Moisture Effects on Protein–Excipient Interactions in Spray-Dried Powders. Nature of Destabilizing Effects of Sucrose

STELIOS T. TZANNIS*,† AND STEVEN J. PRESTRELSKI‡

Contribution from *Biopharmaceutical Research & Development, ALZA Corporation, 950 Page Mill Road, Palo Alto, California 94303.*

Received January 9, 1998. Accepted for publication November 23, 1998.

Abstract □ The preparation of stable solid protein formulations presents significant challenges. Ultimately, the interactions between incorporated excipients and the pharmaceutical protein determine the formulation stability. In this study, moisture was utilized to probe the interactions between a model protein, trypsinogen, and sucrose in the solid state, following spray drying. Through investigation of the physical properties of the spray-dried formulations, we attempted to elucidate the mechanisms underlying the previously observed[1,2] stabilizing and destabilizing effects of the carbohydrate during spray drying. Both dynamic and equilibrium moisture uptake studies indicated the presence of an optimal protein–sugar hydrogen bonding network. At low sucrose contents, a preferential protein–sucrose hydrogen bonding interaction was dominant, resulting in protein stabilization. However, at high carbohydrate concentrations, preferential sugar–sugar interactions prevailed, resulting in a phase separation within the formulation matrix. The preferential incorporation of the sucrose molecules in a sugar-rich phase reduced the actual amount of the carbohydrate available to interact with the protein and thereby decreased the number of effective protein–sucrose contacts. As a consequence, the protein could not be effectively protected during spray drying. We hypothesize that the observed phase separation at this sucrose concentration regime originates from its exclusion from the protein in solution before spray drying, further accompanied by preferential clustering of the sucrose molecules.

Introduction

The development of solid state dosage forms of biopharmaceutical molecules presents considerable challenges since the processing environments impose severe stresses that may destabilize and ultimately denature these labile biomolecules. Previous studies have indicated that lyophilization can extensively perturb the structure and diminish the activity and physical stability of several proteins.[3−5] Similarly, spray drying and precipitation in supercritical antisolvents, methods frequently used for production of protein microparticles, have been shown to disrupt the native structure and compromise the enzymatic activity of various proteins.[1,2,6−10] Indeed, the processes involved in spray drying impose stresses that can potentially have detrimental effects on protein stability: pressure effects and exposure to air−liquid interfaces during atomization, as well as heat and dehydration stresses during the drying process, have all shown capacity to denature proteins.[1,3−5,11]

In many cases, formulation excipients are required to preserve the protein native structure and stability in the solid state and to ensure physical stability during either long-term storage or delivery. Research efforts over the past decade have indicated that carbohydrates, and in particular disaccharides, can provide significant protection during lyophilization by preserving the native protein structure during the dehydration process.[3,4,12] Similarly, sucrose and trehalose have shown efficacy in stabilizing several proteins, such as trypsinogen, lysozyme, and soybean trypsin inhibitor (STI) during spray drying.[1,2,13,14] The stabilization was shown to occur via preservation of the native protein structure in the solid state along with impediment of protein aggregation on reconstitution.[1,2] Although the formation of a glassy matrix has been proposed as a stabilization mechanism, the "water replacement" theory is the only one that accounts for specific interactions between the proteins and carbohydrates in a mechanistic fashion. In terms of the latter, disaccharide-induced protein stabilization during drying is thought to occur via hydrogen bonding between sugar and protein molecules, thereby satisfying the hydrogen bonding requirements of the protein during the dehydration step.[3,4,12,15,16]

However, our previous studies[1,2,13,14] have shown that at high concentrations of both disaccharides, the stabilizing effects were partially dissipated: at increasing sucrose-to-protein mass ratios the activity losses approached those observed in the absence of the excipient. Similar activity profiles were observed for STI and trypsinogen when spray-dried in excess of trehalose.[13,14] The incurred losses could not be accounted by aggregation or major conformational perturbations, leading to the hypothesis of process-mediated, irreversible enzyme destabilization.[1]

In effort to account for the destabilizing carbohydrate effects, moisture was used to probe the interactions between trypsinogen and sucrose in the solid state following spray drying. The utilization of moisture as a probe is of particular interest since water is a key determinant of both the solid matrix integrity and the excipient−protein interactions. Dynamic and equilibrium moisture sorption analyses were used to characterize the nature and strength of trypsinogen−sucrose interactions, while physicochemical and thermal methods of analysis were used to probe the state of sucrose in the solid state. The phenomenon of the reduced stabilization of trypsinogen during spray drying at high sucrose concentrations was also examined. The results presented in this study are expected to improve the understanding of protein−excipient interactions in the solid state and to aid the design of stable spray-dried protein formulations, with implications for other solid-state processing methodologies.

Experimental Section

Chemicals—Trypsinogen (1× crystallized, dialyzed against 1 mM HCl and lyophilized; Lot no. 38E273N) was purchased from Worthington Biochemical Corporation (Freehold, NJ). Ultrapure

* Author to whom all correspondence should be addressed.
† Inhale Therapeutic Systems, 150 Industrial Road, San Carlos, CA 94070. Tel: (650) 631 3276; Fax: (650) 631 3276; email: stelios.tzannis@inhale.com.
‡ Powderject Technologies, Inc., 6511 Dumbarton Circle, Fremont, CA 94555.

10.1021/js9800127 CCC: $18.00
Published on Web 02/04/1999

sucrose (>99.5% purity via HPLC, Lot no. 35H03582) was purchased from Sigma Chemical Company (St. Louis, MO). Lithium chloride, potassium carbonate, potassium phosphate, calcium chloride, and sodium chloride salts were purchased from J. T. Baker (Phillipsburg, NJ). Barium chloride and ammonium chloride were purchased from Mallinckrodt (St. Louis, MO).

Experimental Design—Protein powders were prepared by spray drying formulations at different sucrose-to-protein mass ratios: no sucrose, 0.25:1, 0.5:1, 1:1, 2:1, 4:1, 8:1, and no protein, which correspond to sucrose concentrations of 0, 0.015, 0.029, 0.058, 0.234, 0.47, and 0.53 M, respectively. In all cases, the protein concentration was held constant at 20 mg/mL. All results in this study are reported in terms of sucrose-to-protein mass ratios.

Spray Drying—Trypsinogen particles were prepared by spray drying in a Yamato minispray dryer model ADL-31 (Yamato Scientific, Orangeburg, NY). The proteinaceous solutions were prepared in 1 mM HCl, achieving a pH around 3.1 for all formulations in order to minimize the autolysis of the enzyme.[1,17] The solutions were continuously fed to the spray dryer at an approximate flow rate of 3−5 mL/min and were dried at an inlet temperature of 120 °C; outlet temperatures ranged from 85 to 90 °C. Powders were aliquoted in lyophilization vials in a drybox (Terra Universal, Anaheim, CA) at <1% relative humidity (% RH).

Particle Size Analysis—The particle size of the spray-dried powders was determined with a Malvern Mastersizer S (Southborough, NH) laser-light scattering analyzer. Small amounts of powders were dispersed in an excess of octanol; the suspension concentration was properly adjusted to attain optimal obscuration. The samples were then analyzed in a static mode; measurements were repeated in triplicate over a 20-min period, to ensure that no dissolution or powder agglomeration occurred. The size distribution was expressed in terms of volume median diameter, particle size range, and span.

Moisture Analysis—The moisture content of the spray-dried powders was analyzed via Karl Fisher titration; assays were performed on an Aquatest 10 Karl Fisher Coulorimetric Titrator (Seradyn, Indianapolis, IN), calibrated with anhydrous methanol. The moisture content of the protein microparticles was monitored in triplicate using a methanol extraction protocol.

Moisture Sorption Isotherms—Dynamic moisture sorption isotherms were determined by gravimetric measurement of water uptake using an Integrated Microbalance System, model MB-300G (VTI Corporation, Hialeah, FL), equipped with a digital Cahn ultra-microbalance model 13200-1 (Cahn Instruments, Madison, WI). The microbalance was calibrated with poly(vinylpyrollidone) (PVP) standard. The temperature of the sample chamber throughout the experiment was maintained at 37 °C by circulating water from a constant temperature Neslab circulator, model RTE-100 (Neslab Instruments Inc., Newington, NH) in a water jacket built around the sample compartment. Five to ten milligrams of sample was loaded onto the microbalance, carefully minimizing the sample exposure to ambient conditions. The powder was first subjected to a drying cycle, in which the residual water was removed by vacuum, until equilibrium was attained; the equilibrium criteria were set to reflect sample weight fluctuations of less than 5 μg (representing <1% of the loaded sample) within 5 min. Following drying, the samples were exposed to incremental changes in relative humidity from 0 → 95 → 0% RH at 5% RH intervals. The %RH level was automatically incremented only after complete sample equilibration at each RH level, as dictated by the set equilibrium conditions of less than 3 μg weight change every 10 min with a maximum equilibration time of 400 min. All sorption profiles were determined in triplicate.

The monolayer amount of sorbed water molecules was calculated by a fit of the adsorption data between 0 and 30% RH to the two-state Brunauer−Emmet−Teller (BET) equation[18]

$$W = \frac{W_m C_B (P/P_o)}{1 - (P/P_o)[1 - (P/P_o) + C_B(P/P_o)]} \quad (1)$$

where W is the weight of water adsorbed per unit weight of dry solid at a relative pressure of P/P_o, P and P_o are the vapor pressure and saturation vapor pressure of water, respectively, at the experimental temperature, W_m is the monolayer capacity of the adsorbent, and C_B is a constant related to the heat of adsorption. The latter provides an indication of the affinity of water molecules for the solid sample[19,20] and is given by:[18,21]

$$C_B = k \exp\frac{H_1 - H_L}{RT} \quad (2)$$

where H_1 is the heat of adsorption of the first vapor molecule adsorbed to a substrate site, H_L is the heat of condensation of bulk adsorbate, R is the universal gas constant, T is the absolute temperature, and k is a constant, usually assumed to be close to unity.[21] The approximate water sorption-based sample surface area was calculated from the BET monolayer value assuming 12.5 Å^2 for the cross-sectional area of each water molecule.[19]

BET Surface Area Measurements—The measurements were carried out on a Gemini 2360 surface area analyzer (Micromeritics Instrument Corporation, Norcross, GA) based on the multi-point BET gas adsorption method. For each measurement 0.4−1.5 g of spray-dried powders were loaded onto the sample holder. The surface area was determined using nitrogen as the adsorbate at five different relative pressures (P/P_o range of 0.1−0.3). Prior to the measurements, the samples were degassed under high vacuum at room temperature for at least 24 h, until no further gas or vapor evolved, as judged by the stabilization of the instrument readings.

Equilibrium Sorption Experiments—The spray-dried samples were stored for 10 days at room temperature in sealed desiccators equilibrated with the appropriate saturated salt solutions. The humidity was maintained at 12, 43, 75, and 84% RH with saturated aqueous solutions of LiCl, K_2CO_2, NH_4Cl, and $BaCl_2$, respectively. The physical state of the powders was characterized by X-ray diffraction and thermal analysis.

Powder Thermal Stability—The thermal stability of the spray-dried formulations was determined by the glass transition (T_g) and crystallization exotherms (T_{cryst}) obtained by solid state DSC. The experiments were performed on a TA Instruments (New Castle, DE), Model 2920 DSC, which was calibrated with indium prior to sample analysis. Approximately 10−50 mg of the samples were loaded under environmentally controlled conditions in 50-μL hermetically sealed aluminum pans. After a short preequilibration step at −20 °C, the samples were heated to 200 °C. In effort to deconvolute relaxation events during the glass transition, the samples were analyzed by modulated DSC (MDSC) at a heating rate of 5 °C/min with a modulation frequency of ±2 °C every 60 s. The glass transition temperatures were extracted in triplicate using the transition midpoints.

X-ray Diffraction Experiments—X-ray diffractograms (Coors Ceramics Analytical Laboratory, Golden, CO) were collected using a Scintag Pad X θ-θ diffractometer under the following conditions: copper tube operated at 45 kV, 40 mA; goniometer radius 250 mm; beam divergence slits of 6 and 1 mm and scatter and receiving slits of 0.5 and 0.3 mm; germanium solid state detector bias 1000 V; the PHA was set to accept only K-α radiation. The samples were loaded on a zero-background quartz plate mounted on a special sample holder. Diffractograms were collected between 3° and 90° 2θ at a scan speed of 2.0° 2θ per minute and a chopper increment of 0.03° 2θ. The temperature at the time of the experiments was approximately 23−25 °C. The percent crystallinity of the samples was calculated by numerical integration of the sample and amorphous X-ray diffraction data over the 5−80° range. After background subtraction, utilizing a cubic spline fit, the integrated peak areas were determined using a Lorentzian profile fitting routine, and % crystallinity was assessed by calculating the relative percentage of crystalline peak areas to the total area under the diffractograms.[22]

Results

Particle Size Analysis—The size distribution analysis of the spray-dried particles is given in Table 1. In agreement with our previous observations,[1,14] spray drying of the trypsinogen−sucrose formulations produced a homogeneous monomodal distribution of an approximate diameter of 3−5 μm and a narrow range (0.8−20 μm). The addition of sucrose had a rather minor effect on the particle average diameter: a small increase of the particle size was observed with increasing amounts of added sucrose, however, within the error of the measurements.

Moisture Content—The moisture content of the spray-dried trypsinogen powders as a function of the protein-to-

Table 1—Particle Size and Size Distribution of Spray-Dried Trypsinogen–Sucrose Powders[a]

sucrose:trypsinogen mass ratio	median diameter[b] (μm)	span[c]	$(SSA)_{estimated}$[d] (m^2/g)
no sucrose	3.1 ± 2.2	1.03 ± 0.02	2.3 ± 0.6
0.25:1	3.2 ± 1.3	1.04 ± 0.02	2.1 ± 0.5
0.5:1	3.6 ± 2.3	1.15 ± 0.03	1.5 ± 0.4
1:1	3.7 ± 2.8	1.26 ± 0.03	1.4 ± 0.4
2:1	4.6 ± 1.8	1.11 ± 0.01	0.7 ± 0.2
4:1	4.9 ± 0.9	1.26 ± 0.02	0.6 ± 0.2
8:1	5.2 ± 1.9	1.16 ± 0.03	0.5 ± 0.1

[a] Values represent averages and standard deviations of triplicate measurements. [b] Volumetric median diameter (D(v, 50)). [c] Span = [D(v, 90) − D(v, 10)]/D(v, 50), where D(v, 90), D(v, 50), and D(v, 10) are the equivalent volume diameters at 90, 50, and 10% cumulative volume, respectively. [d] Nominal specific particle surface area estimated based on the particle size distribution.

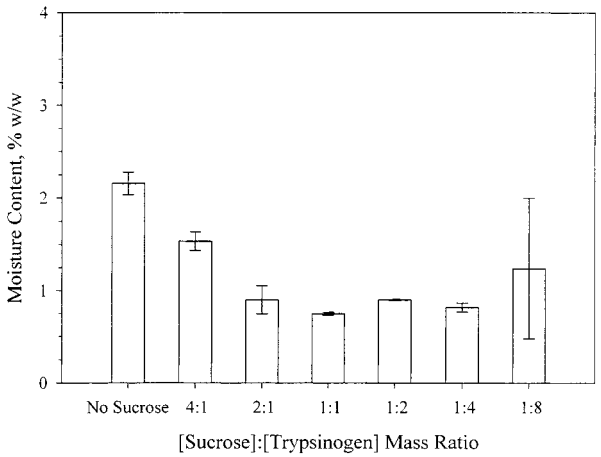

Figure 1—The effect of sucrose on the moisture contents of the spray-dried trypsinogen formulations. Error bars represent (±) one standard deviation of triplicate determinations.

sucrose mass ratio is shown in Figure 1. In agreement with our previous studies,[1,14] spray drying yielded powders of very low residual moisture. The moisture content achieved when trypsinogen was spray-dried in the absence of sucrose was 2.2 ± 0.1% w/w. This is lower than the calculated water monolayer value of 5.4% w/w, which assumes the attachment of one water molecule per charged amino acid, as suggested by Leeder et al.[23]

The addition of sucrose, up to a sucrose-to-protein mass ratio of 1:1, progressively decreased the attained moisture content; thereafter, it remained constant up to the highest sucrose concentration examined. The observed reduction in the final moisture content indicates that the dehydration process is more efficient in the presence of sucrose. This could indicate an increase of the effective heat transfer coefficient as a consequence of the increased surface tension of the droplets, caused by the addition of sucrose. Alternatively, it may reflect facilitation of the removal of water, thereby providing an indirect indication of specific hydrogen bonding interactions between the spray-dried components.

Particle Surface Area Analysis—The nitrogen adsorption analysis results are given in Table 2. The surface area of pure protein particles was 14.5 ± 0.02 m^2/g, which is larger than what would be expected for perfectly spherical particles of this volumetric size distribution (given in Table 1). Since nitrogen sorption is a purely surface phenomenon, this suggests that the particle surface is not perfectly smooth or alternatively the presence of blowholes and cracks. Upon addition of sucrose the surface area decreased: at a sucrose mass ratio of 1:1, it became half of that in the absence of the carbohydrate, while at the highest sugar mass ratio examined, the mean particle surface area decreased 4-fold. A similar trend was observed for the estimated values of W_m, which decreased with increasing sucrose content, thereby providing further evidence of a decrease in the available surface sites for nitrogen adsorption. Scanning electron microscopy (SEM) analysis (data not shown) confirmed that this was due to the increased smoothness of the sucrose-containing particles compared to the wrinkled surface of those in the absence of the carbohydrate; no cracks or blowholes were observed. In contrast, the affinity of the adsorbate for the particle surface did not appear to change with the addition of sucrose, as suggested by the relatively invariable values of the constant C^N_B, indicative of similar heats of nitrogen adsorption to the particle surface.

Dynamic Moisture Sorption Isotherms—The moisture adsorption/desorption isotherms are shown in Figures 2a–f and the BET analysis results are given in Table 2. Spray-dried trypsinogen in the absence of sucrose exhibited a sigmoidal-Type II adsorption isotherm (Figure 2a), which is characteristic of amorphous protein and protein–sugar systems.[24–31] The estimated monolayer of 5.3 ± 0.6 g/100 g solid is in excellent agreement with that calculated by assuming strong adsorption sites on the protein surface (5.4 g/100 g solid). This suggests that at the monolayer, approximately 67 ± 5 water molecules are bound per trypsinogen molecule. The adsorption curve also indicates that the amount of moisture sorbed in the multilayer region (>20–25% w/w) is much larger than what would be expected from the particle surface area. This is also implied by the calculated water sorption-based specific surface area, which is much larger than that determined by the nitrogen adsorption experiments. These observations provide corroborative evidence that moisture actually penetrates into the amorphous solid in contrast to the purely surface-limited adsorptive phenomenon.[20,30] Absorption phenomena have been previously described for lyophilized lysozyme, recombinant bovine somatotropin (rbST), ovalbumin, β-lactoglobulin, and microcrystalline cellulose.[24,30,32,33] The moisture sorption profile of trypsinogen also revealed the presence of hysteresis $[W^{desorption} - W^{adsorption}]_{RH}$ between the sorption and desorption isotherms, which is generally thought to be a consequence of water absorption. The hysteresis loop appeared at 80% RH and closed at 0% RH, indicating the complete reversibility of water sorption, under the experimental conditions.

On addition of low-to-moderate concentrations of sucrose (carbohydrate-to-protein mass ratios of 2:1 and 1:1), the sorption curves retained their Type II profile, as shown in Figures 2b and 2c, respectively. However, the amount of moisture sorbed at low vapor pressures appeared to decrease with increasing sucrose content, and it reached a minimum at a mass ratio of 1:1. This was also reflected in the values of W_m, which decreased to 4.8 ± 0.1, 4.6 ± 0.1, and 4.4 ± 0.1 g H_2O/100 g solid at sucrose-to-protein mass ratios of 0.25:1, 0.5:1, and 1:1, respectively, indicating a reduction of the number of sorbed water molecules per unit weight of the solid at monolayer conditions. The same trend was observed for the water sorption-based particle surface areas, which, however, remained higher than the actual surface areas, indicating that absorption is still the main mode of water interaction with the solid. The gradual addition of sucrose appears to also progressively reduce the magnitude of the hysteresis loop: the loop areas decreased from 135 ± 4.4 units in the absence of sucrose, to 103.2 ± 9.9 and 93.2 ± 3.3 units for sucrose-to-protein mass ratios of 0.25:1 and 0.5:1, respectively. The hysteresis loop disappeared at a mass ratio of 1:1 (area of 1.2 ± 1.1 units).

In contrast, at high sucrose contents (mass ratios > 1:1),

Table 2—Gas and Moisture Sorption BET Analysis of Spray-Dried Trypsinogen–Sucrose Formulations

sucrose:trypsinogen mass ratio	gas sorption analysis			moisture sorption analysis			
	surface area,[a] (m^2/g)	W_m[a] (gr/100 g solid)	C^N_B[a]	app. surface area,[b] (m^2/g)	W_m[b] (gr/100 g solid)	C^W_B[b]	ΔW_m[e]
no sucrose	14.5 ± 0.02	3.3 ± 0.01	32.1 ± 0.01	224.3 ± 18.6	5.3 ± 0.5	12.4 ± 0.9	0
0.25:1	–	–	–	202.3 ± 11.8	4.8 ± 0.1	6.2 ± 0.5	0.5 ± 0.1
0.5:1	9.3 ± 0.01	2.1 ± 0.01	29.4 ± 0.01	199.9 ± 7.4	4.6 ± 0.1	4.7 ± 0.2	0.7 ± 0.1
1:1	7.6 ± 0.01	1.7 ± 0.01	29.3 ± 0.01	185.4 ± 13.4[c]	4.4 ± 0.1[c]	5.6 ± 0.5[c]	0.9 ± 0.1
2:1	6.5 ± 0.01	1.5 ± 0.01	27.6 ± 0.01[4]	196.9 ± 2.8	4.7 ± 0.1	6.2 ± 0.7	0.6 ± 0.1
4:1	5.5 ± 0.01	1.3 ± 0.01	28.1 ± 0.01	228.5 ± 20.1[d]	5.1 ± 0.6[d]	4.4 ± 0.6[d]	0.2 ± 1.1
8:1	3.6 ± 0.01	0.8 ± 0.01	29.9 ± 0.01	244.5 ± 80.8[d]	5.4 ± 0.4[d]	3.5 ± 0.5[d]	–0.1 ± 0.4

[a] Standard errors represent standard deviations from the fit of the five-point gas sorption curve. [b] Values represent averages and standard deviations of triplicate measurements ($n = 3$) except [c]$n = 2$, and [d]$n = 5$. [e] $\Delta W_m = W_m^{protein} - W_m^{protein/sucrose}$.

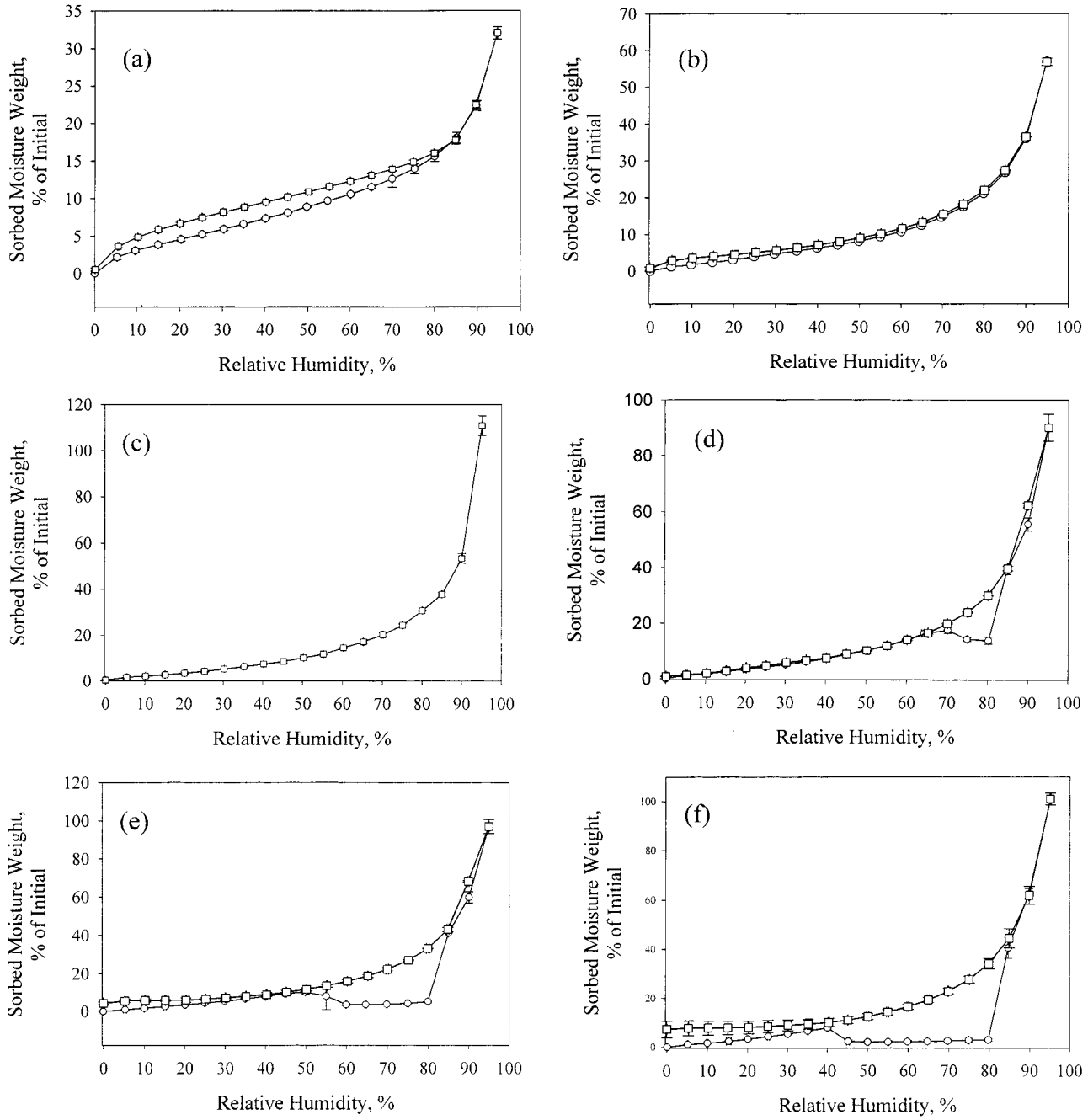

Figure 2—Dynamic moisture absorption (○) and desorption (□) isotherms of spray-dried trypsinogen-sucrose formulations (a) in the absence of sucrose, and sucrose-to-protein mass ratios of (b) 0.25:1, (c) 1:1, (d) 2:1, (e) 4:1, and (f) 8:1. Error bars represent (±) one standard deviation of triplicate determinations.

the amount of sorbed moisture at low vapor pressures increased with increasing sucrose concentration. This was also reflected in the increasing values of the water-based BET surface areas as well as the estimated equivalent monolayer values. The latter gradually increased from 4.4 ± 0.1 g/100 g solid at a mass ratio of 1:1, to 4.7 ± 0.1, 5.1

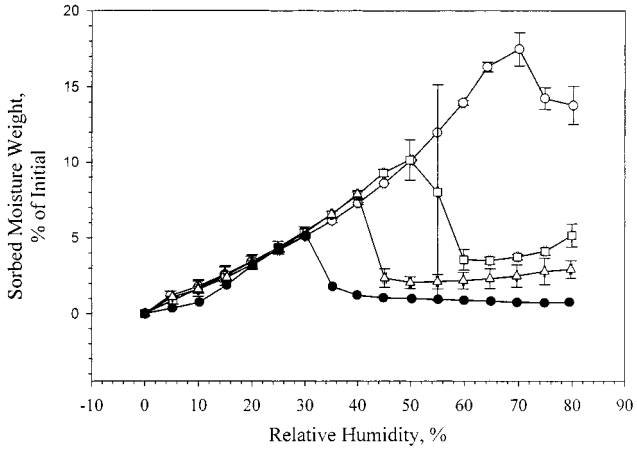

Figure 3—Comparison of the sucrose crystallization phenomenon for the spray-dried formulations at sucrose-to-trypsinogen mass ratios of (a) 2:1, (b) 4:1, (c) 8:1, and (d) in the absence of protein. Error bars represent (±) one standard deviation based on triplicate determinations.

± 0.6 and 5.4 ± 0.4 g/100 g solid at mass ratios of 2:1, 4:1, and 8:1, respectively. Therefore, at this sucrose concentration regime, the available water sorption sites progressively increased with increasing sucrose content. At the highest sucrose-to-protein mass ratio, W_m reached the value determined for the spray-dried pure protein, potentially implying that all the initial sorption sites in the mass of the solid have been reinstated. This is further demonstrated by the decreasing value of the constant C^W_B, which indicates a decline of the heat of water sorption, and thereby decreased affinity of water for the particles, with increasing sucrose concentrations.

One of the most significant features of the sorption isotherms at this sucrose concentration regime is the discontinuity appearing at high vapor pressures. For the 2:1 formulation, the discontinuity occurred at approximately 75% RH. This break is thought to be due to the release of water following the phase transition of sucrose from the amorphous glass to its crystalline state.[24,31,34–36] The formed crystals persisted up to 80% RH; upon further increase of the vapor pressure, the powder resumed its sorptive behavior, as large amounts of water were absorbed: this is indicative of the dissolution of the sucrose crystals and the formation of a saturated solution.[31,35,36] On reversal of the sorption driving force, this formulation exhibited a desorption pathway that followed closely the absorption curve. With the exception of the absence of the discontinuity in the desorption curve, no hysteresis phenomena were observed, and therefore sorption of moisture was fully reversible, as also indicated by the return to the initial conditions.

Crystallization was also observed at all higher sucrose concentrations (Figures 2e,f). However, it occurred at lower critical RH: 55 and 45% for the spray-dried samples at sucrose-to-protein ratios of 4:1 and 8:1, respectively. The crystallization process began at moisture contents of 10.2 ± 1.4 and 7.9 ± 0.3% w/w and completed at 80% RH at 5.1 ± 0.76 and 2.9 ± 0.6% w/w for the 4:1 and 8:1 formulations, respectively. In agreement with the observations with the 2:1 formulation, the sucrose crystals dissolved and the powders resumed their sorptive behavior at 85% RH. However, the desorption isotherms of these samples did not follow the absorption portions, while the loops did not close upon return to the initial conditions, potentially indicating irreversible changes in the state of the powder during the sorption process. In comparison, in the adsorption isotherm of spray-dried pure sucrose (Figure 3) the crystallization discontinuity occurred at approximately 30–35% RH. The crystallization process began at a moisture content of 5.4 ± 0.6% and completed at 0.8 ± 0.3% w/w. This result is very similar to that observed in previous studies of sucrose crystallization by Saleki–Gerhardt et al.,[34] which indicated crystallization of sucrose at approximately 32% RH (equilibrium water content of 6% w/w) at 25 °C, which is within experimental error from our observations.

Table 3—Glass Transitions of the Spray-Dried Sucrose–Trypsinogen Powders before and after Exposure to Controlled Relative Humidity Levels[c]

sucrose:trypsinogen mass ratio	glass transition (T_g)[a]		
	no. exposure	12%	43%
0.25:1	80.4 ± 1.3	44.2 ± 0.8	ND[b]
1:1	75.1 ± 1.9	44.6 ± 0.5	ND[b]
2:1	71.3 ± 1.9	39.2 ± 1.6	5.6 ± 2.7
4:1	69.5 ± 2.7	39.9 ± 2.9	3.1 ± 1.1
8:1	67.4 ± 2.6	42.3 ± 3.4	ND[b]

[a] No glass transitions were detected for the samples exposed to 75 and 84% RH. [b] No glass transitions were detected.

The results, as illustrated in Figure 3, indicate that increased sucrose concentration in the spray-dried mixtures facilitates its moisture-induced crystallization. From a different perspective, the addition of protein inhibits the carbohydrate state transition, as indicated by the decreasing critical crystallization RH with increasing protein concentration in the solid matrix. Similar results have been reported for binary mixtures of sucrose with other proteins, such as recombinant consensus interferon (rConIFN) and recombinant human granulocyte colony stimulating factor (rhG-CSF),[37] rbST,[38] and several polymers, such as starch and celluloses.[39] However, in the case of the rbST–sucrose system, the exact value of the critical %RH of sucrose crystallization at different protein contents was generally higher than that observed in our studies; the different experimental temperatures as well as the differences of the initial states of the spray-dried and lyophilized powders may account for the observed differences.

X-ray Diffraction Analysis—The diffractograms of all spray-dried formulations (results not shown) were characterized by two broad humps and the absence of any sharp peaks over the entire 2θ range, which are indicative of their amorphous nature, confirming the water sorption findings. Even at the highest sucrose-to-protein mass ratio of 8:1, the carbohydrate retained its amorphous character, as would be expected from the nature of the process and the very low moisture contents attained. These results are in agreement with previous studies that indicated the amorphous character of spray-dried rConIFN and rhG-CSF formulations even when they were prepared at high concentrations of trehalose (90% w/w).[37]

Powder Thermal Analysis—The glass transitions of the spray-dried sucrose–trypsinogen formulations, as obtained by DSC thermal analysis, are given in Table 3. The glass transition of the spray-dried pure trypsinogen could not be determined. This is a common problem with protein glasses in the absence of excipients,[37,38] which presumably arises from their large internal heterogeneity and results in an extremely broad distribution of their relaxation times.[40] However, the determination of T_g was possible for the sucrose-containing formulations. In all thermograms, only a single glass transition was observed, implying the complete miscibility of the protein and sucrose in the amorphous matrixes or, alternatively, that a second phase, if present, exists in small amounts that cannot be detected by DSC. Increasing sucrose concentrations gradually decreased the glass transition from 80.4 ± 1.3 °C at a mass ratio of 2:1 to 68.4 ± 3.2 °C for the 8:1 formulation. This observation provides further evidence of the miscibility of

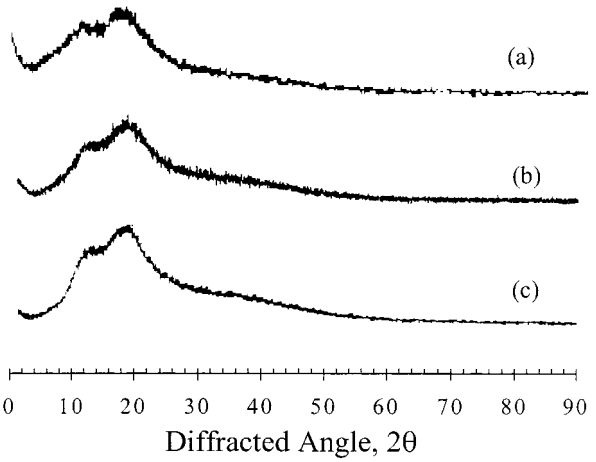

Figure 4—X-ray diffractograms of spray-dried trypsinogen formulations before exposure to elevated moisture: (a) in the absence of sucrose and at sucrose-to-protein mass ratios of (b) 0.5:1, (c) 1:1, (d) 2:1, (e) 4:1, and (f) 8:1.

the two formulation components in the solid matrix.[38] In contrast with the other spray-dried powders, the formulation at a sucrose-to-protein mass ratio of 8:1 exhibited a stress relaxation endotherm, indicating increased molecular mobility of its components.[34]

The formulations at high sucrose concentrations further exhibited an exothermic transition, which is characteristic of sucrose recrystallization. The exact temperature and magnitude of the transition, however, varied with the formulation: 173.9 °C, 160.4 °C, and 142.9 °C for the formulations at sucrose-to-protein mass ratios of 2:1, 4:1, and 8:1, respectively. These results are in agreement with previous studies,[38] and further confirm the hypothesis of protein-induced inhibition of sucrose crystallization, as observed in the dynamic moisture sorption experiments.

Equilibrium Water Sorption Studies—These studies were performed to confirm the dynamic sorption isotherms, which do not represent true equilibrium events; the "non-equilibrium" attributes arise from the limitations of user-defined equilibrium criteria of the "dynamic sorption" experiments prevent the system from reaching true equilibrium. The diffractograms of the moisture-exposed samples are shown in Figures 5–7, and the sample crystallinity analysis is given in Table 4. The X-ray diffraction analysis of the moisture-exposed samples confirmed the dynamic sorption experiments, indicating the appropriateness of this technique. When the spray-dried particles were exposed to 12% RH, the formulations retained their amorphous character; the diffractograms illustrated in Figure 4 are characterized by two broad humps and the absence of any sharp features. This is expected from the dynamic moisture sorption studies, which indicated the absence of any state changes in all formulations at this %RH level. Further, these results are in agreement with moisture sorption studies of protein–disaccharide systems, which demonstrated the absence of state transitions (for both sucrose and trehalose) upon exposure to low relative humidities.[37,38] However, the absorbed water plasticized the solid matrixes, as indicated by the decreased glass transitions given in Table 3. For all examined powders, T_g was reduced to almost half of its original value, while all formulations exhibited a relaxation endotherm during the glass transition.

As indicated in Figure 5, even after exposure to 43% RH most spray-dried powders (sucrose-to-protein mass ratios of 1:1, 2:1, and 4:1) remained amorphous, again in good agreement with the dynamic moisture sorption studies. In contrast, the diffractogram of the highest sucrose-containing powder (8:1) exhibited crystalline patterns dominated

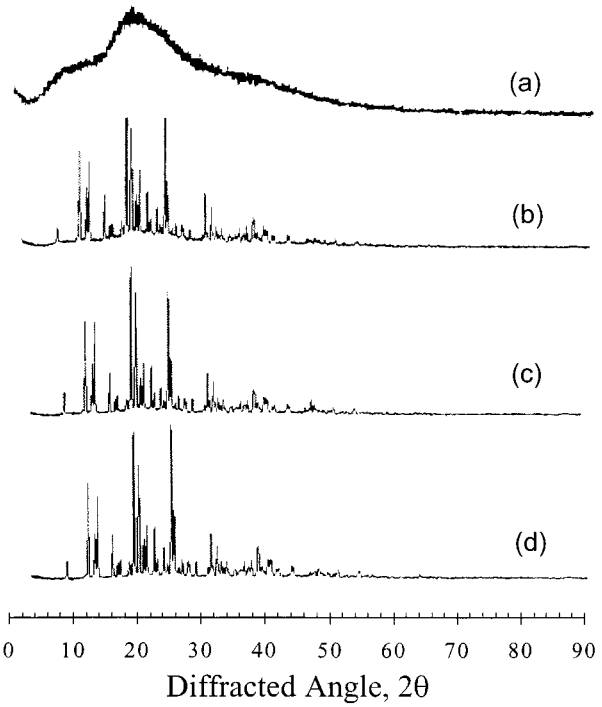

Figure 5—X-ray diffractograms of spray-dried trypsinogen formulations after equilibrium exposure to 12% RH for 10 days. Formulations are at sucrose-to-protein mass ratios of (a) 2:1, (b) 4:1, and (c) 8:1.

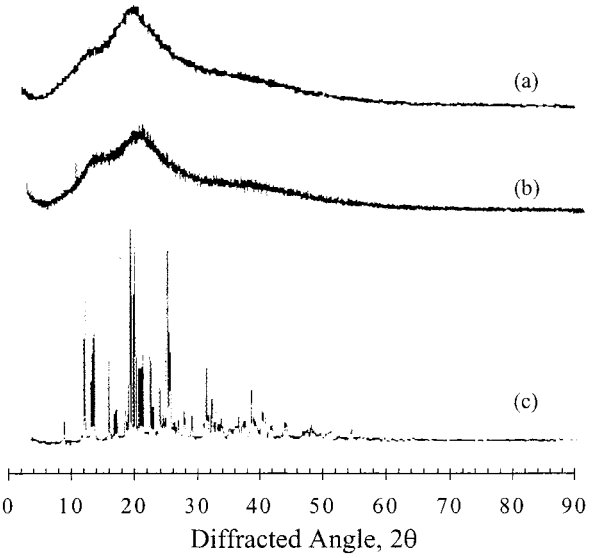

Figure 6—X-ray diffractograms of spray-dried trypsinogen formulations after equilibrium exposure to 43% RH for 10 days. Formulations illustrated are at sucrose-to-protein mass ratios of (a) 2:1, (b) 4:1, and (c) 8:1.

by sharp peaks located at 11.6°, 12.8°, 18.8°, and 24.7°, which are indicative of sucrose crystals.[39] The incurred phase transformation was expected, since the dynamic sorption results indicated the occurrence of a crystallization event at 40–45% RH. Sample crystallinity was determined at 76% w/w solid, indicating that under these conditions, most of the sucrose in the formulation has crystallized. As illustrated in Table 3, the formulation T_gs further decreased, as would be expected by the increased amounts of sorbed water.

Even after exposure to 75% RH, no state transition became apparent for the 1:1 formulation (Figure 6a), indicating that when the protein concentration in the solid matrix exceeds this mass ratio, the crystallization of sucrose can be completely inhibited. These findings are in

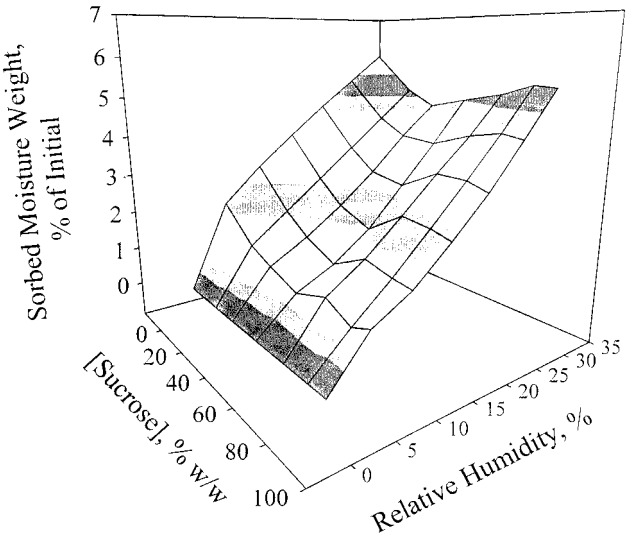

Figure 7—X-ray diffractograms of spray-dried trypsinogen formulations after equilibrium exposure to 74% RH for 10 days. Formulations illustrated are at sucrose-to-protein mass ratios of (a) 1:1, (b) 2:1, (c) 4:1, and (d) 8:1.

Table 4—Crystallinity of Spray-Dried Trypsinogen–Sucrose Formulations before and after Exposure to Controlled Relative Humidity Environments[a]

sucrose:trypsinogen mass ratio	no exposure	% crystallinity			
		12%	43%	75%	84%
1:1	0[a]	ND[b]	ND[b]	0[a]	0[a]
2:1	0[a]	0[a]	0[a]	51	0[a]
4:1	0[a]	0[a]	0[a]	71	44
8:1	0[a]	0[a]	76	85	71

[a] No crystalline peaks were detected. Samples are characterized as 100% amorphous. [b] Sample crystallinity was not determined.

agreement with literature studies which concluded that sucrose crystallization is entirely inhibited when the protein concentration in the spray-dried rConIFN and rhG-SCF formulations exceeded 50%.[37] In contrast, the sucrose in the 2:1 and 4:1 formulations underwent crystallization upon exposure to 75% RH, as indicated by the diffraction patterns shown in Figures 6b-c. These were identical to those observed for the 8:1 formulation at 43% RH. Again, these results conform well with those obtained from the dynamic sorption studies, which showed sucrose crystallization phenomena at 55 and 75% RH, respectively. However, the extent of crystallization varied, as indicated by the quantitative crystallinity estimates of 51%, 67%, and 82% for the 2:1, 4:1, and 8:1 formulations, respectively. These findings suggest that the amount of crystallizable sucrose increases with increasing concentration of the excipient in the solid matrix. No glass transitions were detected for the formulations exposed to 75% RH. This is probably due to the crystallization of most of the sucrose in the formulations, which renders the amount of the remaining amorphous material insufficient for detection of its T_g via DSC.

Finally, upon exposure of the formulations to 84% RH, the crystallinity patterns were partially disrupted, as indicated by the decreased intensity of the diffractogram peaks. As a consequence, the determined crystallinity percentages dropped to 44% and 71% for the 4:1 and 8:1 formulations, respectively, while the crystalline patterns completely disappeared for the 2:1 sample. These findings are in good agreement with the previous studies which demonstrated decreased crystallinity levels of sucrose on exposure to 84% RH, due to the crystal dissolution and the formation of saturated solutions at this vapor pressure.[39]

Discussion

It is well documented that pharmaceutical solids tend to adsorb significant amounts of water over a wide range of relative humidities. The presence of water can accelerate degradation phenomena in the solid state, such as deamidation, oxidation, disulfide cross-linking, and Maillard reactions.[41] In particular for proteins, water can affect a complex matrix of protein motions, ranging from oscillatory and rotational movement of individual amino acid groups, to segmental motions and internal fluctuations that increase their dynamic mobility and thereby decrease their conformational stability.[41,42] Moreover, the sorbed water may have a tremendous impact on both the physical properties of the processed material. In terms of the latter, water sorption plasticizes the solid matrix, reduces its T_g,[43] and may induce excipient crystallization and alter its morphology, physicochemical properties, and physical characteristics,[44] phenomena that can all deteriorate product stability or even diminish proper delivery.

In agreement with previous studies,[37,45] the X-ray diffraction results suggest that spray drying of trypsinogen, even in the presence of increasing amounts of sucrose, results in the formation of amorphous microparticles. In this state, proteins are devoid of long-range intermolecular interactions or orientation, although they maintain their specific short-range interactions.[41] Upon exposure to different relative humidity levels, the protein-based powders take up large amounts of water, illustrative of their amorphous nature. Water sorption confers increased mobility, which may be regarded as an increase in the dynamic fluctuations of the protein, as demonstrated for trypsin, a protein that has a significant degree of homology with trypsinogen.[46]

Similarly with amorphous polymers, water sorption in all spray-dried trypsinogen−sucrose formulations occurs via penetration into the disordered solid and is not limited to surface adsorption.[41] This was demonstrated by the large excess of moisture taken up by the solid at high vapor pressures and the lack of dependence of the water uptake on the specific surface area of the samples.[43] The absorption process is further elucidated by comparison of the surface areas obtained from the nitrogen adsorption studies with those calculated from fits of the BET equation to the moisture sorption isotherms; although the former are highly dependent on particle size and physical state of the solid, the latter are unaffected by these properties. The very large H_2O/N_2 surface area ratios obtained for all spray-dried formulations are consistent with the water penetration hypothesis.

This explanation is also consistent with the sorption hysteresis, observed for formulations at the low sucrose concentration regime. Hysteresis has been described for many amorphous systems.[27] Although it was first suggested to originate from capillary condensation, it was later thought to be caused by chemisorption in rigid materials, and by water−polymer interactions in nonrigid solids (such as proteins).[27] Specifically for proteins, hysteresis phenomena have also been attributed to conformational rearrangements that involve a structural relaxation or even a phase change, which can be facilitated by increasing free volume and mobility of the protein during sorption.[44,47] It has been previously hypothesized that it provides an indication of the existence of the protein in a kinetically metastable, dynamically constrained state, which arises from the macroscopic swelling due to the penetration of water in the solid matrix.[25,41,44,47] The extent of the hysteresis loop in our experiments, decreased with increasing sucrose concentration, indicating the decreased plasticization of the amorphous solid by water. Alternatively, the protein con-

formational changes should be less dramatic in the presence of sucrose; this is confirmed by our previous studies, which indicated that trypsinogen gradually maintains a more nativelike conformation in the presence of increasing amounts of sucrose.[1] Finally, since hysteresis is related to the glass transition of the solid at the specified RH,[44] it is possible that its decreased magnitude may also relate to variations of $\Delta T = T - T_g(\mathrm{RH})$ during the sorption process for the different trypsinogen−sucrose formulations. The disappearance of the hysteresis loop at a sucrose-to-protein mass ratio of 1:1, formulation at which the protein fully recovers its native structure and activity,[1] further underscores the utility of this parameter in correlating solid behavior with protein conformation and structural dynamics.

The hygroscopic nature of both trypsinogen and sucrose would forecast a cumulative water sorption capacity of their physical mixtures. However, comparison of the actual amounts of sorbed water at low (<35%) relative humidities indicated a concentration-dependent effect of sucrose. At low mass ratios of the disaccharide, the sorptive capacity of the powders actually decreased, in good agreement with literature studies.[26,48] This observation indicates that addition of sucrose contributes to the blockage of potential sorption sites in the binary solid matrix, providing evidence of specific hydrogen bonding interactions between the two components. This analysis further supports the expressed "water replacement" hypothesis.[3,4,12] The interaction is also confirmed by the decreased heat of sorption with increasing sucrose concentration. Moreover, the facility of the replacement of protein−sucrose interactions by water, as represented by the amount of sorbed water at low vapor pressures (<35%) can provide an estimate of the extent (and perhaps the strength) of their interactions. The latter is illustrated in the three-dimensional mesh plot of Figure 7 as a function of the sucrose concentration and vapor pressure. It becomes apparent that the extent of interaction increases with increasing sucrose concentration and becomes maximal at equal mass ratios; thereafter in decreases, as the sorptive capacity of the formulation increases, potentially implying the preferential replacement of the stabilizing protein−sugar contacts.

The existence of specific protein−sucrose interactions was further confirmed by examination of the estimated BET monolayer of water. The importance of this parameter stems from its correlation with the onset of internal protein flexibility.[49,50] Moreover, the water content at the monolayer has been directly linked to the free energy changes induced by water sorption and its concomitant changes in the protein structure,[43] while it can provide information on the relative affinity of the water for the substrate. The decreased value of W_m, upon sucrose addition, confirmed the specific protein−sucrose hydrogen bonding interactions. Similar to our previous observations, W_m became minimal at a sucrose-to-protein mass ratio of 1:1, while it increases at higher sucrose contents, reaching its value for the pure protein at the highest sucrose content.

This explanation is in agreement with our enzymatic activity results,[1] which indicated the sucrose-induced stabilization of trypsinogen up to a mass ratio of 1:1, with subsequent reduction of its protective effects at excess concentrations of the carbohydrate. Moreover, the profile of monolayer values closely follows that of the activity losses of the protein during spray drying, potentially indicating that sucrose−protein interactions in the solid matrix are responsible for both stabilizing and destabilizing effects of the disaccharide. Assuming that the relative values of W_m are proportional to the number of component−water interactions, a relative estimate of the sucrose-occupied sites on the protein surface could be obtained by

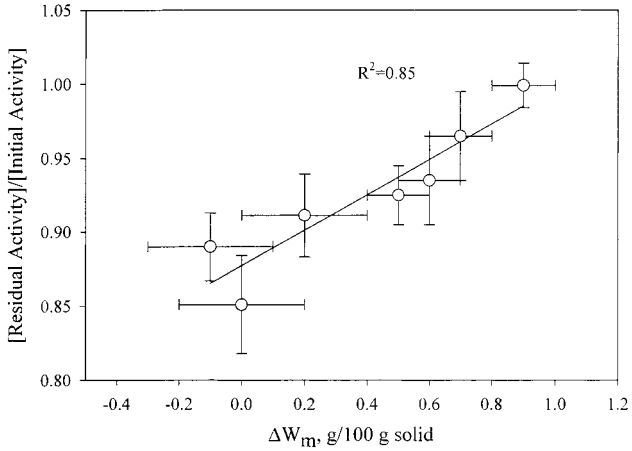

Figure 8—3-D mesh plot of the effect of sucrose and %RH of exposure on the moisture uptake by the spray-dried trypsinogen–sucrose powders.

subtracting the number of water-accessible hydrogen bonding sites of the protein/sucrose mixture ($W_m^{\text{protein/sucrose}}$) from the total number of water-accessible hydrogen bonding sites on the protein in the absence of sucrose (W_m^{protein}), $\Delta W_m = W_m^{\text{protein}} - W_m^{\text{protein/sucrose}}$. From the values of ΔW_m, which are given in Table 2, it follows that at conditions of excess sucrose, the hydrogen bonds between the two components actually decrease and finally reach depletion. This interpretation would support the finding of reduced stability of trypsinogen at the high sugar concentration regime.[1] Indeed, as illustrated in Figure 8, a good correlation ($R^2 = 0.85$) exists between ΔW_m and the residual activity of trypsinogen after spray drying, confirming this hypothesis and implying the replacement of the stabilizing protein−sugar interactions.

Sucrose Crystallization and Inhibition Phenomena—Our moisture sorption experiments indicated that pure, amorphous sucrose can undergo an amorphous-to-crystalline state change at a moisture level of 5.8% w/w. Sucrose crystallization was initiated presumably by sorption of sufficient moisture that reduced its T_g below the operating temperature of 37 °C; in the spray-dried particles crystallization is thought to start at the particle surface and rapidly propagate in the interior.[51] The crystals persisted until approximately 85% RH, which is close to the saturation water activity ($a_w = 0.86$) of sucrose.[27] It is interesting to consider the structure of sucrose in the crystals. In sufficiently diluted solutions, all eight available hydroxyl groups of sucrose form bonds with water molecules.[52−54] However, in concentrated solutions, the high flexibility of the bond joining the two glucose and fructose units promotes the formation of two intramolecular and several intermolecular hydrogen bonds.[53] At this concentration regime, aggregation phenomena occur between sucrose molecules, which now exist in clusters, and under the appropriate conditions will create the stable three-dimensional nucleus.

Although crystallization events were absent at sucrose-to-protein mass ratios lower than 1:1, they became apparent at higher mass ratios of the disaccharide, after exposure to intermediate or high relative humidities. The critical RH of crystallization of these formulations were higher than that observed for the pure compound, denoting the inhibitive role of the protein: increasing concentrations of the protein more effectively inhibit the state transition of the disaccharide, as also indicated by the increasing T_{cryst} with increasing trypsinogen content. From a different perspective, and as illustrated in Figure 3, increasing sucrose concentrations facilitate crystallization, approaching that of the pure compound. Despite the small differences in the

actual critical RH levels, similar results on the inhibitive role of protein in the growth of sucrose crystals were previously described for the rbST−sucrose lyophilized system.[38] It is well documented that impurities decrease the overall growth rate of crystals and change their morphology, as they can block the crystal faces, or even modify the solution characteristics and affect the supersaturation level of sucrose.[52,53] The inhibition of sucrose crystal growth in the presence of high amounts of trypsinogen could reflect the absence of nuclei or even their poisoning by the protein. At low relative protein concentrations, there is less protein available for growth inhibition, while the capacity of the protein phase to compete for water sorption is reduced. In this case, and when adequate water is sorbed to increase the mobility of the sucrose molecules, nuclei can form and grow in the absence of inhibiting interactions. Once nucleation takes place, crystallization occurs rapidly.[55]

For crystallization to proceed, the presence of significant sucrose−sucrose contacts in the solid matrix is imperative. It is therefore conceivable that these are responsible for replacing the stabilizing protein−sugar interactions and could account for the altered interactions of the solid with water. We further assessed the validity of this hypothesis to investigated the nature of this phenomenon.

Phase Separation Phenomena and Origin Hypothesis—Quantitative estimates of the maximum amount of crystallizable sucrose in the formulations can be obtained by the X-ray estimates at conditions of maximal crystal content, 75% RH. Even under these conditions, the X-ray-estimated fractions of crystalline material (within the 5−10% error of the method) cannot fully account for the total amount of sucrose in the formulations, indicating the simultaneous presence of amorphous sugar. Since sucrose crystallization would be inhibited by the presence of protein, it can be assumed that the crystallizable sugar matrix is devoid of protein. This provides evidence for the separation of the dried solid into two distinct phases: a protein-rich and a sugar-rich phase. In the former, amorphous phase, sucrose molecules are in contact with protein, which inhibits their crystallization. In contrast, the second, crystallizable phase, is devoid of protein, so when the system acquires sufficient mobility upon moisture sorption, crystallization is induced. This implies that in the spray-dried material, the phase-separated, crystallizable sucrose does not contribute to the stabilization of the protein, due to its exclusion from it. Assuming that the determined crystallinity fractions provide, within error, a representation of the mass fraction of the crystallized sucrose, we could then obtain via a mass balance an estimate of the actual amounts of amorphous sucrose in the formulations; the analysis is illustrated in Table 5. Using this approach, the amount of protein-interacting, amorphous sucrose was calculated at 15.7, 13.0, and 6.9% w/w for formulations 2:1, 4:1, and 8:1. The analysis indicates that as the mass fraction of sucrose in the formulation increases, there is less available carbohydrate to interact with the protein. The actual amounts of active, protein-interacting sucrose are smaller than expected, based on the formulated mass ratios, as indicated in Table 5. In fact, the effective mass ratios are below the maximally stabilizing mass ratio of 1:1, indicating the inadequate protection of the protein, in agreement with the decreased stability of trypsinogen at these sucrose mass ratios.[1] Moreover, despite the small variability of the observed effects, the phenomenon of partial dissipation of the protective effects of disaccharides has been observed with other proteins,[13,14] indicating a global phenomenon rather than an isolated, protein-specific event.

The onset of the phase separation and crystallization phenomena, with respect to the sucrose content, in the solid formulations coincides with that of the excluded volume effects of the sugar in the solutions before spray drying.[1] Excluded volume phenomena arise from the preferential separation of the carbohydrate from the protein surface; they represent by default phase-separation events at the molecular level. We hypothesize that this distribution of phase-separated formulation components in solution before spray drying is not significantly disturbed during the atomization step, resulting in the formation of protein-rich and sugar-rich phases in the formed particles. The low moisture contents achieved during spray drying prohibited sucrose crystallization in the formulations; however, when the viscosity of the solid matrix decreased upon water sorption, crystal formation was promoted. Further, as discussed above, the protein-excluded sucrose molecules in the solution phase may exist as aggregates, promoting nuclei formation. The presence of aggregate clusters is favored at high concentrations of the disaccharide, as reflected in the reduced hydration number of sucrose: it decreases from 10 to 12 (mol of water/mol of sugar) at sucrose concentrations < 2% w/w, to 5 (mol of water/mol of sugar) at concentrations between 10 and 50% w/w.[54] While the formulated solutions at low sucrose concentrations (mass ratios < 1:1) reside in the former, those prepared at high sucrose concentrations (mass ratios > 1:1) lie in the latter regime, indicating the possible presence of phase-separated, aggregated sucrose in the pre-spray drying solutions.

The proposed mechanisms are in agreement with previous studies on the destabilization of β-galactosidase during lyophilization in the presence of an excess amount of mannitol and inositol;[56,57] however, our results indicate that it is not crystallization per se, but the preceding phase separation event that is responsible for the destabilizing phenomena. It is important to note, however, that the initial protein concentration during spray drying may play an important role, as it may dramatically influence both the starting solution equilibria and the interactions with the excipient in the solid state. More work is required in this direction to understand the concentration dependence of the protein−excipient interactions in the solid state.

Our results suggest that utilization of high concentrations of excipients during preparation of solid state protein formulations may not always be desirable, as even ordinarily stabilizing excipients (such as the commonly utilized disaccharides) may not guarantee the expected stabilization. This becomes particularly important when excipients

Table 5—Nominal and Effective Sucrose Concentrations in the Spray-Dried Trypsinogen–Sucrose Powders

[sucrose: TGN]$_{nominal}$ mass ratio	sucrose[a] (% mass$_{total}$)	free sucrose[b] (% mass$_{total}$)	bound sucrose[c] (% mass$_{total}$)	[sucrose: TGN]$_{effective}$[d] mass ratio
no sucrose	0	0	0	no sucrose
0.25:1	20.0	0	20.0	0.25:1
0.5:1	33.3	0	33.3	0.5:1
1:1	50.0	0	50.0	1:1
2:1	66.7	51.0	15.7	0.47:1
4:1	80.0	71.0	9.0	0.45:1
8:1	88.9	85.0	3.9	0.35:1

[a] Nominal sucrose concentration (% w/w) on a percent total solids basis ($f^{sucrose}_{set}$). [b] Crystallizable sucrose, percent total solids basis. The value represents X-ray diffraction percent crystallinity estimates, under the assumption that sucrose is the only crystallizable formulation component ($f^{sucrose}_{free} = f^{XRD}_{crystalline}$). [c] Noncrystallizable sucrose, percent total solids basis. The value is extracted from a mass balance from the crystallizable sucrose X-ray diffraction estimates ($f^{sucrose}_{bound} = (f^{sucrose}_{set} - f^{sucrose}_{free})$. [d] The "effective" sucrose-to-protein mass ratio is calculated from the mass ratio of noncrystallizable (protein-bound) sucrose to total protein ($f^{sucrose}_{bound}/f^{protein}_{total}$).

that exert excluded volume effects are utilized. Formulation components that exhibit increased capacity for exclusion from the protein surface will have a high tendency to phase separate and will preferentially interact with themselves rather than the protein. Moreover, they may undergo undesirable phase transformations that may decrease product stability. This becomes of particular significance during the administration of pharmaceutical products with sustained release delivery systems, such as polymeric injectable depots or implantable micropumps, because of the exposure of the formulation to increased water levels at the physiologic temperature of 37 °C.

References and Notes

1. Tzannis, S. T.; Prestrelski, S. J. Activity-Stability Considerations of Trypsinogen during Spray Drying: Effects of Sucrose, *J. Pharm. Sci*. **1999**, *88*, 351−359.
2. Tzannis, S. T. Stabilization of Proteins during Spray Drying, Protein Stability Conference, Breckenridge, CO, July 16−18, 1998.
3. Prestrelski, S. J.; Arakawa, T.; Carpenter, J. F. Structure of Proteins in Lyophilized Formulations Using Fourier Transform Infrared Spectroscopy. In *Formulation and Delivery of Proteins and Peptides*, Cleland, J. L., Langer, R., Eds.; ACS Symposium Series 567; American Chemical Society: Washington, DC, 1994; pp 148−169.
4. Prestrelski, S. J.; Tedeschi, N.; Arakawa, T.; Carpenter, J. F. Dehydration-Induced Conformational Transitions in Proteins and Their Inhibition by Stabilizers. *Biophys. J*. **1993**, *65*, 661−671.
5. Griebenow, K.; Klibanov, A. M. Lyophilization-induced Reversible Conformational Changes in the Secondary Structure of Proteins. *Proc. Natl. Acad. Sci. U.S.A*. **1995**, *92*, 10969−10976.
6. Winters, M. A.; Knutson, B. L.; Debenedetti, P. G.; Sparks, G.; Przybycien, T. M.; Stevenson, C. L.; Prestrelski, S. J. Precipitation of Proteins in Supercritical Carbon Dioxide. *J. Pharm. Sci*. **1996**, *85*, 586−594.
7. Mumenthaler, M.; Hsu, C. C.; Pearlman, R. Feasibility Study on Spray-Drying of Protein Pharmaceuticals: Recombinant Human Growth Hormone and Tissue-Type Plasminogen Activator. *Pharm. Res*. **1994**, *11*, 12−20.
8. Labrude, P.; Rasolomanana, M.; Vigneron, C.; Thirion, C.; Chaillot, B. Protective Effect of Sucrose on Spray Drying of Oxyhemoglobin. *J. Pharm. Sci*. **1989**, *78*, 223−229.
9. Broadhead, J.; Edmond Rouan, S. K.; Hau, I, Rhodes, C. T. The Effect of Process and Formulation Variables on the Properties of Spray-Dried β-Galactosidase. *J. Pharm. Pharmacol*. **1994**, *46*, 458−467.
10. Broadhead, J.; Edmond-Rouan, S. K.; Rhodes, C. T. The Spray Drying of Pharmaceuticals. *Drug Dev. Ind. Pharm*. **1996**, *22*, 813−822.
11. Tzannis, S. T.; Hrushesky, W. J. M.; Wood, P. A.; Przybycien, T. M. Irreversible Inactivation of Interleukin-2 in a Pump-Based Delivery Environment. *Proc. Natl Acad. Sci. U.S.A*. **1996**, *91*, 5460−5465.
12. Carpenter, J. F.; Prestrelski, S. J.; Anchordoquy, T. J.; Arakawa, T. Interactions of Stabilizers with Proteins During Freezing and Drying. In*Formulation and Delivery of Proteins and Peptides*. Cleland, J. L., Langer, R., Eds.; ACS Symposium Series 567; American Chemical Society: Washington, DC, 1994; pp 134−147.
13. Tzannis, S. T.; Meyer, J. D.; Prestrelski, S. J. Secondary Structure Considerations during Protein Spray Drying. 213th ACS National Meeting, San Francisco, CA, April 13−17, 1997; American Chemical Society: Washington, DC, 1997.
14. Tzannis, S. T. Spray Drying of Protein Pharmaceuticals: Powder Characteristics and Stability Considerations. Presented at Conference of Formulations and Drug Delivery II, La Jolla, CA, October 5−8, 1997.
15. Carpenter, J. F.; Crowe, J. H. An Infrared Spectroscopic Investigation of the Interactions of Carbohydrates with Dried Proteins. *Biochemistry* **1989**, *28*, 3916−3922.
16. Carpenter, J. F.; Prestrelski, S. J.; Arakawa, T. Separation of Freezing- and Drying-Induced Denaturation of Lyophilized Proteins Using Stress-Specific stabilization. I. Enzyme Activity and Calorimetric Studies. *Arch. Biochem. Biophys*. **1993**, *303*, 456−464.
17. Prestrelski, S. J., Byler, D. M., Liebman, M. N. Comparison of Various Molecular Forms of Bovine Trypsin: Correlation of Infrared Spectra with X-ray Crystal Structures. *Biochemistry* **1991**, *30*, 133−143.
18. Brunauer, S.; Emmett, P. A.; Teller, E. Adsorption of Gases in Multimolecular Layers. *J. Am. Chem. Soc*. **1938**, *60*, 309−319.
19. Teng, C. D.; Zarrintan, M. H.; Groves, M. J. Water Vapor Adsorption and Desorption Isotherms of Biologically Active Proteins. *Pharm. Res*. **1991,** *8*, 191−195.
20. Zografi, G. States of Water Associated with Solids. *Drug Dev Ind. Pharm*. **1988**, *14*, 1905−1926.
21. Kontny, M. J.; Zografi, G. Sorption of Water by Solids. In *Physical Characterization of Pharmaceutical Solids*; Brittain, H. G., Ed.; Marcel Dekker Inc.: New York, 1995; pp 387−418.
22. Andres, M. C.; Bugay, D. E.; McKenzie, A. T.; Remick, D. M.; Stahly, G. P.; Thomas, A. S. Quantitative X-ray Powder Diffraction Analysis: A Comparison of Data Analysis Techniques. *PharmSci* **1998**, *1*, S-23.
23. Leeder, J. D.; Watt, J. C. The Stoichiometry of Water Sorption by Proteins. *J. Colloid Interface Sci*. **1974**, *48*, 339−344.
24. Hageman, M. J. The Role of Moisture In Protein Stability. *Drug Dev. Ind. Pharm*. **1988**, *14*, 2047−2070.
25. Bizot, H.; Buleon, A.; Mouhous-Riou, N.; Multon, J. L. Some Factors Concerning Water Vapour Sorption Hysteresis on Potato Starch. In *Properties of Water in Foods*; Simatos, P., Multon, J. L., Eds.; NATO-AST Series E: Applied Sciences, No. 90, Martinus Nijhoff Publishers: Dordrecht, The Netherlands, 1985; pp 83−93.
26. Chinachoti, P.; Steinberg, M. P. Interaction of Sucrose with Gelatin, Egg Albumin and Gluten in Freeze-Dried Mixtures as Shown by Water Sorption. *J. Food Sci*. **1988**, *53*, 932−934.
27. Chinachoti, P.; Steinberg, M. P. Moisture Hysteresis is due to Amorphous Sugar. *J. Food Sci*. **1986**, *51*, 453−455.
28. Chinachoti, P.; Schmidt, S. J. Solute-Polymer−Water Interactions and their Manifestations. In *Water Relationships in Foods: Advances in the 1980s and Trends for the 1990s*; Levine, H., Slade, L., Eds.; Plenum Press: New York, 1991; pp 561−583.
29. Kuntz, I. D.; Kauzmann, W. Hydration of Proteins and Polypeptides. *Adv. Prot. Chem*. **1974**, *28*, 239−345.
30. Hageman, M. J.; Possert, P. L.; Bauer, J. M. Prediction and Characterization of Water Sorption Isotherm for Bovine Somatotropin. *J. Agric. Food Chem*. **1992**, *40*, 342−347.
31. Saltmarch, M.; Lanuza, T. P. Influence of Relative Humidity on the Physicochemical State of Lactose in Spray-Dried Sweet Whey Powders. *J. Food Sci*. **1980**, *45*, 1231−1236.
32. Benson, S. W.; Ellis, D. A.; Zwansig, R. W. Surface Areas of Proteins. III. Adsorption of Water. *J. Am. Chem. Soc*. **1950**, *72*, 2102−2105.
33. Nakai, Y.; Fukuoka, E.; Kakajima, S.; Hasegawa, J. Crystallinity and Physical Characterization of Microcrystalline Cellulose. *Chem. Pharm. Bull*. **1977**, *25*, 96−101.
34. Saleki-Gerhardt, A.; Zografi, G. Non-Isothermal and Isothermal Crystallization of Sucrose from the Amorphous State. *Pharm. Res*. **1994**, *11*, 1166−1173.
35. Makower, B.; Dye, W. B. Equilibrium Moisture Content and Crystallization of Amorphous Sucrose and Glucose. *Agric. Food Chem*. **1956**, *4*, 72−77.
36. Warburton, S.; Pixton, S. W. The Moisture Relations of Spray Dried Skimmed Milk. *J. Stored Prod. Res*. **1978**, *14*, 143−158.
37. French, D. L.; McAuley, A. J.; Chang, B.; Niven, R. W. Moisture Induced State Changes in Spray-Dried Trehalose/Protein Formulations. *Pharm. Res*. **1995**, *12*, S83.
38. Sarciaux, J.-M. E.; Hageman, M. J. Effects of Bovine Somatotropin (rbST) Concentration at Different Moisture Levels on the Physical Stability of Sucrose in Freeze-Dried rbST/Sucrose Mixtures. *J. Pharm. Sci*. **1997**, *86*, 365−371.
39. Chinachoti, P.; Steinberg, M. P. Crystallinity of Sucrose by X-ray Diffraction as Influenced by Absorption versus Desorption, Waxy Maize Starch Content and Water Activity. *J. Food Sci*. **1986**, *51*, 456−459.
40. Green, J. L.; Fan, J.; Angell, C. A. The Protein-Glass Analogy: Some Insights from Homopeptide Comparisons. *Biophys. Chem*. **1994**, *98*, 13780−13790.
41. Hageman, M. J. Water Sorption and Solid-State Stability of Proteins. In *Stability of Protein Pharmaceuticals. Part A: Chemical and Physical Pathways of Protein Degradation*. Ahern, T. J., Manning, M. C., Eds.; Plenum Press: New York, 1992; pp 273−309.
42. Bone, S. Dielectric Studies of Native, Unfolded and Intermediate Forms of β-lactamase. *Phys. Med. Biol*. **1994**, *39*, 1801−1809.
43. Zografi, G.; Hancock, B. Water-Solid Interactions in Pharmaceutical Systems. In *Proceedings of 53rd International Congress of Pharmaceutical Sciences*; Tokyo, Japan, Elsevier: New York, 1994.

44. Levine, H.; Slade, L. Water as a Plasticizer: Physicochemical Aspects of Low Moisture Polymeric Systems. In *Water Science Reviews*; Franks, F., Ed.; Cambridge University Press: Cambridge, UK, 1987; Volume 3, pp 79−185.
45. White, G. W.; Cakebread, S. H. The Glassy State In Certain Sugar-Containing Foods. *J. Food. Technol.* **1966**, *1*, 73.
46. Parak, F. Correlations of Protein Dynamics with Water Mobility: Mössbauer Spectroscopy and Microwave Absorption Methods. *Methods Enzymol.* **1986**, *127*, 196−206.
47. Morozov, V. N.; Morozova, T. Y.; Kachalova, G. S.; Myachin, E. T. Interpretation Of Water Desorption Isotherms Of Lysozyme. *Int. J. Biol. Macromol.* **1988**, *10*, 329−336.
48. Costantino, H. R.; Curley, J. G.; Wu, S.; Hsu, C. C. Water Sorption Behavior of Lyophilized Protein−Sugar Systems and Implications for Solid-State Interactions. *Int. J. Pharm.* **1998**, *166*, 211−221.
49. Poole, P. L.; Finey, J. L. Sequential Hydration of Dry Proteins: A Direct Difference IR Investigation of Sequence Homologues Lysozyme and α-Lactalbumin. *Biopolymers* **1984**, *23*, 1647−1666.
50. Rupley, J. A.; Gratton, E.; Careri, G. Water and Globular Proteins. *Trends Biochem. Sci.* **1983**, *8*, 18−22.
51. Palmer, K. J.; De, W. B.; Black, D. X-ray Diffractometer and Microscopic Investigation of Crystallization of Amorphous Sucrose. *Agric. Food Chem.* **1956**, *4*, 77−81.
52. Vaccari, G.; Mantovani, G. Sucrose Crystallization, In *Sucrose: Properties and Applications*; Mathlouthi, M., Reiser-Cedus, P., Eds.; Chapman & Hall: New York, 1995; pp 33−74.
53. Mathlouthi, M. Amorphous Sugar. In *Sucrose: Properties and Applications*; Mathlouthi, M., Reiser-Cedus, P., Eds.; Chapman & Hall: New York, 1995; pp 75−100.
54. Flink, J. M. Structure and Structure Transitions in Dried Carbohydrate Materials. In *Physical Properties of Foods*; Peleg, M., Bagley, E. B., Eds.; AVI Publishing Co.: New York, 1984; pp 473−521.
55. Van Scoik, K. G.; Carstensen, J. T. Nucleation Phenomena in Amorphous Sucrose Systems. *Int. J. Pharm.* **1990**, *58*, 185−196.
56. Izutsu, K.; Yoshioka, S.; Terao, T. Decreased Protein Stabilizing Effects of Cryoprotectants due to Crystallization. *Pharm. Res.* **1993**, *10*, 1233−1238.
57. Izutsu, K.; Yoshioka, S.; Kojima, S. Physical Stability and Protein Stability of Freeze-Dried Cakes during Storage at Elevated Temperatures. *Pharm. Res.* **1994**, *11*, 995−999.

Acknowledgments

The authors thank Wei Zhang, Joan Huey-Dow, and Tom Piercy (ALZA Corporation) for their assistance in the modulated DSC and surface area measurements.

JS9800127