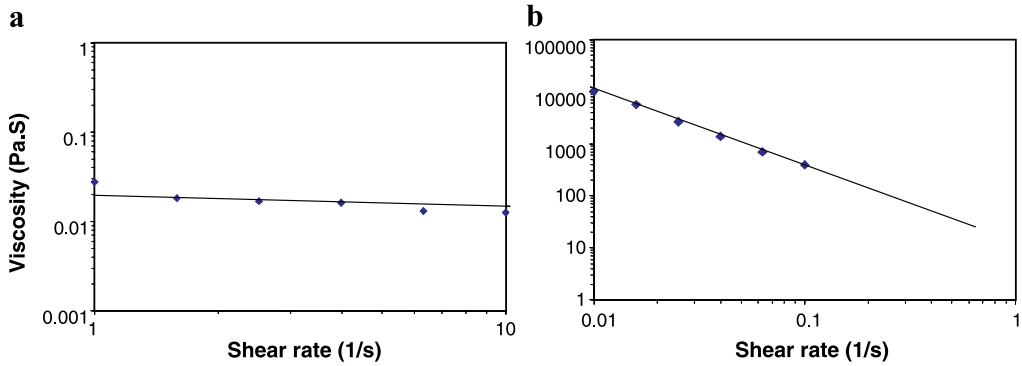

Fig. 5. Viscosity and shear rate. (a) System viscosity *vs*. shear rate at low concentration of sucrose (28% sucrose). The slope of the linear relationship is −0.2. (b) System viscosity *vs*. shear rate at high concentration of sucrose (55.6% sucrose). The slope of the linear relationship is −1.4.

lowing the Arrhenius law. That is, the ln(η) is linear in the reciprocal of temperature (data not shown). From the slope, the activation energy was calculated as Ea = 8.41 kcal/mol.

Our data indicate the sucrose solutions are shear-thinning systems, particularly at high concentrations. A shear-thinning slope less than −1 is considered to have its origins in weak bond breaking (such as H-bond dissociation) during shearing. Our results showed large negative slopes especially at high concentration of sucrose and low temperatures suggesting that the high sucrose concentration systems have very strong hydrogen-bonded networks, which break down during shearing (24). A highly H-bond system certainly is plausible and also explains the high viscosity for these systems.

Coupling Coefficient Between Unfolding Kinetics and System Viscosity

The unfolding half-lives of both PGK and β-lactoglobulin were plotted against system viscosity in Fig. 7a and b. We note that the viscosity data were determined in the actual formulations (including stabilizer and denaturants if any), although the impact of denaturants on the system viscosities was very small. However, the impact of protein (up to 5%) was not investigated, but given the low level of protein relative to the other solutes, the protein effect should be negligible. Equation (7) was fit to the data of unfolding half-life as a function of system viscosity.

$$t_{1/2} = A \cdot \eta^{\alpha} \tag{7}$$

where A is a constant of proportionality, η is the system viscosity, and α is the coupling coefficient ($\alpha \geq 0$). Both sets of data were well represented by Eq. (7) with positive values of α. The coupling coefficients for PGK and β-lactoglobulin systems were 1.94 and 0.68, respectively. The type of motion measured by viscosity is "global" or "α-type" motion involving whole molecule or large segment rotational and translation motion. One might argue that the mobility required for protein unfolding might be just "local" or "β-type" motion especially in the system where solutes (e.g., sucrose) are preferentially excluded from its microenvironment. However, it is also well known that sucrose behaves as water substitute, hydrogen bonding to the protein as water is removed, which would suggest that motion of the protein and motion of the surrounding water and sucrose are highly coupled. Indeed, we speculate that the protein, as a macromolecule, might require the coordination of motion between multiple sites involving solutes and solvent molecules during the unfolding process, at least for the major unfolding involved in denaturation. In other words, the motion should demand a high degree of cooperativity. The coupling coefficients for PGK and β-lactoglobulin are relatively high confirming our speculation that protein unfolding requires "global" motion. The coupling coefficient of PGK (1.94) was larger than unity, suggesting that PGK unfolding requires more free volume and a greater amplitude of motion than does viscous flow (25). Conversely, the smaller coupling coefficient of β-lactoglobulin (less than unity) demonstrates that the unfolding of β-lactoglobulin requires less free volume than viscous flow. One might speculate that here, the rate determination step of protein unfolding involves molecular motion more internal to the tertiary structure for β-lactoglobulin. Conversely, the PGK unfolding movement involves a more cooperative process with greater involvement of the "solvent" and "solute" molecules.

The time scales of protein unfolding can be extrapolated to low temperatures by using the fitted parameters (A and α). Suppose that both proteins (PGK and β-lactoglobulin) are freeze-dried in sucrose formulations. The glass transition

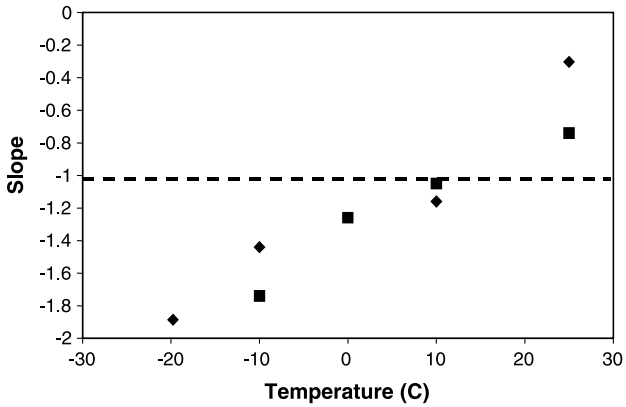

Fig. 6. The effect of temperature on shear rate dependence of viscosity. The slope of shear thinning at different temperatures. Diamonds = 55.6% sucrose and 7 M guanidine; squares = 55.6% sucrose.

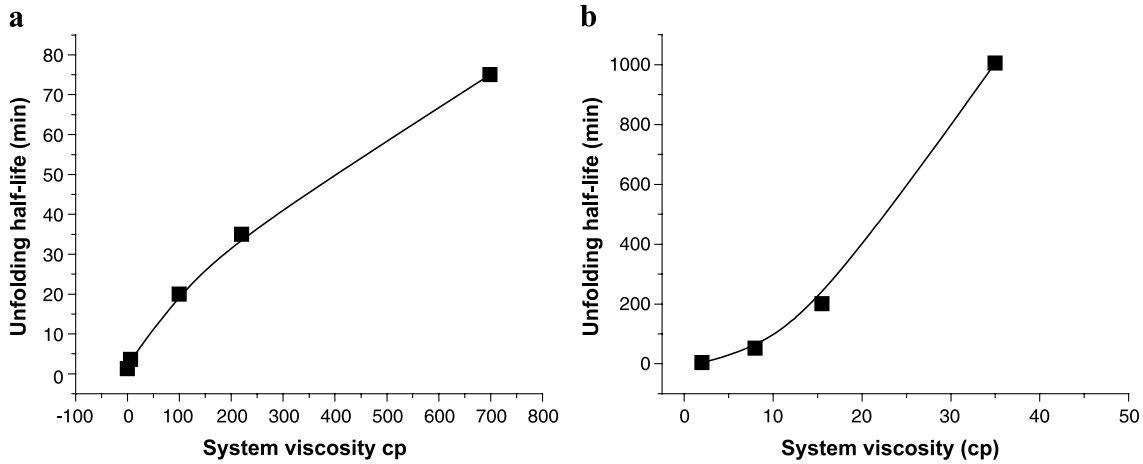

Fig. 7. Coupling between unfolding kinetics and system viscosity. Equation: $t_{1/2} = A*\eta^{\alpha}$, fit to data, where α is the coupling coefficient ($\alpha \geq 0$). The smooth lines are smoothed results from the fit. Both proteins show good coupling between unfolding kinetics and system viscosity, with PGK having stronger coupling than β-lactoglobulin. (a) β-Lactoglobulin, α = 0.68. (b) PGK, α = 1.94.

temperature of the freeze concentrate (T_g') is about −35 or −45°C if T_g'' is considered as the only real glass transition temperature (26–28). The viscosity of a system at the glass transition temperature is about 10^{12} Pa s. The collapse temperature in freeze-drying is often related to the higher transition temperature at −35°C, but there is not general agreement on whether or not T_g' is also a glass transition. The fitted parameters for β-lactoglobulin were A = 0.89 and α = 0.68 [Eq. (7)]; thus, the unfolding half-life can be calculated to be 2.5×10^4 years at the glass transition (i.e., $\eta = 10^{12}$ Pa s). The viscosity of concentrated sucrose solutions can be obtained from the literature [29]. Even at −15°C (viscosity from literature [29]), which is 20°C higher than the T_g' (−35°C) or 30°C higher than the T_g'' (−45°C), the estimated unfolding half-life of β-lactoglobulin is 2.2 years. The same estimations were performed for PGK, giving unfolding half-lives of 2.4×10^{23} years at the glass transition temperature and 5.5×10^{11} years at −15°C, respectively. Therefore, at least with these proteins, the time scales of protein unfolding are projected to be much longer than the process time of freeze-drying, which means the proteins (PGK and β-lactoglobulin) are kinetically stable in the freeze concentrate even at tempera-tures 20°C or more above the glass transition temperature. We admit that the extrapolation to T_g' or 20°C above T_g' is very long, and probably not highly accurate, but the results are so conclusive (i.e., $t_{1/2}$ so long) that the conclusion seems valid. These results strongly suggest that it should not be necessary to freeze-dry a protein formulation below the T_g' to avoid unfolding-related degradation, at least for those proteins with unfolding kinetics similar to the proteins studied here.

Application of Kinetic Data to the Freeze-Drying of PGK

Two PGK formulations were freeze-dried to test the above predictions of protein kinetic stability against unfolding. In formulation 1, 4 mg/ml PGK was dissolved in 20 mM phosphate buffer pH 6.5 with 0.2 M guanidine hydrochloride and 2% sucrose. The cold denaturation temperature of PGK in formulation 1 is below −20°C before ice formation and is above 20°C after ice formation in the freeze concentrate, where the concentration of guanidine hydrochloride is about 6 M and the concentration of sucrose is about 60%. Therefore, thermodynamically, PGK would not be destabilized during supercooling (about −12°C determined by thermocouple response) in dilute solution before ice formation but would be thermodynamically unstable after ice formation and cooling to −40°C in the freezing step. The question is, "Are the kinetics of unfolding fast enough for thermodynamics to prevail?" Our projections based on Eq. (7) say, "No!"

The freeze-drying was conducted at high product temperature (from −5 to −1°C) during primary drying, and the product was totally collapsed because the T_g' of the formulation is about −75°C. The dry product was in the amorphous state, as confirmed by polarized light microscopy.

The dry product was quickly reconstituted, and PGK tryptophan emission fluorescence spectroscopy peak positions were collected as a function of time to allow extrapolation of the peak position to zero time and thereby provide a measure of structure in the solid prior to reconstitution. This extrapolation is presented in Fig. 8a, in which the peak position extrapolated to zero time was in the range of native structure. Thus, the solid was freeze-dried to yield the native conformation; that is, in spite of thermodynamic instability and freeze-drying well above T_g', native structure was preserved as predicted by our analysis of unfolding kinetics. This experiment was repeated and the same results were obtained for both experiments.

The other formulation (formulation 2) of PGK (3.2 mg/ml) in 20 mM phosphate buffer pH 6.5 with 0.75 M guanidine hydrochloride and 5% sucrose was also freeze-dried. The cold denaturation of the PGK in formulation 2 was 8°C, determined by DSC, and would be higher than 20°C in the freeze concentrate after ice formation (about 6 M guanidine hydrochloride and 60% sucrose). The sample was held at −5°C for 1 h before freezing to allow unfolding and was then freeze-dried at the product temperature of −33°C. PGK refolding was detected by tryptophan fluorescence emission spectroscopy (Fig. 8b). That is, a sharp decrease in peak

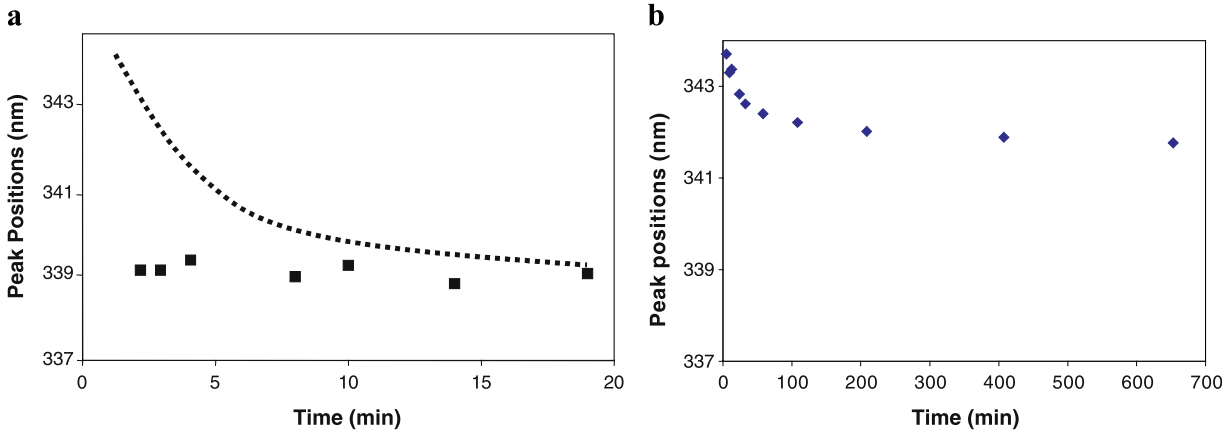

Fig. 8. Measurement of solid structure by extrapolation to time of reconstitution. Fluorescence spectroscopy after reconstitution of freeze-dried PGK formulations as a function of time. (a) Protein was thermodynamically stable before ice formation but thermodynamically unstable during freeze-drying. The dashed line is the calculated refolding of denatured protein (assuming $t_{1/2}$ is about 4 min as expected for a dilute solution), and the symbols are the experimental data. It is obvious that the peak position in solution (and structure) is independent of time and consistent with the native conformation. (b) The protein was unfolded by annealing at −5°C before ice formation and then freeze-dried; the sharp decrease peak position immediately after reconstitution from a value consistent with the unfolded state indicates that the unfolded structure was frozen-in during freeze-drying.

position occurs from an initial value characteristic of the unfolded state to a value approaching the native state. Although for unknown reasons the peak position was not shifted back to 339 nm, which was typical of "native structure" for all the PGK refolding experiments in this study, the extrapolation back to zero time clearly indicates that the PGK structure was unfolded in the solid state after freeze-drying. Thus, the results indicated that PGK was unfolded during freeze-drying. That is, PGK was cold denatured or unfolded during the −5°C hold in solution before ice formation (ice formed at −13°C, as determined by a thermocouple), and the unfolded structure was trapped in the freeze concentrate during freezing and drying. Thus, the results (Fig. 8) support our conclusion that the PGK unfolding time scale is much longer than the freeze-drying process time even at a temperature 20°C higher than the T'_g. Therefore, freeze-drying of PGK below the T'_g is not necessary.

Given the fact that some proteins do lose activity during freeze-drying, the remaining question would be "How can a protein unfold during freeze-drying?" One plausible explanation of unfolding is that unfolding only occurs when the protein is not dispersed in a viscosity-enhancing stabilizer and/or is without strong coupling between unfolding rate and "viscosity" in the quasi-solid phase. It has been reported that proteins, including β-lactoglobulin, totally or partially lost secondary structure (both α-helix and β-sheet regions by FT-IR) after freeze-drying without using stabilizers, whereas the proteins retained most of their native structure in co-lyophilization with sucrose (30). Yoshida *et al.* (31) reported loss of PGK activity after freeze-drying without using stabilizers. We also observed β-lactoglobulin unfolding in a pure protein solution (with 20 mM phosphate buffer) in the frozen state at −20°C using tryptophan fluorescence emission spectroscopy (data not shown). Secondly, conformational changes that significantly impact protein activity may involve much less motion than unfolding and, therefore, may occur much faster in freeze-drying systems. Further studies are needed to clarify this issue.

It should be emphasized that collapsed lyophiles may not be pharmaceutically acceptable, although the protein integrity is intact. Grossly collapsed products tend to have longer reconstruction time and higher product moisture content as well as present major cosmetic concerns. However, a bulking agent (such as mannitol or glycine) with a high eutectic temperature and/or high collapse temperature (T_c) can be used in the protein formulations to allow freeze-drying at a product temperature significantly above T'_g (but below T_c) without producing macroscopic collapse (4).

The significance of this research is that the protein unfolding rate (as a result of any stress, including surface, and pH shift induced unfolding) in the freeze concentrates studied are so slow that freeze-drying may be conducted at much higher temperatures than the T'_g. Therefore, to the extent that these observations may be generalized, protein drugs can be formulated with bulking agent and disaccharide and freeze-dried far above the T'_g of the amorphous phase without fear of in-process degradation. While we cannot predict if a given protein formulation will behave as the formulations studied here, it does seem advisable that one should at least challenge a protein formulation by freeze-drying above T'_g to determine if stability really is compromised. Our data suggest that stability may well be no different than if dried much more slowly at much lower temperatures.

CONCLUSIONS

Protein unfolding kinetics are highly coupled with system viscosity, and the coupling coefficients, which potentially provide information about protein unfolding mechanisms, are protein dependent. The kinetics of protein unfolding in viscous systems can be determined by both tryptophan fluorescence emission spectroscopy and high-sensitivity modulated DSC. The system viscosities were high in systems with high concentration of stabilizers, such as sucrose at low temperature, because of very strong hydrogen-bonded networks.

Protein unfolding is very slow on the time scale of freeze-drying, even when the system is freeze-dried well above T'_g. Therefore, it is not always necessary to freeze-dry

protein formulations at temperatures below T_g' to avoid unfolding.

ACKNOWLEDGMENTS

This project was funded by a grant from the National Science Foundation's Center for Pharmaceutical Processing Research. The authors also thank Leonard Thomas for his help in using the high-sensitivity modulated differential scanning calorimeter, MDSC (T_{zero}, TA Instruments, Newcastle, DE).

REFERENCES

1. X. C. Tang and M. J. Pikal. The Effects of Stabilizers and Denaturants on the Cold Denaturation Temperature of Proteins and Implications for Freeze-Drying. *Pharm. Res.* **22**(8):1177–1185 (2005).
2. M. J. Pikal. Freeze-drying of proteins. Part I: process design. *BioPharm* **3**:22–24, 26–28. (1990).
3. M. J. Pikal. Freeze-drying of proteins part II: formulation selection. *BioPharm* **3**:26–30 (1990).
4. X. Tang and M. J. Pikal. Design of freeze-drying processes for pharmaceuticals: practical advice. *Pharm. Res.* **21**:191–200 (2004).
5. P. L. Privalov. Cold denaturation of proteins. *Crit. Rev. Biochem. Mol. Biol.* **25**:281–305 (1990).
6. 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).
7. 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).
8. H. A. Kramers. Brownian motion in a field of force and the diffusion model of chemical reactions. *Physica* **7**:284–304 (1940).
9. A. P. Demchenko. Concepts and misconcepts in the analysis of simple kinetics of protein folding. *Curr. Protein Pept. Sci.* **2**:73–98.
10. R. P. Bhattacharyya and T. R. Sosnick. Viscosity dependence of the folding kinetics of a dimeric and monomeric coiled coil. *Biochemistry* **38**:2601–2609 (1999).
11. T. Y. Tsong and R. L. Baldwin. Effects of solvent viscosity and different guanidine salts on the kinetics of ribonuclease. A chain folding. *Biopolymers* **17**:1669–1678 (1978).
12. P. L. Privalov, Y. V. Griko, S. Y. Venyaminov, and V. P. Kutyshenko. Cold denaturation of myoglobin. *J. Mol. Biol.* **190**:487–498 (1986).
13. Y. V. Griko and P. L. Privalov. Calorimetric study of the heat and cold denaturation of .beta.-lactoglobulin. *Biochemistry* **31**:8810–8815 (1992).
14. 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).
15. 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).
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. V. Griko lu and P. L. Privalov. Calorimetric study of the thermal denaturation of beta-lactoglobulin in the presence of urea and phosphate ions. *Mol. Biol.* **26**:150–157 (1992).
18. B. Wang and F. Tan. Calorimetric study of cold and thermal denaturation of bovine .beta.-lactoglobulin B. *Chin. Chem. Lett.* **4**:971–974 (1993).
19. B. Wang and F. Tan. DSC study of cold and heat denaturation processes of .beta.-lactoglobulin A with guanidine hydrochloride. *Sci. China, Ser. B Chem.* **40**:316–322 (1997).
20. D. Q. Wang, J. M. Hey, and S. L. Nail. Effect of collapse on the stability of freeze-dried recombinant factor VIII and a-amylase. *J. Pharm. Sci.* **93**:1253–1263 (2004).
21. X. Ma, D. Q. Wang, R. Bouffard, and A. MacKenzie. Characterization of murine monoclonal antibody to tumor necrosis factor (TNF-MAb) formulation for freeze-drying cycle development. *Pharm. Res.* **18**:196–202 (2001).
22. K. Gekko and H. Ito. Competing solvent effects of polyols and guanidine hydrochloride on protein stability. *J. Biochem. (Tokyo)* **107**:572–577 (1990).
23. 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).
24. M. O. Portmann, S. Serghat, and M. Mathlouthi. Study of some factors affecting intensity/time characteristics of sweetness. *Food Chem.* **44**:83–92 (1992).
25. M. J. Pikal. Mechanisms of protein stabilization during freeze-drying and storage: the relative importance of thermodynamic stabilization and glassy state relaxation dynamics. In L. Rey and J. C. May (eds.), *Drugs and the Pharmaceutical Sciences 96: Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products*, Marcel Dekker, Inc., New York, 1998, pp. 161–198.
26. L. Chang, X. Tang, M. J. Pikal, N. Milton, and L. Thomas. The origin of multiple glass transitions in frozen aqueous solutions. *Proc. NATAS Annu. Conf. Therm. Anal. Appl.* **27**:624–628 (1999).
27. E. Y. Shalaev and F. Franks. Structural glass transitions and thermophysical processes in amorphous carbohydrates and their super-saturated solutions. *J. Chem. Soc., Faraday Trans.* **91**: 1511–1517 (1995).
28. S. A. Knopp, S. Chongprasert, and S. L. Nail. The relationship between the TMDSC curve of frozen sucrose solutions and collapse during freeze-drying. *J. Therm. Anal. Calorim.* **54**: 659–672 (1998).
29. E. Maltini. Estimation of the viscosities of the frozen sucrose–water system from glass transition temperatures, *Ital. J. Food Sci.* **5**(4):371–377 (1993).
30. K.-I. Izutsu and S. Kojima. Excipient crystallinity and its protein-structure-stabilizing effect during freeze-drying. *J. Pharm. Pharmacol.* **54**:1033–1039 (2002).
31. A. Yoshida, S. Watanabe, S.-H. Chen, E. R. Giblett, and L. A. Malcolm. Human phosphoglycerate kinase. II. Structure of a variant enzyme. *J. Biol. Chem.* **247**:446–449 (1972).