Impact of Sucrose Level on Storage Stability of Proteins in Freeze-Dried Solids: II. Correlation of Aggregation Rate with Protein Structure and Molecular Mobility

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ABSTRACT: The purpose of this study is to investigate the impact of sucrose level on storage stability of dried proteins and thus better understand the mechanism of protein stabilization by disaccharides in lyophilized protein products. Five proteins were freeze dried with different amounts of sucrose, and protein aggregation was quantified using Size Exclusion Chromatography. Protein secondary structure was monitored by FTIR. The global mobility was studied using Thermal Activity Monitor (TAM), and fast local dynamics with a timescale of nanoseconds was characterized by neutron backscattering. The density of the protein formulations was measured with a gas pycnometer. The physical stability of the proteins increased monotonically with an increasing content of sucrose over the entire range of compositions studied. Both FTIR structure and structural relaxation time from TAM achieved maxima at about 1:1 mass ratio for most proteins studied. Therefore, protein stabilization by sugar cannot be completely explained by global dynamics and FTIR structure throughout the whole range of compositions. On the other hand, both the fast local mobility and free volume obtained from density decreased monotonically with an increased level of sucrose in the formulations, and thus the local dynamics and free volume correlate well with protein storage stability. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: protein stability; aggregation; stabilizer; freeze-drying; lyophilization; glass dynamics; molecular mobility; fast dynamics; local motion; protein structure; specific interaction; protein–sugar interaction; water substitute; structural relaxation; free volume; protein stabilization; fusion protein; cytokine

INTRODUCTION

The objective of this study is to investigate the impact of sucrose level on storage stability of five freeze-dried proteins in glassy solids and better understand the mechanism of protein stabilization by disaccharide during storage. Protein aggregation rate was quantitatively correlated to protein–sugar specific interaction model, protein secondary structure (monitored from FTIR), global mobility (τ^β from TAM), local mobility (monitored by neutron backscattering) and free volume (estimated from density data). In addition, the relative importance of FTIR structure and glass dynamics on protein stability was systematically evaluated.

The storage stability of proteins is of prime importance to the pharmaceutical industry.[1–5] If the liquid formulation has inadequate stability due to the fast degradation, proteins are commonly dried with a freeze-drying (lyophilization) method.[3–7] Sugars such as sucrose and trehalose have been frequently used as stabilizers to protect proteins during lyophilization and storage.[4–7] Two main hypotheses have been proposed to explain the stabilization mechanism of sugar on proteins during drying and storage. One is the "water substitution" hypothesis[8–10] and the other is the "glass dynamics" hypothesis.[11,12] Most of the reported studies on protein stabilization focused on only one mechanism when interpreting the data, and in such cases it is not obvious if the alternate interpretation is also consistent with the data. However, it must be recognized that either mechanism may dominate depending on the application, and it is possible that both mechanisms play a role in any given application.[13] The "water substitution hypothesis," which may be classified as a "specific interaction" mechanism, states that stabilizers can form hydrogen bonds at specific sites on the surface of the protein and thus substitute for the stabilization function of water that is lost during drying. Protein unfolding during the drying process is inhibited thermodynamically (i.e., the hydrogen bonding increases the free energy of protein denaturation); thus this hypothesis proposes a purely thermodynamic mechanism.[13] Assuming that the native conformation is more resistant to degradation during storage than a partially unfolded form, enhanced storage stability may be a result of native structure preservation in the dried solid. However, it is important to recognize that preservation of native structure during lyophilization could also be attributed to immobilization of protein molecules in a glass matrix during freezing and drying and "solute exclusion" hypothesis operating during the freezing step.[10,13,14]

There are many studies supporting the importance of hydrogen-bonding interaction in the preservation of native structure of proteins.[5,8,9,15–19] For example, the H-bonding interactions between the protein and sugar in the solid state has been characterized with Infrared spectroscopy.[8,9] The peak at 1580 cm^{-1} in Infrared spectrum, which is ascribed to the H-bond formed with a carboxylate group, was observed in the presence of sugar even though it was not detectable in the dried pure lysozyme.[9,15] These observations suggest that H-bonding between sugar and protein induced preservation of native protein structure during lyophilization. In addition, stability of several lyophilized monoclonal antibodies and small proteins correlate with the preservation of native secondary structure studied by FTIR and Raman spectroscopy.[8,16] While native structure preservation may well be important for protein stability, we also note that there are several reports of failure to correlate stability with the degree of retention of the native structure.[20–23]

The second hypothesis, the glassy dynamics hypothesis, states that the sugar forms a rigid, inert matrix in which the protein is molecularly dispersed, and the limited mobility in the glassy matrix dampens the protein mobility necessary for movement along the degradation pathway.[11,12] Thus, if the motion of reactants and protein in the solid are sufficiently slow on the time scale of experiment, the protein will not significantly degrade regardless of what the free energy of unfolding or chemical degradation might become, and stabilization is kinetically controlled.[24] The implicit assumption of this hypothesis is that the dynamics of the protein molecule (and reactant) couples with the dynamics of the glass, and the dynamics of this combination can be either viscosity-coupled global dynamics (often referred to as structural relaxation or α-relaxation) or local dynamics (β relaxation). It was originally thought that the higher the T_g (glass transition temperature) of the glass, the greater the level of protection the glassy formulation would offer the protein. However, it was found that a good glass former with high T_g, such as dextran, does not necessarily provide better stability than glass with a low T_g.[25,26] Thus, the T_g of the glass may not be a good predictor of molecular mobility. It might be expected, however, that the structural relaxation time would be a better indicator of global dynamics than "$T-T_g$". Global dynamics does not necessarily track with T_g, owing to the fact that the fragility and fictive temperature of the formulation may vary with excipient and method of preparation.[28,29] Recent studies demonstrated that stability correlates with structural relaxation time for some therapeutics.[27–29] A formulation with a longer structural relaxation time would mean greater stability, assuming degradation rate and structural relaxation time are strongly coupled. However, failure to correlate protein stability with global motion in some cases suggests that the local motion may also play an important role in stability.[30–32] Local mobility measured with neutron scattering spectrometry

and NMR was recently reported to correlate with stability of some proteins.[31–33]

Exploration of these hypotheses over the past two decades has improved our general understanding of key stability parameters, and some minimal criteria have been proposed for the development of stable protein products.[4,7] While protein structure-related parameters such as those taken from FTIR spectra, and parameters related to dynamics, such as T_g and structural relaxation time are useful for the guidance of formulation development, optimization of the protein formulation remains, in part, an empirical exercise, and the mechanisms of protein stabilization by sugars during storage are still not completely understood. Because of difficulties associated with exploring the properties of glass dynamics, the structural relaxation time of the protein formulation is not often reported in the literature, and local dynamics studies on protein/sugar system are even more rare. Therefore, a systematic study designed to investigate the impact of sucrose on both global and local dynamics of protein systems is needed.

The present work aims to investigate the impact of sucrose level on storage stability of several freeze-dried proteins in glassy solids and better understand the importance of both secondary FTIR structure and glass dynamics on protein stability. Sucrose was chosen because it is a widely used excipient in commercial protein products, and has been reported to provide higher stability than trehalose for several proteins at low temperature.[28,34–36] The aggregation rates of sucrose–protein formulations upon storage were studied as a function of sucrose level at temperatures below T_g, and correlations were investigated between storage stability and protein–sugar interactions, protein FTIR structure, global mobility (such as T_g and structural relaxation time), and fast local dynamics (measured by neutron backscattering) and free volume.

In this work we use five different therapeutic proteins including three fusion proteins and two cytokines on which to perform this extensive study on the effect of sucrose level on protein stability. Recently, fusion proteins have emerged as important therapeutics. These proteins are the results of a genetic fusion of two biologically relevant domains, typically a dimeric molecule in which each chain contains the Fc potion of an IgG fused to the soluble extracellular ligand-binding domain of a receptor.[37] The properties of fusion protein (such as stability, molecular mobility etc.)

as a function of sucrose level have not been well studied. It is our intention that with an extensive study of different proteins with molecular weight ranging from 19 to 185 kDa, a better understanding of mechanism of protein stabilization in glassy solids can be achieved.

MATERIALS AND METHODS

Materials

Five proteins including three IgG1 fusion proteins and two recombinant cytokines were provided by Wyeth Biopharma (Andover, MA). The molecular weights ranged from 19 to 185 kDa (Tab. 1). All protein-containing solutions were buffer exchanged through a regenerated cellulose membrane filter (Millipore, Bedford, MA[1], area of 50 cm^2, with a 30 kDa Molecular Weight cut off value used for three fusion proteins, 10 kDa for protein D and 5 kDa for protein E). Protein solutions (10 mg/mL) were prepared after dialysis against two buffer solutions: one buffer was 10 mM histidine, pH 6.5; the other was 10 mM histidine and 40 mg/mL of sucrose, pH 6.5. By mixing two stock solutions at different ratios, variation in the mass ratio of sucrose to protein of 0, 0.5, 1, 2, and 4 was achieved in the final protein solutions. L-histidine, sodium chloride, poly-L-lysine, 2-hydroxylpropyl-β-cyclodextrin, pluronic F68 (a hydrophilic nonionic surfactant), polysorbate-80, morpholinoethane sulfonic acid (MES) and glycine were purchased from Sigma (St. Louis, MO). α-Sucrose with reduced heavy metal (<5 ppm) content was purchased from Calbiochem of EMD Biosciences (San Diego, CA). These reagents were of the highest grade available, and were used without further purification.

Lyophilization Procedures

The protein formulations with different mass ratios of sucrose to protein were prepared as described above while maintaining the protein concentration as a constant (10 mg/mL for five proteins). All the protein solutions were buffered with 10 mM histidine at pH 6.5. This pH was

Table 1. Thermal Properties of the Freeze Dried Protein Formulations Containing Different Content of Sucrose

| Protein | Description | M_w (kDa) | P10S0 (°C) | | P10 S5 (°C) | P10S10 (°C) | P10 S20 (°C) | P10S40 (°C) | | |
			T'_g	T_g	$T_g{}^b$	$T_g{}^c$	T_g	T'_g	T_c	T_g
A	Fusion protein of human IgG1	150	−4	a	120	105 (0.38)	92 (0.36)	−28.6	−26	77°C (0.38)
B	Fusion protein of human IgG1	125–130	−4	a	101	94 (0.37)	80 (0.39)	−28.2	N/D	72 (0.37)
C	Fusion protein of human IgG1	185	−6	a	106	101 (0.24)	84 (0.32)	−25.3	N/D	76 (0.33)
D	Cytokine	75	N/D	a	104	95 (0.27)	87 (0.31)	−23.8	N/D	76 (0.35)
E	Cytokine	19	N/D	a	89	81 (0.27)	77 (0.28)	−29.0	N/D	73 (0.29)

T'_g, T_c, T_g are shown, and the change of heat capacity at T_g (ΔC_p, J/g/°C) are included in the bracket. Notice that the sucrose level increases in formulation from P10S0 to P10S40, where P represents the protein, S represents sucrose and the number is the mass (mg) of the component in the formulation that was freeze dried. N/D, not determined.
[a]The transition of pure protein could not be detected using DSC.
[b]T_g measured from MDSC is the middle-point of the glass transition.
[c]The maximum standard deviation in T_g was ±0.6°C, and the maximum standard deviation in ΔC_p was ±0.02 J/g/K.

selected because it is close to the optimal pH for stability in the solution state (range from 6 to 7.2 for all proteins), which should reduce the degree of structure perturbation during the freeze drying process.

Glass transition temperature of freeze-concentrate (T'_g) values were determined using Modulated DSC Q1000 (TA Instruments, New Castle, DE). Approximately 15 µL samples for each formulation were hermetically sealed in an aluminum pan, frozen to −60°C and heated at 2.5°C min^{-1} to 20°C, using modulation with a ±0.5°C amplitude every 100 s period. T'_g reported here is the mid-point of the transition.

The collapse temperature (T_c) was measured using freeze-drying microscopy (Linkam FDCS-196 stage attached to Nikon Eclipse E600 microscope, McCrone, Chicago, IL) equipped with a liquid nitrogen cooling system and programmable temperature controller. The sample was frozen to −40°C and the pressure reduced to about 6.6 Pa (50 mTorr). The stage temperature was immediately changed to −35°C. A 30-min hold at −35°C resulted in formation of zone of product dried with retention of structure against which structure at higher temperatures could be compared to assess collapse. The temperature of stage was increased slowly in 1°C increment with a 10-min holding step at each temperature, and the frozen sample was observed with polarized light microscopy. The collapse temperature (T_c) is the temperature at which the sample starts to lose structure in the dried region adjacent to the ice. The T_c and T'_g results for all formulations are listed in Table 1. Based on these data, a target product temperature of −30°C was used for the design of lyophilization cycle for the protein formulations.

The protein samples were lyophilized in a Genesis 25 EL (Virtis, SP Industries, Gardiner, NY) freeze-dryer. All the protein formulations were filled into 2 mL tubing glass vials (1 mL fill volume). The products were first dried at a shelf temperature of −25°C and a chamber pressure of 6.6 Pa (50 mTorr) for 20 h, the pressure was then decreased to about 4 Pa (30 mTorr), and the sample was further dried at this lower pressure and a shelf temperature of −25°C for 35 h. Primary drying was conducted below the T'_g of all formulations. Secondary drying was carried out for 6 h at 25°C. After the drying process was finished, nitrogen was backfilled into the vials. All formulations for a given protein were freeze-dried as one batch and sealed with Daikyo Fluorotec stoppers (West Pharmaceutical, Lititz, PA). The

thermocouple data from vials positioned in the center of shelves showed that the product temperature profiles were very similar in all the batches. All samples freeze dried with full retention of cake structure.

Residual Water Measurement

The water content for each freeze-dried formulation was determined using Karl Fischer coulometric titration (Aquastar C3000 Coulometric Titrator). At least two vials of each formulation were used. All the samples were amorphous after freeze-drying as evidenced by the absence of birefringence when examined with Polarized Light Microscopy (PLM). Even after storing the freeze-dried vials for 6 months at 50°C, no crystallization was noticed (PLM) in any of the formulations. However, after exposing the formulation with a sucrose/protein ratio of 4 to air for 2 days, crystallization was observed with PLM, which was confirmed to be sucrose by X-ray diffraction (Bruker AXS model D8/Advance).

Differential Scanning Calorimeter (DSC) Measurements

The glass transition temperature (T_g) and the change of heat capacity at T_g (ΔC_p) of each formulation were measured using Modulated DSC (TA Instruments, Q1000). Approximately 5–10 mg samples for each formulation were first compressed to form a powder compact, and then hermetically sealed in an aluminum pan. All these operations were carried out in a nitrogen-purged glove box with relative humidity <2%. Before use, the DSC instrument was first calibrated using sapphire samples for baseline calibration; indium and water were used for temperature calibration. Finally, crystalline sucrose was used to calibrate the heat capacity based on the published data.[38] The samples were run at a ramp rate of $2°C\,min^{-1}$, modulated with a $\pm0.5°C$ amplitude and a 100 s period. The DSC cell was purged with nitrogen at a flow rate of 50 mL/min. The relaxation enthalpy at infinite time (ΔH_∞), which is used for the measurement of structural relaxation time at temperature T, was calculated using the following equation:

$$\Delta H_\infty = (T_g - T) \times \Delta C_p \qquad (1)$$

Investigation of the Global Mobility in the Glassy Solid by Thermal Activity Monitor (TAM)

Background

An amorphous solid will relax toward the equilibrium state and release heat when stored at temperatures below T_g. The rate of the heat released from the sample can be directly measured with isothermal calorimetry (i.e., TAM). The derivative version of the Kohlraush–Williams–Watts (KWW) equation can be used to describe the resulting power-time curve.[29]

$$P = 277.8 \cdot \Delta H(\infty) \cdot \left(\frac{\beta}{\tau}\right)$$
$$\cdot \left(\frac{t}{\tau}\right)^{\beta-1} \cdot \exp\left[-\left(\frac{t}{\tau}\right)^{\beta}\right] + C \qquad (2)$$

where P is power in µW/g, t is time in hours, τ is most probable relaxation time, β ($0 < (\leq 1)$ is a measure of the width of distribution of relaxation times, ΔH_∞ is the relaxation enthalpy at infinite time at the experimental temperature calculated from Eq. (1), and C is a constant included to represent possible heat flow from a degradation reaction.

The KWW equation is a poor approximation for the actual kinetics at small times since the first derivative of this equation approaches infinity as time approaches zero.[39] The modified stretched exponential equation (MSE) is usually recommended when the initial relaxation behavior of an amorphous material is analyzed.[29,39,40]

$$P = 277.8\frac{\Delta H_\infty}{\tau_0}\left(1+\frac{\beta t}{\tau_1}\right)$$
$$\times \left(1+\frac{t}{\tau_1}\right)^{(\beta-2)}\exp-\left(\frac{t}{\tau_0}\right)\left(1+\frac{t}{\tau_1}\right)^{(\beta-1)}\Bigg]$$
$$+ C \qquad (3)$$

where τ_0 and τ_1 are relaxation times. τ_D is equivalent to τ of the KWW equation and is calculated as:

$$\tau_D = (\tau_0\tau_1^{\beta-1})^{1/\beta} \qquad (4)$$

When an amorphous sample is annealed during the TAM study, the structural relaxation time constantly increases with time due to the depopulation of fast relaxation species. Thus, neither kinetic expression above (Eq. 2 or Eq. 3) can be correct since both assume a constant value of τ throughout the annealing process. The net result of this change in τ is that the value of τ and

β obtained by analysis of the power-time curve according to either Eq. (2) or Eq. (3) are too large and too small, respectively.[40,41] However, τ^β (or τ_D^β) is an accurate representation of the initial state of the sample.[39,41] Therefore, the τ_D^β value is reported in this study.

Thermal Activity Monitor (TAM) Measurements

The rate of enthalpy relaxation (dH/dt) was directly measured using a Thermal Activity Monitor (TAM III) (minicalorimeter, Thermometric, Jarfalla, Sweden). Approximately 150–200 mg samples of each formulation were sealed into dry stainless steel vessels in a glove box purged with nitrogen. The built-in Alumina insert was used as the inert reference. Before the baseline was determined, zero calibration and electrical calibration of 60 μW were performed with TAM Assistant software (Thermometric). After slowly loading the sample ampoules into the equilibration positions of TAM chambers for a 30 min thermal equilibration, the signal came back to baseline. The sample ampoules were then gently lowered into the measuring positions. To avoid artifacts due to the thermal disturbance, only data after the first hour in the measurement position were used in the data analysis. All samples were run in the TAM at 50°C for about 3 days, and then the original data was exported for data analysis with Origin software (OriginLab Corp., Northampton, MA). Since the T_g and C_p could not be detected for pure protein from DSC, the value of ΔH_∞ for pure protein was obtained as an adjustable parameter in the nonlinear regression analysis. The results were found to be consistent with ΔH_∞ for the other formulations.

Determination of Protein Aggregation by Size Exclusion Chromatogram

Assays for chemical degradation were not utilized because the samples studied did not show sufficient degradation to allow a quantitative analysis of chemical instability. Size Exclusion Chromatography (SE-HPLC) was used to assess the physical stability of proteins with respect to aggregation during lyophilization and storage. The initial assays were run immediately after freeze-drying for each protein formulation. The remaining sample vials were then transferred to incubators with controlled humidity, and stored in sealed vials at 25°C/60% relative humidity (RH) for (12 months), 40°C/75% RH (1, 3, 6, and 12 months) and 50°C (1, 3, and 6) months. It is recognized that since the vials were sealed, the relative humidity during storage was irrelevant, but these humidity conditions were the conditions available for 25 and 40°C. After storage for the appropriate time, the samples were reconstituted with water and analyzed with SE-HPLC. All protein solutions for stability study were clear and no "hazy" appearance was noticed after reconstitution; thus the proteins studied did not show significant insoluble aggregate.

For protein E analysis, a TSK-2000SWxl column (7.8 mm × 300 mm, Tosoh Biosep, Montgomeryville, PA) with a flow rate of 1.0 mL/min was utilized The mobile phase consisted of 50 mmol/L MES, 0.1 mmol/L glycine and 0.5 mol/L NaCl (pH 6.0). The column was run at 5°C, and the UV absorption was monitored at 225 nm. For the other proteins, a column of YMC DL20S05-3008WT (8.0 mm × 300 mm, Waters, Milford, MA) was used at room temperature with a flow rate of 1 mL/min. The mobile phase consisted of 20 mmol/L NaH_2PO_4 and 0.2 mol/L NaCl, pH 7.2. The standard error in the percentage of aggregate for all sample replicates was <0.2%.

Reconstitution of lyophilized protein formulations after storage was also carried out using reconstitution media containing 0.5% (w/w) additives such as 2-hydroxylpropyl-β-cyclodextrin, poly-L-lysine, pluronic F68, Polysorbate-80 or Polysorbate-20. Also, the impact of sucrose level on aggregation during reconstitution was tested by reconstitution with water containing different concentrations of sucrose. To be consistent with the literature study on another cytokine (IL-2),[42] protein E solution after reconstitution was filtered with a 0.22 μm syringe filter (PVDF membrane, Millipore), and the concentration of the filtered protein was detected with UV absorbance at 275 nm. However, it was noticed that the filtering step does not impact the reconstitution result. For other proteins, the SE-HPLC analysis was used to estimate the amount of aggregate in the reconstituted sample.

Powder Density Measurement Using Gas Pycnometer

The density of dried protein formulations was measured using an Accupyc 1330 helium pycnometer at room temperature (Micromeritics,

Norcross, GA). The pycnometer was calibrated with an iron sphere standard of known volume before the density measurement, and the measurement was carried out inside a glove bag with controlled humidity (below 2% RH) to prevent the moisture uptake by the hygroscopic freeze dried samples. Approximately 100 mg of amorphous protein sample was loaded into a 1 mL aluminum sample cup, and the cup was then transferred into the measurement chamber. The sample was purged with helium for about 30 min with a purge fill pressure of 134 kPa (19.5 psig), and then sample density was measured with number of runs fixed at 20 and the helium equilibration rate of 34 Pa/min (0.005 psig/min). The gas pycnometer was tested for accuracy and precision using crystalline sucrose. The published density value of crystalline sucrose is 1.587 g/mL,[43] and our measurements gave a density value of 1.588 ± 0.003 g/mL ($n = 5$, replicates on 5 different days). The reproducibility of density measurement on four different amorphous sucrose samples gave 1.509 ± 0.005 g/mL.

Fast Dynamics from Elastic Incoherent Neutron Scattering Spectrometer

The high flux backscattering (HFBS) spectrometer at the National Institute of Standards and Technology (NIST) Center for Neutron Research on the NG2 beam line was used to study the fast dynamics. The HFBS spectrometer operates with an incident neutron wavelength of 6.271 Å and a kinetic energy of 2.08 meV.[32] The accessible momentum transfer (Q) range is 0.25–1.75 Å^{-1}. About 300 mg of freeze dried samples were loaded into sealed neutron scattering sample cells in a dried glove box. In these studies, the spectrometer operates in the fixed-window scanning mode where the elastic scattering intensity (I) is recorded as a function of Q while the sample is heated at 1 K/min from 40 K to about 330 K (all temperatures were below the glass transition temperature of the tested formulations).

The elastic scattering intensity was analyzed in terms of the Debye–Waller factor, assuming a harmonic oscillator model, using DAVE software (available at http://www.ncnr.nist.gov/dave). The hydrogen-weighted mean-square atomic displacement ($<u^2>$) was obtained at temperatures throughout the range reported above.[32] In order to remove small artifacts of the coherent scattering signal, I was normalized to the intensity at the lowest temperature available, which is about 40 K. Performing this normalization is equivalent to assuming that $<u^2>$ is zero at 40 K. We compensate for this effect by fitting a straight line through the $<u^2>$ versus T data between 40 K and 100 K, and vertically offsetting the data with the intercept of this line so that $<u^2>$ is zero at temperature of 0 K. A larger $<u^2>$ means a higher local mobility of the system.

The Debye–Waller formalism is a harmonic approximation for the high Q data ($Q^2 > 0.5$ Å^{-2}), where the motions are smaller and the harmonic approximation is most appropriate.[33] It is important to realize that only those motions faster than 200 MHz (or faster than a 5 ns time constant) can be measured with this instrument, and slower motion with a timescale longer than 5 ns is not detected. Fundamentals of the technique can be found in the literature.[44]

RESULTS AND DISCUSSION

Protein Stability

During the filtration process used during buffer exchange, protein molecules were exposed to air/protein interface and some shear stress, which might cause protein aggregation. The level of aggregation after the filtration process was analyzed, and the sucrose/protein formulations showed lower aggregation than the protein formulation without sucrose (data not shown). Thus, it appears that sucrose protect proteins during the filtration process, perhaps due to "solute exclusion" as reported elsewhere.[14,16] Immediately after freeze-drying, the protein/sucrose formulations exhibited lower levels of aggregation than pure protein samples (data not shown), and sucrose stabilize proteins during the lyophilization process.

It has been reported that for lyophilized interleukin-2 (IL-2) and keratinocyte growth factor, the amount of aggregates measured was impacted by using different reconstitution media.[42,45] The presence of polyanions with high charge density such as poly-L-lysine resulted in the decrease in the level of aggregation, while surfactants such as pluronic caused an increase in the amount of IL-2 aggregate. Thus, the reconstitution media impacts the formation of aggregate, presumably by changing the balance between refolding and aggregation of partially unfolded species during reconstitution. This reconstitution media effect was also investigated in this research, and Table 2 shows the impact of

Table 2. Effects of Reconstitution Medium on Recovery of Soluble Cytokine in Protein E Formulation with Sucrose Mass Fraction of 0.8 and IgG1 Monomer Peak Area in Protein C Formulation with Sucrose Mass Fraction of 0.5

Reconstitution Medium	Stability Ratio for Protein E[a]	Monomeric Peak Area Ratio for IgG1[b]
Phosphate buffer	1.00	0.99 (0.3)
2-hydroxylpropyl-β-cyclodextrin	0.99	0.98 (0.1)
Poly-L-lysine	0.94	0.97 (1.3)
Polysorbate-20	1.01	0.97 (0.1)
Pluronic F68	0.97	0.97 (0.1)
Polysorbate-80	1.01	0.97 (0.2)

0.5 mass % additives unless specified.

[a]Stability ratio is defined as the recovery of soluble protein when the lyophilized protein was reconstituted with an additive solution to that reconstituted with pure water. The maximum standard deviation in stability ratio was about ± 0.02.

[b]Monomeric peak area ratio is the monomeric peak area for IgG1 when the sample was reconstituted with an additive solution to that reconstituted with water. Values in brackets represent standard deviation ($n = 6$).

reconstitution medium composition on the relative stability of a cytokine. The stability ratio is defined as the recovery of soluble protein when the lyophilized protein was reconstituted with a given additive solution to that reconstituted with pure water.[42] None of the additives significantly impacted the amount of aggregate. The two IgG1 proteins gave similar results; the amount of monomer was not impacted by the reconstitution media composition. Thus, the data suggest that aggregation of these proteins occurs mainly during the storage of lyophilized formulations, even at temperature well below T_g. Similarly, reconstitution in water containing different amounts of sucrose did not change the amount of aggregate (data not shown). Therefore, differences in storage stability between protein/sucrose formulations are mainly the result of chemical and dynamic processes that occur during storage in the solid state, at least for the proteins investigated. Essentially the same results were obtained for β-galactosidase.[35] It has been suggested that aggregation of protein in the solid state can occur between protein molecules which are nearest neighbors even though large-scale translational diffusion motion is largely absent in the rigid glass.[13,35,46]

When the level of aggregation is plotted against storage time, a nonlinear relation was observed for all proteins studied. However, when the level of aggregation was plotted against the square root of time, as illustrated in Figure 1, a straight line was observed. Square root of time degradation kinetics are commonly observed in glassy

materials.[2,40,46] The square-root of the time (stretched time) kinetics presumably results from a superposition of reactions from a number of sub-states, each of which have a different degradation rate.[36,41] A key point is that in a glass, the sub-states are not in structural equilibrium. Thus, when the faster reacting sub-states are depopulated by reacting, only the slower reacting states remain. The aggregation rate constants were obtained as the slope of the straight line when
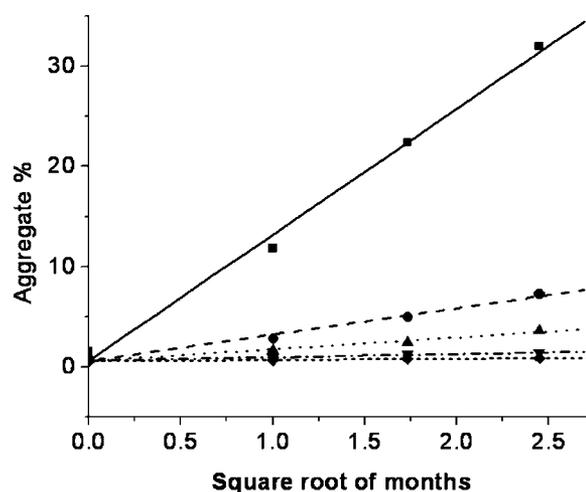
Figure 1. An illustration of the aggregation kinetics using aggregation data of protein B after storage at 50°C. Linearity was observed when aggregation is plotted as a function of square root of time for sucrose/protein mass ratio of 0 (■), 0.5 (●), 1(▲), 2 (▼), and 4 (□). The line is the best fit of Eq. (5) to the aggregation data. Error bar is smaller than the size of the symbol.

fitting the stability data to the following equation,

$$P = P_0 + k\sqrt{t} \qquad (5)$$

where P_0 is the initial percentage of aggregate, P is the percentage of aggregate after storage for time t, and k is an empirical aggregation rate constant.

Table 3 lists the effect of sucrose level on the aggregation rate constant of the freeze-dried proteins. As evident from the table, the rate constants decrease monotonically with an increase of sucrose level at both 50 and 40°C. Similar qualitative trends in the aggregation rate constant with sucrose level have been observed with other proteins.[46] The rate constants for different protein formulations at 50°C were plotted against the mass fraction of sucrose in Figure 2. Figure 2A displays the rate constants on a linear scale. In this format, it appears that a plateau is developing near the 0.8 sucrose mass fraction point, and one might be tempted to conclude that the degradation rate is approaching a plateau. However, the linear format can be misleading when the ordinate values become small compared to the range of the plot axis. Figure 2B displays this same data on a logarithmic scale. In this presentation it is clear that no plateau is observed. It seems plausible that the activation energy for protein aggregation would be a relatively simple function of sucrose fraction, perhaps even a linear function; thus, $\log k$ would be linear in sucrose fraction. Indeed, a log-linear dependence of rate constant on the sugar fraction has been observed in several literature reports.[35,46]

However, there are several reports that a protein appeared to reach maximum in stability at a certain level of sugar in the dried formulations.[1,47] Specifically, physical stability increased as sugar level increased but beyond a critical point, stability remained approximately constant. However, these reports typically only showed the percent aggregation at one time point versus composition, and the claims of no change seemed to be a result of limited assay sensitivity and/or the method of data plotting. Indeed, if the data are replotted in the form of log of rate constant as a function of concentration, the apparent plateau may no longer appear.[48] Even though these formulations may be sufficiently stable from a practical viewpoint to meet the shelf-life requirement,[49] the usage of the term "maximum stability" in this context may cause confusion. Our results are consistent with previous findings that

Table 3. Effect of Sucrose Level on the Aggregation Rate Constant of Freeze-Dried Proteins at 50 and 40°C

Mass Fraction of Sucrose, X_s	Protein A		Protein B		Protein C		Protein D		Protein E	
	50°C	40°C	50°C	40°C	50°C	40°C	50°C	40°C	50°C	40°C
0	9.5±0.7	5.5±0.5	12.5±0.7	6.2±0.4	15±0.7	7.5±0.5	4.9±0.6	0.46±0.04	0.95±0.1	0.31±0.13
0.333	3.4±0.2	1.8±0.14	2.6±0.2	1.2±0.07	4.3±0.4	2.0±0.2	1.4±0.1	0.23±0.13	0.3±0.06	0.11±0.1
0.5	1.4±0.1	0.3±0.05	1.2±0.1	0.48±0.02	1.8±0.2	0.6±0.16	0.55±0.08	0.14±0.11	0.2±0.08	a
0.667	0.4±0.01	0.05±0.02	0.3±0.02	0.13±0.01	0.3±0.06	0.16±0.05	0.23±0.05	0.10±0.07	0.1±0.04	a
0.8	0.09±0.01	0.03±0.02	0.1±0.01	0.05±0.02	0.1±0.02	a	0.16±0.03	a	0.02±0.01	a

The rate constants (%/month$^{1/2}$) were obtained using square root of time kinetics as in Eq. (5). The standard error which is the uncertainty in the best fit is also shown.
a The rate constant is not available because insufficient aggregation observed in the formulation does not allow accurate calculation of the rate constant.
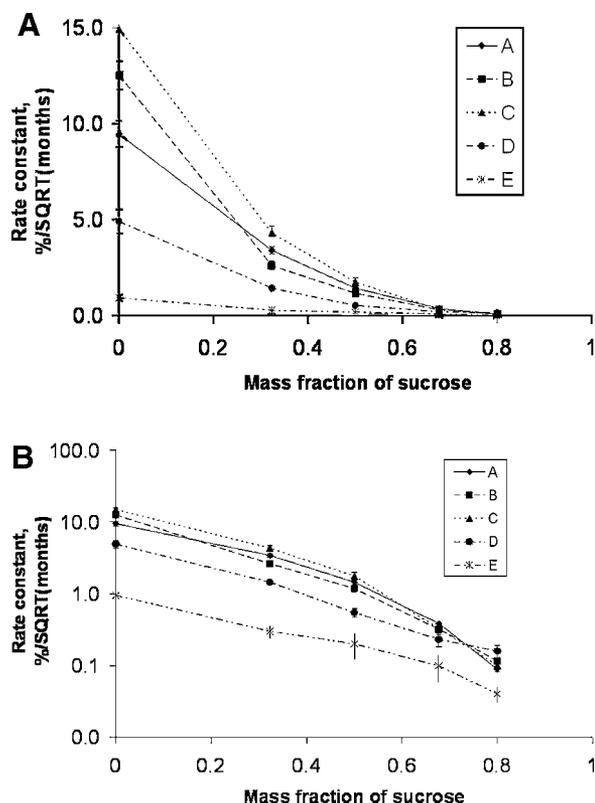
Figure 2. Effect of sucrose level on the long-term storage stability of freeze-dried proteins at 50°C. The rate constant was plotted in linear scale in (A) and in logarithmic scale in (B). The rate constant was calculated from the square root of time kinetics, and error bars are standard error in rate constant as given by regression analysis.

Figure 3. Comparison of predicted and experimental levels of protein aggregation after the protein formulations were stored at 25°C for 12 months. Predicted aggregation levels were calculated based on square-root-of-time kinetics and Arrhenius behavior in the rate constants obtained from accelerated stability study. Error bars are smaller than the symbol size. The multiple entries for the same protein represent the five formulations with different sucrose/protein mass ratios. The lower is the sucrose/protein mass ratio, the higher the percentage of the protein aggregation.

aggregation rate constants decreased monotonically with increasing sugar level (even up to 95% of sugar in the formulation).[35,40,46]

Based on an Arrhenius plot using aggregation rate constants obtained at 40 and 50°C, the rate constant at 25°C was estimated. In order to assess the validity of the extrapolation from high temperature to room temperature, we compared the predicted aggregation rates to experimental aggregation data from samples stored at 25°C for 12 months. The predicted aggregation level was calculated based on Eq. (5). Figure 3 gives the comparison of the experimental versus predicted aggregation at room temperature after 1-year storage. The solid line represents the expected values of aggregation. The predicted and experimental data correlate very well, indicating that aggregation follows Arrhenius behavior and square-root-of-time kinetics and demonstrating that reliable predictions of stability at a lower temperature can be made from accelerated stability test data, at least in the systems studied. We do note that one point strays away from the correlation line, where the predicted aggregation level in the pure protein A formulation is greater than the experimental value. The exact reason for this difference is not known; however, the deviation may be related to the elevated water content in that sample. It is reported that one IgG antibody demonstrated a minimum in degradation rate at about 3% (w/w) residual water.[50] For pure protein A, the water content is elevated after 12 months storage, and the elevated water content may bring the sample within the water content range for optimal stability, thus conferring greater stability than expected from extrapolation from dryer samples.

Physical Stability and Dilution Effect

One possible mechanism for the stabilization of protein in sucrose is the dilution effect. It can be argued that as the protein molecules are diluted in a solid matrix, the probability of close contacts between two proteins decreases, and thus the probability of aggregation decreases.[5,46] This

"dilution effect" does not demand any particular interaction between protein and sucrose. Based on a derivation similar to "regular solution" interaction model, empirical reaction rates (k_1), was found be proportional to the mass fraction of protein (X_p) in the formulation[46]

$$k_1 = k_0 \times Xp, \qquad (6)$$

where k_1 is the rate constant and k_0 is the relative rate constant for pure protein sample.

From Eq. (6), the expected stabilization factor due to dilution (k_0/k_1) can be determined as a function of sucrose mass fraction. For example, at a sugar/protein mass ratio of 2:1, the mass fraction of protein is about 0.33. Therefore, we expect the aggregation rate constant in the pure protein to be three times greater than that of the sugar/protein ratio of 2:1 formulation. However, from the rate constants at 50°C, we find that the stabilization factor for a sugar/protein ratio of 2:1 formulation is 25 for protein A, 39 for protein B, 46 for protein C, 21 for protein D and 10 for protein E. Since all of these values are much higher than 3, the stabilization effect from a sugar matrix is much greater than that which can be accounted by the dilution effect alone. Other mechanisms must be important for the stabilization of protein molecules in the sugar matrix.

Interpretation of Stability Trends with a Stoichiometric Binding Model

The specific interaction between the sucrose and protein suggests that this interaction must involve binding of the sugar to the protein in some stoichiometry, but there must be a limited number of binding sites on the protein surface to form hydrogen bonds with the stabilizer. Therefore, there should be a level of sucrose beyond which further addition of sugar has no measurable effect on protein structure, but unless the binding constant is effectively infinite, the level of sucrose needed to reach saturation will be larger than the minimum required by stoichiometry. The water substitute concept[8–10] suggests that sugar replaces the water lost during secondary drying, and thus the number of sucrose molecules required to saturate the water binding sites on the protein surface should be less than or equal to the number of waters required to do so, since sugars are multi-valent with respect to H-bond donation and acceptance. Therefore, a given sugar could occupy one or more water binding sites, assuming steric constraints are not severe. The water binding sites of the proteins have been studied by water sorption experiments,[51] and the number of water molecules required to occupy all of the strong water-binding sites is listed in Table 4. Note that the water binding sites based on water sorption experiments are similar to those predicted from protein primary structure, including both amino acid sequence and glycosylation information.[51] FTIR results showed that the protein native structure reached a plateau at 1:1 sugar/protein mass ratio.[51] The mole ratio of sugar to protein at this 1:1 mass ratio is comparable to the number of water binding sites on a single protein (range of 1.0–1.4) as measured from water sorption experiments. Thus, based on

Table 4. Comparison of the Binding Sites Per Protein Molecule Based on Protein Structure, Water Monolayer, and Stoichiometric Binding Model

Protein	Binding Sites From Protein Structure[a]	Binding Sites (m) From Measured Monolayer[b]	Sugar/Protein Molar Ratio (l) at 1:1 Mass Ratio[c]	Binding Number Per Sucrose at 1:1 Mass Ratio[d]	K From Stoichiometric Binding Model	n From Stoichiometric Binding Model[e]	m/n
A	688	600	439	1.4	24	323	1.9
B	563	477	380	1.3	31	211	2.3
C	586	668	541	1.2	24	342	2.0
D	[f]	263	219	1.2	23	138	1.9
E	72	57	56	1.0	14	29	2.0

[a]See Ref.[51]
[b]Binding sites can be estimated from measured water monolayer (M_0) using the following expression: $m = (M_0 \times M_w/18)/100$.
[c]Sugar/protein molar ratio(l) at 1:1 mass ratio can be calculated using the following expression: $l = (1/342)/(1/M_w)$.
[d]Binding number per sucrose molecule at 1:1 mass ratio is estimated using the ratio of m/l.
[e]Binding constant (K) and Binding sites (n) was calculated based on the fit of stoichiometric model (see Ref.[46]) to aggregation rate constant as a function of sucrose level.
[f]Not available due to lack of glycosylation information.

this calculation, it appears that each sucrose molecule occupies approximately one water-binding site when the protein/sugar binding reaches saturation. Even though there appears to be a saturation level for both the protein structure and sugar/protein interaction, at least within the sensitivity of these measurements, there is no corresponding saturation effect in stability for any of the proteins over the sucrose concentration range studied (see Tab. 3 and Fig. 2B). However, the absence of saturation in stability does not necessarily rule out a stoichiometric binding interpretation if the binding constant is of modest strength.

In order to quantitatively evaluate the impact of stoichiometric binding, experimental stability data were fit to a previously described stoichiometric interaction model[46] to estimate the binding stoichiometry and binding constants. MLAB (Civilized Software, Silver Spring, MD) was used to fit the theory to the aggregation rate constants vs. sugar content data (see Appendix II in Ref.46). A key assumption in the model is that protein molecules that have all binding sites fully occupied are essentially un-reactive, where the binding event likely occurs during formation of the product (i.e., during freeze-drying). The experimental stability data fit the model well, suggesting that the stoichiometric binding model is at least consistent with the data. The calculated number of binding sites per protein, n, and the thermodynamic equilibrium constant for binding, K are also listed in Table 4. It seems that n values are reasonable in that all are less than the number of water binding sites. Strikingly, the ratio of water binding site per sucrose is approximately 2 for all proteins studied. Thus, based on this model, sucrose seems to occupy two water-binding sites at full saturation. Also, notice the binding constant K is in the range of 10–30, which is smaller than expected. One might expect $K \approx 300$ based on the contribution of a typical hydrogen-bond to the binding free energy.[46] However, accepting the small binding constant as a reflection of reality, a small binding constant demands a large excess of sugar to drive the equilibrium to saturation and thereby decrease the fraction of un-bound (and reactive) protein to negligible levels. Thus, a stoichiometric binding model does appear consistent with the stability data.

While this model does fit the stability trend and does provide some insights to the protein structure preservation during the freeze-drying process, some ambiguities need to be discussed. First,

the model assumes that the aggregation rate is negligible with full saturation of the binging sites.[46] Since the fraction of "bound" protein will always be <1 even at a high binding constant K, the binding between protein and sugar will never saturate even at extremely high sucrose levels. This behavior seems inconsistent with the experimental results from FTIR structure, water sorption, and solution calorimetric study in that the protein-sugar interaction DOES saturate when measured by these techniques.[51] Perhaps these experimental techniques simply do not have the sensitivity to detect the impact of a very small fraction of the protein in the reactive state, but then why does the secondary structure never attain "full native" structure, within the sensitivity of the FTIR technique, even at the highest sucrose levels studied? Second, the model assumes that the number of binding sites for a given protein is a constant, regardless of structural state. However, the native structure of the pure protein is significantly perturbed as shown in FTIR spectrum,[51] and the degree of native structure preservation improves upon adding more sucrose, at least in the range up to 1:1 mass ratio. The impact of variable structure on binding thermodynamics is not known. Third, the binding constants obtained are much smaller than the expected values, making it difficult to argue that the specific interaction between protein and sugar is responsible for the stability trends. That is, more plausible binding constants, on the order of 100, do not fit the data!

Correlation of Stability with Native Structure Preservation

In order to study the structural changes of proteins freeze dried from formulations with different sucrose levels, the FTIR spectra of all protein formulations were investigated.[51] A brief summary of the FTIR results are given in Table 5. The peak height of the major band in the solid protein formulation was divided by that in aqueous solution, and this peak height ratio was used as the measure of the degree of native structure preservation. When sucrose was added into the formulations, the extent of the structure perturbation decreased as shown by the increase in the peak height. When the mass fraction of sucrose reached 0.5, the degree of structure protection by sucrose begins to plateau, and further increases in sucrose level does not

Table 5. Impact of Sucrose Level on the FTIR Secondary Structure in the Protein Formulations

Mass Fraction of Sucrose, X_s	Protein A	Protein B	Protein C	Protein D	Protein E
0	0.69	0.81	0.79	0.79	0.82
0.333	0.76	0.85	0.86	0.88	0.87
0.5	0.81	0.88	0.89	0.92	0.95
0.667	0.81	0.92	0.89	0.92	0.95
0.8	0.81	0.92	0.89	0.92	0.94

The value shown is the ratio of peak height of the major band in dried solid to that of liquid solution. The major band for protein A, B, C, and D is the β-sheet peak at about 1641 cm^{-1} and for protein E is the α-helix peak at about 1655 cm^{-1}. The standard error in three replicates for the same sample is about 0.01.

significantly improve the native structure preservation. Such saturation in protein structure at similar sugar levels has been observed in other proteins.[34,46,52]

Our purpose is to correlate secondary structure, as determined by FTIR, to protein stability. We acknowledge that the measure of protein structure in the solid state using FTIR spectroscopy has limitations. First, in this study, the quantification of the degree of structure preservation was based on peak height comparison (the ratio of peak height of dried solid to that of liquid solution). It is possible that other measures of spectral similarity between dried solid and liquid solution would give somewhat different results.[51] Secondly, FTIR can only probe the secondary structure of a protein, but it is certainly possible that changes in tertiary structure could impact stability.

Protein-sucrose interaction leading to "water replacement" was also studied from water vapor sorption.[51] The degree to which sucrose replaced water at the protein surface was determined using the difference between water required to create a "monolayer" at the protein surface in the colyophilized protein/sugar formulation and physical mixture of the same composition.[51] The nonideal behavior in the water "monolayer" is a reflection of protein-sugar interaction in the colyophilized protein formulations.[53] In Figure 4 we plot data for protein A comparing FTIR secondary structure, degree of protein–sucrose interactions and aggregation rates corrected for the dilution effect. As Figure 4 shows, the difference in water "monolayer" reached a maximum value at 0.5 mass fraction of sucrose. This was essentially the same for all the five proteins studied.[51] Thus, it seems that the solid–state interaction between

protein and sucrose reaches saturation at a sucrose mass fraction of 0.5, which is consistent with the finding from a solution calorimetric study on other protein/sugar systems.[54]

We see that, inconsistent with the behavior of the secondary structure metric and the degree of water replacement, the normalized aggregation rate constants continue to decrease through the whole sugar composition range. Thus, while it could be said that the storage stability of the proteins correlates with FTIR structure and
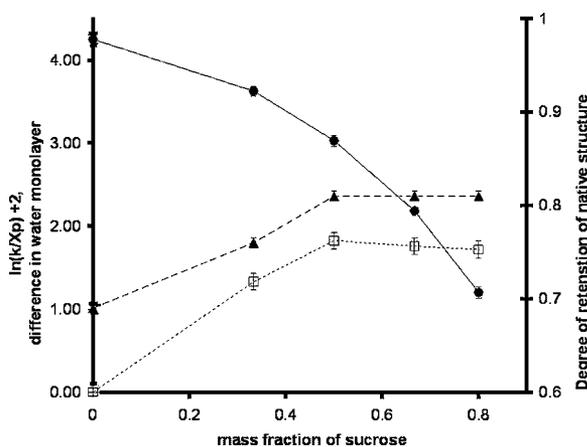
Figure 4. The relationship between normalized aggregation rate constant of protein A at 50°C (●), the degree of retention of "native" structure (▲) and difference in water monolayer between colyophilized protein/sugar formulation and physical mixture of the same composition (□). Normalization means that the aggregation rate constant (k) was divided by the mass fraction of protein (X_p) to account for the dilution effect. The normalized rate constant decreases with increasing sucrose fraction in the formulation, indicating more stabilization mechanism above the "dilution" effect. Error bar represents standard errors.

protein–sugar interaction in a protein-rich system (sucrose mass fraction less than 0.5), dilution and/ or sugar–protein enthalpic interactions can not completely explain the stability trends observed through the whole range of compositions studied, and we look to dynamics as potential mechanisms that may dominate the stability trends.

Correlation of Storage Stability with Glass Dynamics

The glass transition temperatures, T_g, of the protein/sucrose formulations were measured and are listed in Table 1. T_g decreases monotonically with the addition of sugar to protein; thus the formulation with sugar/protein ratio of 4:1 exhibited the lowest T_g. The temperature dependence of dynamics above T_g can generally be analyzed by the Williams–Landel–Ferry (WLF) Model,[55] and it has been reported that the chemical degradation rate correlates with $T-T_g$ for several systems.[55,56] It is assumed in the WLF model that increased T_g would lead to better stability, but such universal relationship between $T_g-T_{storage}$ and stability does not exist at temperature well below T_g.[25,26] As we see in this study, stability does not improve as T_g of the formulation increases. Certainly, T_g is an important parameter, and the protein/excipient formulation needs to remain in the glassy phase to ensure product elegance. While storage near and above T_g may indeed result in poor stability, at storage temperatures well below T_g, a formulation with high T_g does not necessarily demonstrate better stability than a formulation with a low T_g.[5,25,28]

TAM for Global Mobility Measurement

Since T_g is not a good predictor of molecular mobility at temperatures well below T_g, it was suggested that structural relaxation time may be a better indicator of global dynamics.[27–29] Thus structural relaxation time was determined with the TAM at 50°C. A representative power-time relaxation curve of a protein E formulation is shown in Figure 5. The structural relaxation time was calculated using MSE fit of Eq. (3) to the experimental data. The MSE fit is extremely good, as demonstrated by the overlap of data with the fitted line and the small chi-square (χ^2) value.

Table 6 lists the structural relaxation times for protein/sucrose formulations at 50°C. The "stretched" structural relaxation time constant
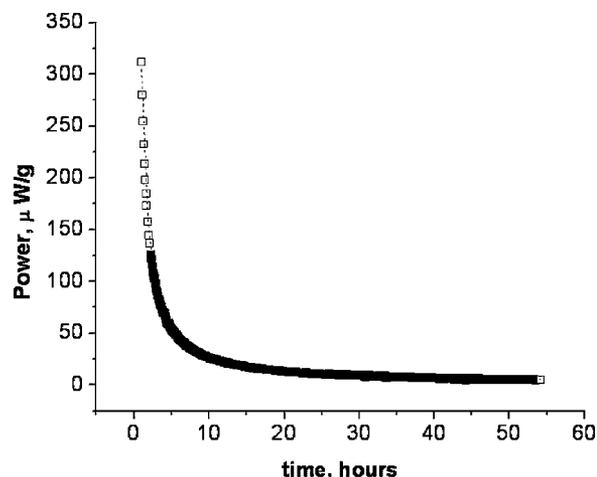
Figure 5. Power-time data from isothermal microcalorimetry for protein E formulation with sucrose mass fraction of 0.8 at 50°C. The dashed line was generated by fitting Eq. (3) to the TAM data. $\tau_0 = 2.550 \pm 0.005$; $\tau_1 = 0.875 \pm 0.003$; $\beta = 0.309 \pm 0.001$; chi-square (χ^2) = 0.044.

(τ^β) increases when sucrose is initially added into pure protein, reaches a maximum at a sucrose mass fraction of 0.5, and then decreases with the further addition of sucrose. Similar trends were observed for all proteins studied. These observations are qualitatively consistent with previous results on different proteins such as an IgG1 antibody and HSA.[46] Structural relaxation time passes through a maximum value at about a 1:1 mass ratio, which indicates that the structural relaxation is not a monotonic function of the sucrose concentration in the formulation. Since stability continues to improve beyond 1:1 ratio, it is obvious that τ^β does not correlate with stability over the whole sugar range studied. Given the fact that both structural relaxation time and FTIR structure reached maximum values at about 1:1 ratio, it is possible that the τ^β trend is also a reflection of protein–sugar interaction in the dried solid formulation.

Study of Local Dynamics as Determined from Neutron Scattering

In addition to global diffusional motion, some local motions such as rotation of the side chains and small amplitude fluctuations may also be important to storage stability.[57] Recently, Cicerone et al. found that the stability of some enzymes (HRP, NADH) and an IgG1 antibody at

Table 6. The Structural Relaxation Time Constant (τ^β) of Protein Formulations Measured at 50°C

Formulation	Mass Fraction of Sucrose, %	Protein A[a]	Protein B	Protein C	Protein D	Protein E
P10S0	0	2.2	2.2 ± 0.1	2.7	3.2	1.2 ± 0.1
P10S5	33.3	5.9 ± 0.1	3.2 ± 0.6	4.8 ± 0.4	3.9 ± 0.5	3.6 ± 0.7
P10S10	50	9.7 ± 1.5	4.2 ± 0.5	5.0 ± 0.6	6.1 ± 1.2	4.4 ± 1.0
P10S20	66.7	3.3 ± 0.8	3.8 ± 0.8	2.3 ± 0.6	5.0 ± 0.5	3.3 ± 0.6
P10S40	80	2.2 ± 0.2	3.2 ± 0.1	[b]	2.0 ± 0.2	2.8 ± 0.6

The value was obtained using Eqs. (3) and (4).
[a]Uncertainties in structural relaxation time constant are standard error based on replicates ($n = 2$). For some samples of pure protein, only one TAM has been run due to limitation imposed by sample availability.
[b]Not determined due to sample availability for the TAM experiment.

temperatures well below T_g correlates with fast motions on a length scale of Angstroms and timescale of nanoseconds, as probed by neutron backscattering.[32,33] We reason, therefore, that fast local dynamics may be important to the stability of protein/sugar systems at temperatures well below T_g. Neutron scattering experiments were performed to test this hypothesis.

Table 7 shows the mean-squared amplitude ($<u^2>$) of local motions in the protein/sugar formulations of protein E at different temperatures. The decrease in the amplitude of the fast motion corresponds to a decrease in the local mobility of the system. It was found that the pure protein exhibited the largest amplitude of fast local motion at all examined temperatures, and the amplitude of the fast local motion decreased sharply with a small increase of sucrose level from 0 to 33 mass%. Further increase of sucrose level results in a continued monotonic decrease in the local mobility. All protein formulations studied showed a higher mobility than sucrose alone, consistent with previous neutron scattering results on an IgG1 antibody.[40] It appears that local motion of the protein is coupled to that of sugar matrix and sucrose seems to act by suppressing such motion of the protein, thereby stabilizing the protein. Similar to the effect of glycerol on protein stability at an intermediate

level,[32] sucrose seems to plasticize by decreasing the T_g, but anti-plasticize the beta relaxation through the decrease in the local motion.

Figure 6 illustrates the correlation of protein stability with both global and local dynamics for protein E. τ^β increases with increasing sucrose level initially, and then reaches a maximum value at about 0.5 mass fraction sucrose, and further increase in sucrose level results in a decrease in τ^β. Thus, while sucrose lowers T_g at all levels, sucrose additions up to a 1:1 mass ratio actually slow down the alpha motion as measured by enthalpy relaxation kinetics, and in this range of sucrose concentrations, τ^β and stability are well correlated. However, at higher levels of sucrose, the correlation disappears. However, both the aggregation rate constant and the amplitude of local motion decrease with increasing fraction of sucrose over the entire range of sugar studied. Thus, local dynamics correlate well with aggregation rate at temperatures well below T_g.

The concept that storage stability on a time scale of months correlates better with local motion ($<u^2>$) than global motion (τ^β from TAM) requires explanation. As proposed earlier, there may be at least two possible pathways from reactant to product.[36] One pathway would primarily involve the highly cooperative α-process motions, which has high activation energy, long timescales, and

Table 7. Fast Local Mobility ($<u^2>$) of Protein E as Measured by Neutron Backscattering Spectrometer at 25, 40, and 50°C Where the Stability was Studied

T (°C)	E10S0	E10 S5	E10S10	E10S20	E10S40	Sucrose
25	0.434 ± 0.005	0.324 ± 0.006	0.315 ± 0.006	0.263 ± 0.005	0.226 ± 0.005	0.114 ± 0.005
40	0.463 ± 0.006	0.353 ± 0.005	0.335 ± 0.007	0.283 ± 0.005	0.251 ± 0.005	0.118 ± 0.007
50	0.471 ± 0.006	0.374 ± 0.006	0.349 ± 0.007	0.299 ± 0.005	0.272 ± 0.006	0.132 ± 0.006

$<u^2>$ is the mean-squared displacement of motions on a nanosecond time scale. The standard error in $<u^2>$ was shown as the uncertainties in the fit of data to the harmonic model. The fast local mobility of pure sucrose is also shown.
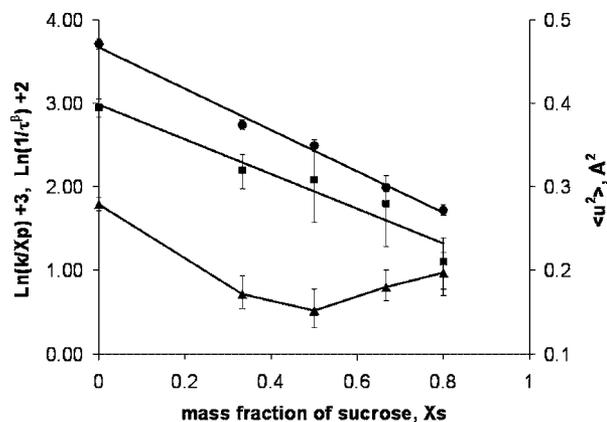
Figure 6. The relationship between global mobility from TAM (▲) and fast local mobility from neutron backscattering (●) with normalized aggregation rate constant (■) for protein E. All data in the graph refer to data at 50°C. The aggregation rate constant (k) was divided by the mass fraction of protein (X_p) to normalize for the dilution effect. The straight line is the best fit of a linear model to the data. Error bar represents standard errors.

dominates at high temperature near and above T_g. A second pathway may largely involve a great number of rapid, small-amplitude local motions with low activation energy.[32] This latter pathway, involving β-relaxation would become dominant at temperatures well below T_g where the structural relaxation time in the α-process becomes very long. In this scenario, one could expect that the rate determining step for degradation would change from the cooperative α-process at high temperature to a process related to local dynamics at temperature well below T_g.

Characterization of Free Volume Using Gas Pycnometer

The density of the protein formulation was measured using a gas pycnometer operating with helium gas. Figure 7 shows the density data of freeze dried protein C formulations as a function of sucrose level. The density of pure protein is low compared to that of freeze-dried pure sucrose. Also, the density increases monotonically with increasing fraction of sucrose in the dried protein formulation. Similar trends in density have also been observed in an IgG1 antibody.[40] The measured density data of colyphilized protein-sucrose samples were different from the calculated values of a physical mixture based on the
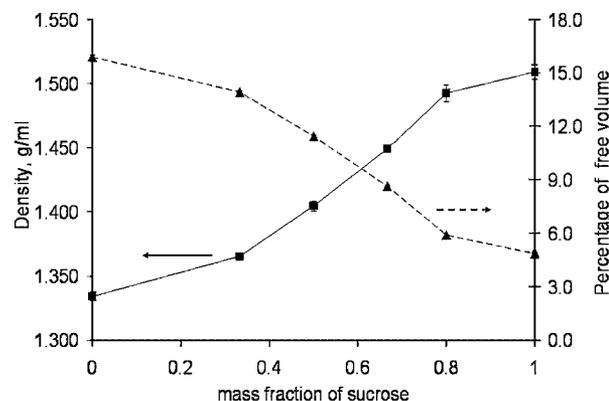
Figure 7. Density (■) and percentage of free volume (▲) as a function of sucrose level in protein C formulation. Density of amorphous solids was measured from helium pycnometer. Error bar represents standard errors, and sometime it is smaller than the size of the symbol.

linear contribution of pure protein and sucrose component, which also indicates the presence of solid–state interactions in the colyophilized formulations.

Since density is the inverse of specific volume, which is directly related to free volume of the system, the free volume in the protein formulations can be estimated from density data. For amorphous sucrose, the (excess) free volume can be calculated as the difference in specific volume between amorphous and crystalline sucrose. This procedure defines the zero point of free volume as the free volume of the crystalline state. Assuming mean atomic masses of pure protein are the essentially the same as sucrose (calculated to be ∼7.6 for pure sucrose and ∼8 for pure IgG1), the free volume of protein formulation can also be estimated using crystalline sucrose as the reference. Thus the ratio of free volume to specific volume can be defined as the free volume fraction. As shown in Figure 7, pure amorphous sucrose has a much lower free volume fraction than that of dried pure protein, which is presumably due to the tight packing pattern of sucrose molecules in the amorphous matrix. The range of the free volume fraction values we find (5–20%) is consistent with literature reports on fractional free volume in polymers, as determined using positron annihilation lifetime spectroscopy (PALS).[58–61] In addition, the free volume percent of the protein samples were found to decrease with increasing fraction of sucrose in the protein formulation. Thus it seems that the (relatively) small sucrose molecules can act to decrease the

fractional free volume of the system by filling the voids between large protein molecules.

Correlation of Protein Stability with Free Volume and Molecular Mobility in the Glass

Within the free-volume framework, molecular transport occurs by the movement of a molecule into a void within a cage delineated by the immediate neighbors of the moving particle, and the probability of a transport event will depend on the number and size of voids, which in turn is directly related to the free volume of the system.[62] Thus, high free volume is associated with high probability of transport and therefore high molecular mobility. The increase in mass fraction of sucrose in the protein formulation results in a monotonic decrease in the free volume percent in the system (Fig. 7), and since the protein stability improves monotonically as the sucrose fraction increases (i.e., as free volume decreases), the free volume data correlates well with stability data.

In addition, the free volume and the fast dynamics (i.e., $<u^2>$) are highly coupled. As sucrose mass fraction in the protein formulation increases, both local mobility ($<u^2>$) and percentage of free volume of the system decrease monotonically. This parallel relationship is consistent with correlations between free volume measured by PALS and fast local mobility measured by neutron and Raman scattering on several organic glasses.[63–65] PALS studies and computer simulations suggest that the excess free volume appears in form of many irregularly shaped holes of sub-nanometer size, which arise from the disordered molecular packing in the amorphous phase (static and pre-existing holes) and molecular relaxation among the molecular chains and terminal ends (dynamic and transient holes).[60,61] The diffusion of small molecules and ions through glassy polymers and small molecule organic glasses, as observed in mechanical or dielectric relaxation and viscosity experiments, were also related to the excess free volume.[66,67] These observations suggest that both global mobility and local mobility depend on the creation of holes and thus are sensitive to the free volume. However, while both global and local motion may be sensitive to free volume, the relationship between mobility and free volume is obviously different for local mobility than for global mobility, and it is the local mobility (fast dynamics) that is well correlated with free volume (and

stability) over the entire sugar range studied at temperatures well below T_g.

Evaluation of Relative Importance of FTIR Structure and Glass Dynamics on Protein Storage Stability

Reports in the literature indicate that both protein structure and glass dynamics seem to play a role in the protein stability,[5,8,12,17] and that both factors may be used to interpret the stability in a given study. However, protein structure and glass dynamics may contribute to stability to different extents, and it is instructive to investigate the relative importance of both factors to the storage stability of proteins in our studies. Our results showed that the trend in degradation rate of proteins as a function of sugar level follows the trend of glass dynamics (local dynamics, or free volume) rather than FTIR structure. Thus it seems that glass dynamics is more important than FTIR structure in controlling the storage stability of these proteins.

In order to address the question quantitatively, statistical analysis using multiple linear regression was performed on stability data ($\ln(k/X_p)$) at 50°C. The stepwise regression with a significance level of 0.15 was used for all glass dynamics parameters (τ^β, $<u^2>$) and FTIR structure parameters (correlation coefficient, area of overlap and normalized peak height).[2] The first parameter entered the model was the $<u^2>$ for all five proteins, indicating that local dynamics is the single best parameter for stability prediction. In addition, the general linear model (GLM) using both $<u^2>$ and FTIR structure was constructed, and the P-values of each parameter are listed in Table 8. As we notice, the P-value of FTIR structure from peak height is larger than that of $<u^2>$ for all proteins investigated, and FTIR structure is generally not a significant predictor of stability when $<u^2>$ is already in the model. A similar qualitative result was obtained when other measures of FTIR structure were used,

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[2]Our unpublished results from a different IgG1 monoclonal antibody showed the similar $<u^2>$ trend as protein E as a function of sucrose level, and also the differences in $<u^2>$ between these significantly different proteins are small at same sucrose/protein mass ratio (relative difference in $<u^2>$ is about 8%), Thus the $<u^2>$ value of protein E can be used as approximate $<u^2>$ for these proteins not subject to the neutron scattering experiment in the current study. It was found that the qualitative conclusions obtained are not changed even with the use of neutron scattering data of IgG1 monoclonal antibody.

Table 8. Result of Statistical Analysis of the Relative Importance of Glass Dynamics ($<u^2>$) and FTIR Structure (Peak Height) on Protein Stability ($\ln(k/X_p)$) at 50°C

Parameter	Protein A	Protein B	Protein C	Protein D	Protein E
$<u^2>$	0.02	0.07	0.01	0.13	0.09
FTIR structure	0.16	0.61	0.06	0.18	0.63

The value shown is *P*-value of the parameter in a two variable model obtained using General linear model (GLM) procedure. The parameter with smaller *P*-value has larger significance to the protein stability.

suggesting that local glass dynamics is more important than FTIR structure to the storage stability of these proteins.

We further explore the relationship between the aggregation stability and local dynamics of the proteins in Figure 8, where the plots of normalized aggregation rate constant versus $1/<u^2>$ at 50°C are provided. The motivation of using $1/<u^2>$ was due to the report that the timescale of local relaxations of the glass should scale exponentially with $1/<u^2>$.[32,68] It seems that, within the uncertainty of data, there is a linear relationship between $1/<u^2>$ and $\ln(k/X_p)$ over the entire sucrose range for each of the proteins studied. Given the fact that protein native structure improves significantly with increasing sucrose only at lower sucrose level (Tab. 5), one might expect that at low sucrose level (i.e., low $1/<u^2>$), the correlation with $1/<u^2>$ would be poor or at least different than at high sucrose (i.e., the slope would undergo a large change). However, the data in Figure 8 suggest that there is no significant

Figure 8. Relationship between the normalized aggregation rate constant and fast local mobility ($1/<u^2>$) at 50°C for five proteins. Error bars are the standard errors in the rate constants as given by the regression analysis. The straight line is the best fit of a linear model to the data.

change in the relationship between fast dynamics and stability in spite of changes in protein native structure as the systems proceed from pure protein to high sugar content. Thus we are forced to conclude that, within our ability to measure, the FTIR-derived structure has, at most, a minimal effect on protein stability. Therefore, glass dynamics is, by far, the more important factor in controlling the stability of dried proteins in our studies.

Since changes in protein structure and glass dynamics are convoluted in the current study (i.e., both change with sugar level in the protein formulations), it is complicated to accurately evaluate the relative importance of both factors. Efforts have been made to separate the effect of protein structure and glass dynamics on storage stability of one IgG1 antibody.[50] By adding different amounts of plasticizer, such as sorbitol, to a protein/trehalose formulation in which the H-bond requirement was met with a high sugar fraction, the initial protein structure after lyophilization was maintained as a constant while the glass dynamics varied with the amount of plasticizer. Thus, if structure were dominant for protein stabilization, then the stability should not change significantly as a function of sorbitol fraction. However, it was found that stability improved with small additions of sorbitol,[50] and the improved stability correlated qualitatively to the fast local dynamics.[33] Our recent study showed similar result in IgG1/trehalose system with glycerol as a plasticizer.[69] Thus, it seems that glass dynamics is more important than FTIR structure in controlling the storage stability of the protein at temperature well below T_g, at least in the systems studied. However, it needs to be admitted that the relative importance of FTIR structure and glass dynamics could vary under different conditions, so not all studies with all protein formulations will necessarily give the same results we find here.
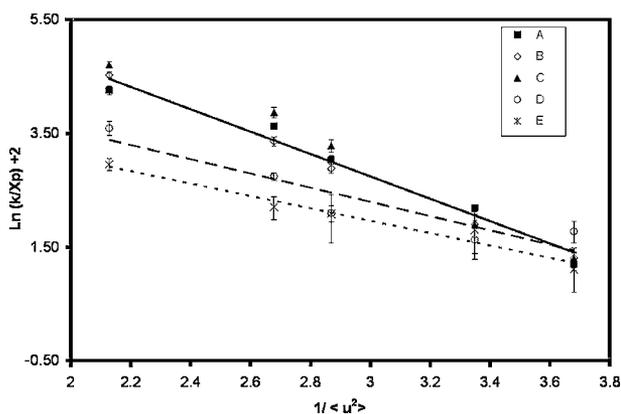
CONCLUSION

The physical stability of the proteins improves monotonically with increasing levels of sucrose in the formulation over the entire sugar range studied. Stability data are consistent with a stoichiometric binding model only if the binding constants are quite small, perhaps unrealistically small. FTIR structure and structural relaxation time measured by TAM correlate with protein stability only up to sucrose/protein mass ratio of 1:1, where the native structure preservation reaches a plateau and the structural relaxation time reaches a maximum. Thus, neither could fully explain the observed stability trends. However, the mean square amplitude of fast local dynamics, as measured by the neutron back-scattering, was found to be linearly related to the log of the rate constant for aggregation over the entire sucrose range studied. We conclude that it is fast local dynamics that is the best predictor of stability, at least for our systems which were studied well below their T_g. In addition, free volume obtained from density data correlates very well with the local dynamics data, and therefore also with the stability data, suggesting that density measurement may be a promising method for the rational development of stable protein formulations.

ACKNOWLEDGMENTS

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