Prediction and Characterization of the Water Sorption Isotherm for Bovine Somatotropin†

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The sorption of water by recombinant bovine somatotropin (rbSt) solids was described by a sigmoidal-shaped isotherm. Isotherm predictions based on rbSt's primary sequence and the H_2O binding capacity of various functional groups provided reasonable estimates of the isotherm, depending on the rbSt salt used. The isotherms were described mathematically by the BET and GAB equations, resulting in "monolayer" values of 5–8 g of H_2O/100 g of protein (60–100 mol of H_2O/mol of protein), depending on the salt of rbSt. The isotherms were independent of particle surface area, and H_2O/N_2 surface area ratios were greater than 1, both consistent with the penetration of water into the solid. The hysteresis in the sorpion–desorption isotherms was consistent with kinetically metastable states typically observed in amorphous polymeric systems. The importance of moisture on rbSt stability was demonstrated and discussed with respect to potential plasticization of the solid by water.

INTRODUCTION

The inherent instability of protein molecules in aqueous media precludes their long-term storage in solution. This difficulty is generally overcome by dehydration of the protein through lyophilization or spray-drying. The stability of protein solids is especially sensitive to residual moisture in the solid and moisture uptake from the environment by the hygroscopic powder (Pristoupil et al., 1985; Hageman, 1988, 1992; Pikal et al., 1991; Roy et al., 1991). Due to the importance of the protein–water interaction in food products, numerous investigators have explored the relationship between moisture and stability of proteins in the solid state (Slade and Levine, 1989; Wolf et al., 1985).

Recombinant bovine somatotropin (rbSt), a 191 amino acid, 22 000 molecular weight protein, is produced in *Escherichia coli* and is subsequently isolated and purified (Evans and Knuth, 1987; Olson et al., 1987). rbSt is of particular interest for development as a veterinary agent to enhance lactation in dairy cows (Bauman et al., 1985). The development of a stable, easily administered dosage form is highly desirable. Dosage forms of this sort are typically lyophilized formulations, which can be reconstituted at the time of administration, or dispersions of protein solids in nonaqueous vehicles and polymer matrices (Ritschel, 1973; Pikal, 1990a,b; Pitt, 1990).

To consistently formulate and manufacture a protein product that meets defined specifications, many critical variables need to be identified and characterized. A key variable that affects the processing and stability of proteins is moisture (Hageman, 1988, 1992). This paper explores the experimental and semiempirical characterization of water sorption in solid samples of rbSt, including a discussion on limitations in the interpretation of the data. The impact of moisture on stability is demonstrated and discussed in terms of potential mechanistic factors that account for moisture-induced instability of protein solids.

MATERIALS AND METHODS

Materials. Recombinant DNA-derived bovine somatotropin (rbSt) was obtained by expression in *E. coli* carrying a temperature-sensitive runaway plasmid into which the bovine somatotropin gene sequence and a tryptophan promoter system had been inserted (Olson et al., 1987). The rbSt was purified according to the method of Evans and Knuth (1987) with biological activity confirmed by a HYPOX rat growth bioassay (Langley et al., 1987). All other chemicals used were of analytical reagent grade or better.

Preparation of rbSt Salts. The sodium salt of rbSt was prepared by dialysis against a NaOH solution, followed by lyophilization or spray-drying (Yamato Model GA31). The internal salt of rbSt was prepared by dialysis against a volatile buffer, followed by lyophilization. The zinc salt of rbSt was prepared by zinc chloride induced precipitation of rbSt from solution, removal of excess zinc by successive centrifugation and pellet washes and, finally, lyophilization of the solid pellet. Locations of potential glass transition temperatures for the amorphous solids were investigated using a differential scanning calorimeter (Perkin-Elmer DSC-4).

Chromatographic Analysis. Reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out using either a Bakerbond Wide Pore C-4 or a Vydac 214TP (C4) column. A gradient system (Varian Vista 5500) with acetonitrile and water mobile phases, both containing 0.1% trifluoroacetic acid (TFA), was used. The protein was eluted with a linear gradient of the acetonitrile phase from 40% to 55% over 30 min, with detection at 215 nm (Kratos 783). Size exclusion high-performance liquid chromatography (SE-HPLC) was run with a Du Pont Zorbax GF-250 column and a pH 7.4 glycine/phosphate/NaCl mobile phase that contained 0.1% sodium dodecyl sulfate (SDS) (Stodola et al., 1986). Detection was by UV 280 nm.

Generation of Sorption Isotherms. Isotherm measurements were determined gravimetrically by weighing approximately 100 mg of the indicated protein salt into a vial and placing it into a desiccator at room temperature (24 ± 1 °C) containing either saturated salt solutions or specified solutions of concentrated sulfuric acid (Dean, 1979; Weast, 1983). Dry weights were determined by storage over phosphorus pentoxide for 3–5 days. Samples were incubated until no further increase/decrease in weight could be noted. This generally required approximately 3–4 days at each condition. Although not reported in this paper, other studies have shown that Karl Fischer analysis agrees quite well with weight gained numbers for water content. Rates of moisture sorption were determined by spreading approximately 100 mg of the internal salt of rbSt in a weighing boat of 21.6 cm^2 to provide maximum contact with the vapor phase. The sample was equilibrated at the respective relative vapor pressure (rvp), weighed, and transferred to a balance at the new rvp. Nonlinear least-squares analysis for purposes of curve fitting was carried out using PCNONLIN, reporting results with 95% confidence limits.

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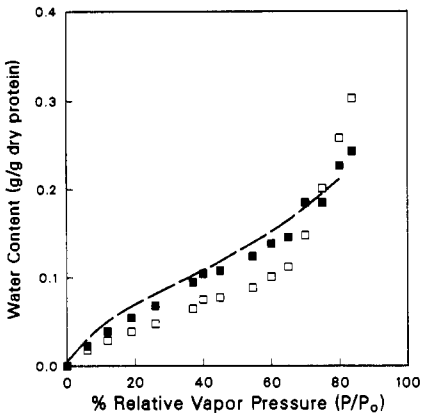

Figure 1. Comparison of experimentally determined sorption isotherms at 24 °C for the sodium (■) and internal (□) salts of rbSt to the predicted isotherm (- - -) generated according to the method of Leeder and Watt (1974).

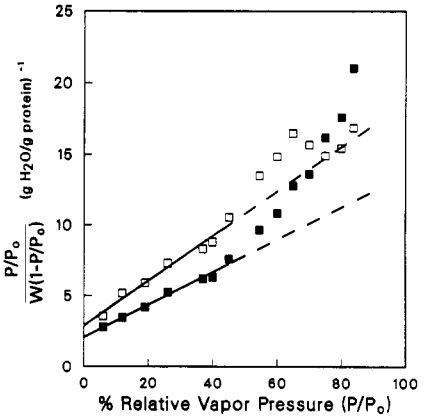

Figure 2. Plot of BET equation (eq 2) for the sodium (■) and internal (□) salts of rbSt generated by least-squares analysis of data at less than or equal to 40% rvp.

Determination of Surface Areas. A Quantasorb dynamic flow system was used to obtain nitrogen gas (N_2) adsorption data. The samples were outgassed by repeated adsorption/desorption of N_2. A three-point BET method was used to determine the monolayer of N_2. The monolayer value was then converted to surface area using a cross-sectional surface area of 16.2 Å^2 molecule for N_2 (Lowell and Shields, 1984). Similarly, "monolayer" levels of water were obtained from application of a multipoint BET method to the water sorption isotherm data (described in detail under Results). This monolayer was then converted to an apparent surface area using a cross-sectional surface area of 10.8 Å^2/molecule for water (Lowell and Shields, 1984).

Stability Studies on Lyophilized rbSt. Lyophilized formulations of rbSt were exposed to rvp of 10–45%, sealed, and stored at 47 °C. Samples were pulled at various time points, reconstituted with water, and diluted with 0.5% (v/v) TFA for RP-HPLC assay. Initial rates of loss of rbSt were calculated as percent lost, with respect to time zero. Residual water levels were determined by the coulometric Karl Fischer method (Mitsubishi CA05) and converted to grams of H_2O per gram of dry weight of the formulation.

RESULTS

The sorption of water vapor by the internal and sodium salts of rbSt increased with increasing rvp (Figure 1). The hygroscopic properties of both salts were described by the typical sigmoidal-shaped or type II isotherm. The internal salt, which was prepared from volatile buffers, appeared to have a slightly lower affinity for water at low ρ/ρ_0 and a larger water capacity at higher ρ/ρ_0 than the sodium salt.

A theoretical isotherm, up to approximately 80% rvp, was generated using the method of Leeder and Watt (1974). This involved calculating the molar fraction of the different functional groups and then summing up the corresponding estimated amounts of water to be adsorbed by that particular functionality at that given rvp. The theoretical curve appeared to correlate reasonably well with the isotherm for the sodium salt; however, it did not correlate well with that of the internal salt (Figure 1).

An adsorption model for gases to solid flat surfaces, developed by Brunauer, Emmett, and Teller (1938), has been extensively used to describe water vapor sorption isotherms for proteins (Van den Berg and Bruin, 1981; Schnepf, 1989). This model, which treats sorbed water vapor as existing in either strongly interactive or free states, is commonly referred to as the BET model.

$$W = C_b W_m(\rho/\rho_0)/[1 - (\rho/\rho_0)][1 - (\rho/\rho_0) + C_b(\rho/\rho_0)] \quad (1)$$

In the BET equation, W is the mass of gas adsorbed at a particular ρ/ρ_0, W_m is the amount of gas necessary to saturate the binding sites related to monolayer coverage,

Table I. Water Monolayers Determined Using the BET Equation

protein	g of H_2O/100 g of protein[a]	mol of H_2O/mol of protein
rbSt (Na salt)		
lyophilized	7.3 (6.7–8.0)[b]	88
spray dried	5.7 (3.3–8.1)*	69
rbSt (internal salt)		
lyophilized	5.1 (4.7–5.6)[c]	62
rbSt (zinc salt)		
ppt/lyophilized	6.3 (3.8–8.8)*	76
bovine somatotropin[d]		
Pauling (1945)	7.4	90
Leeder and Watt (1974)	4.8	58

[a] 95% confidence limits on $n = 8$ in parentheses, on $n = 5$ in parentheses with an asterisk. [b] $W_m = 7.8$ (6.7–8.9) from GAB equation. [c] $W_m = 4.5$ (3.8–5.1) from GAB equation. [d] Predicted using the method referenced.

and C_b is a constant related to the heat of sorption at the site and the liquification of water upon adsorption. Values for W_m can be obtained from a linear plot of the rearranged BET equation as in eq 2 (Figure 2). The questionable

$$\frac{\rho/\rho_0}{W(1 - \rho/\rho_0)} = \frac{1}{W_m C_b} + \frac{C_b - 1}{W_m C_b}(\rho/\rho_0) \quad (2)$$

validity of the BET model to describe isotherms above 50% rvp was apparent. BET monolayer values obtained for the sodium and internal salts of rbSt are reported in Table I. No significant differences in W_m were noted for rbSt prepared by spray-drying vs lyophilization or when prepared as a poorly soluble zinc salt (Table I).

Improved fits of the isotherms over the entire range of ρ/ρ_0 have been obtained by using extensions of the BET model. One of the more common examples is an equation developed independently by Guggenheim, deBoer, and Anderson (GAB model) (Zografi, 1988). This model (eq 3) introduces an additional state of interacting water which

$$W = \frac{W_m C_G K(\rho/\rho_0)}{[1 - K(\rho/\rho_0)][1 - K(\rho/\rho_0) + C_G K(\rho/\rho_0)]} \quad (3)$$

is intermediate to the strongly interacting and free water states used in the BET model (Van den Berg and Bruin, 1981; Zografi, 1988).

Definitions of ρ/ρ_0, W, and W_m are previously described for the BET equation. The constants C_G and K, similar to C_b, are related to thermodynamic measures of sorption for strongly and weakly interacting water. The values of

Table II. Surface Areas Calculated from Nitrogen and Water Sorption Isotherms Using the BET Equation

protein/polymer	N_2 area, m^2/g	H_2O area, m^2/g	ratio H_2O/N_2 areas
rbSt (Na salt)			
lyophilized	1.3	264	211
spray-dried	4.5	206	51
ovalbumin[a]			
air-dried	20.4	218	11
lyophilized	5.8	200	35
microcrystalline cellulose[b]			149

[a] Benson et al. (1950). [b] Nakai et al. (1977).

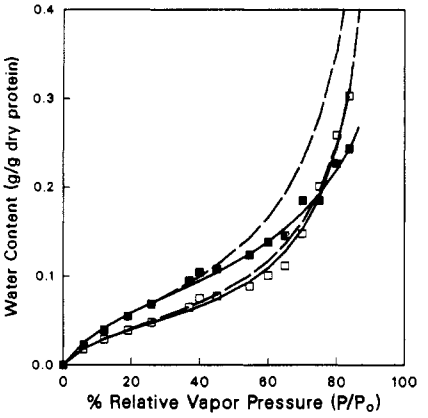

Figure 3. Comparison of fits obtained by the BET (- - -) and GAB (—) models for the sorption isotherms of the sodium (■) and internal (□) salts of rbSt.

W_m obtained through a PCNONLIN fit of the GAB equation differed little from those obtained from the BET equation (Table I).

A comparison of the fits afforded by the BET vs the GAB equations is shown in Figure 3. Interestingly, the BET model fits the sorption isotherm for the internal salt reasonably well. To the contrary, only the GAB model adequately describes the isotherm of the sodium salt at rvp greater than 50%.

Predictions of the monolayer levels were made for comparison to experimentally determined values. One approach suggested by Pauling (1945), wherein every polar group is proposed to interact with a single molecule of water, provided a reasonable estimate (Table I). Alternatively, the method of Leeder and Watt (1974) was used to predict the isotherm. This predicted isotherm was subsequently fitted to the BET equation, providing a slightly lower estimate for the monolayer (Table I).

The monolayer values, W_m (Table I), were used to calculate the apparent surface area as previously described under Materials and Methods (Table II). A three-point nitrogen sorption isotherm was used in combination with the BET equation to determine monolayer levels of nitrogen for lyophilized and spray-dried rbSt solids (R. Meury, 1988; The Upjohn Co., personal communication). The monolayer values of 1.33×10^{-5} and 4.60×10^{-5} mol of N_2/g of protein were determined for lyophilized and spray-dried solids, respectively. Surface areas were then determined as previously described (Table II).

Apparent surface areas determined from water sorption isotherms were significantly greater than those determined from nitrogen sorption isotherms (Table II). Consequently, the H_2O/N_2 ratios of these areas were significantly greater than 1. Other amorphous polymers such as ovalbumin and microcrystalline cellulose also have H_2O/N_2 ratios greater than 1 (Benson et al., 1950; Nakai et al., 1977). These large ratios are indicative of absorption

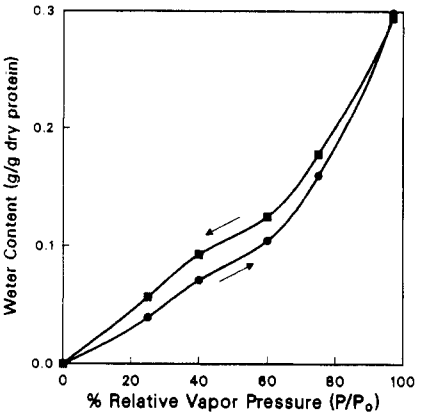

Figure 4. Sorption (●)/desorption (■) hysteresis observed for rbSt at room temperature.

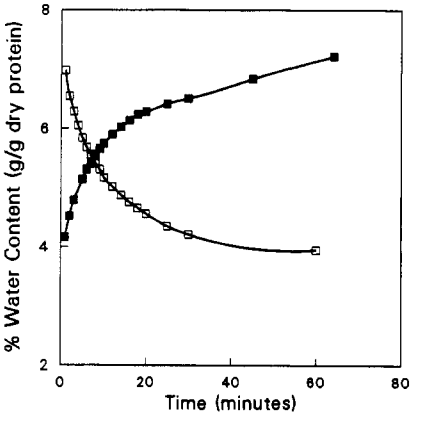

Figure 5. Changes in water content upon a change in relative vapor pressure from 10% to 47% (■) or from 47% to 10% (□) for the internal salt of rbSt.

Table III. Sorption/Desorption for Various rbSt Lots As Expressed in Weight Percent

	relative vapor pressure			
lot	0% → 31%	→ 75%	→ 31%	→ 0% → 31%
1	7.7	16.9	7.8	7.3
2	5.8	15.7	6.7	5.4
3	5.0	17.7	5.4	4.8
4	6.1	16.7	7.5	6.1
5	6.5	18.3	8.0	6.9

of the gas into the solid, i.e., penetration of water into the amorphous solid, as opposed to surface limited sorption (Zografi, 1988). Due to this additional absorption, the term sorption is typically used to account for both adsorption and absorption.

One consequence of this absorption phenomenon was the presence of hysteresis in the sorption isotherms (Figure 4). Various rbSt samples exposed to an increase in rvp from 31% to 75% showed evidence of hysteresis upon returning to 31% rvp (Table III). However, if the samples exposed to 75% rvp were placed over phosphorus pentoxide prior to returning to 31% rvp, the hysteresis effect was nearly eliminated (Table III).

The rates of sorption and desorption were very rapid if the surface area of the powder is readily accessible (Figure 5). The rates for sorption and desorption were similar.

Lyophilized formulations of rbSt incubated at various relative vapor pressures from 10% to 43% resulted in water contents that varied from 0.015 to 0.05 g/g of dry weight of formulation. RP-HPLC analysis of sealed samples incubated at 47 °C clearly demonstrated the impact of increased moisture content on stability (Figure 6). Sim-

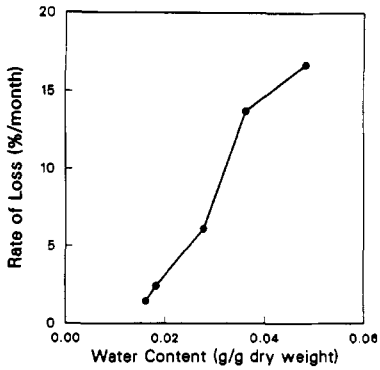

Figure 6. Rate of decomposition, measured by RP-HPLC, of a lyophilized rbSt formulation with varying water content following incubation in sealed vials at 47 °C.

ilarly, SE-HPLC analysis of the samples with water contents of 0.028 and 0.048 g/g of dry weight resulted in losses of monomeric material at rates of 5.2% and 14.6% monomer/month, respectively. There were not sufficient data to discern whether a real discontinuity existed in the stability of rbSt between 0.028 and 0.036 g of H_2O/g of dry weight of formulation as suggested in Figure 6. DSC scans of the lyophilized formulations of rbSt were not able to detect any indication of a phase transition with increasing temperature or water content.

DISCUSSION

The sorption of water by rbSt was typical of other amorphous polymers and was described in terms of a type II, sigmoidal-shaped isotherm (Figure 1) (Kuntz and Kauzmann, 1974). This familiar isotherm for proteins has been characterized in terms of three relatively distinct stages of hydration: (1) water content (W) < 6–8% with strong interactions at high affinity sites, i.e., charged and highly polar groups; (2) 6–8% < W < 20–25%, with weaker interactions of water clustering around the hydrated charged groups and interaction at less polar sites such as the amides; and (3) W > 20–25% with capillary-type condensation of water onto weakly interacting sites resulting in deformation phenomena within the amorphous polymer (Rupley et al., 1983; Poole and Finney, 1984; Morozov et al., 1988). The prediction of the amount of water associated with the protein at each successive stage becomes increasingly difficult. Reasonable approaches, based on primary sequence of the protein, can be used for predictions in stage 1 and stage 2 hydration. However, prediction in stage 3 becomes more difficult due to the influence of physicomechanical properties of the amorphous polymer.

Pauling (1945) obtained reasonable predictions for the amount of water sorbed to the high-affinity sites by considering the interaction of one water molecule per polar group of the protein (Table I). Other investigators have attempted to describe the first two stages of sorption by stoichiometrically relating the functional group composition of the protein to the degree of hydration at any given rvp (Leeder and Watt, 1974).

The method of Leeder and Watt (1974) was used in conjunction with the primary sequence of bSt to predict an isotherm up to 80% rvp (Figure 1). The data for the sodium salt of rbSt agreed reasonably well, but the data from the internal salt had a much lower water affinity than predicted. There is a tendency for these models to overestimate the amount of sorbed water, primarily because they do not take into account the inaccessibility of many intramolecular hydrogen binding sites involved in maintenance of the secondary and tertiary structure of the protein.

The predictability, as noted with rbSt, was also limited as a result of molecular state of ionization and/or counterions present (Rüegg and Blanc, 1976; Rochester and Westerman, 1976). Consequently, the use of such predictive methods can only provide an initial estimate with the need for supporting data. However, depending on the strategy to be pursued in formulation, prediction and data collection can emphasize certain stages of hydration. For example, lyophilized formulations for reconstitution should emphasize stages 1 and 2, while sustained release formulations should probably emphasize stages 1 and 2 for shelf-life purposes and stages 2 and 3 for protein stability following administration (Hageman, 1992; Hageman et al., 1992).

The practical value in the characterization of isotherms lies in the ability to effectively describe the water content over the vapor pressure range of interest and apply that information during future formulation and processing functions. Consequently, the use of a mathematical model can be of great benefit. The BET (eq 1) and GAB (eq 3) models were developed by assuming that sorbed water exists primarily in two (BET) or three (GAB) states of interaction with the protein (Van den Berg and Bruin, 1981; Zografi, 1988; Schnepf, 1989). The mathematical modeling of the sigmoidal sorption isotherms is predictably better with three-variable equations (GAB) vs the two-variable BET equation (Figure 3). However, assignment of physical significance to each of the variables is difficult (Zografi, 1988). Regardless, the models can be used to provide a method for describing the relationship between water sorption and environmental conditions.

The BET equation is typically used to calculate the amount of water necessary to provide monolayer coverage on a solid adsorbent containing homogeneous binding sites. However, in the case of proteins, this "BET monolayer" can be as high as 200 m^2/g (Kuntz and Kauzmann, 1974), consistent with numbers obtained here (Table II). Therefore, monolayer most likely refers to absorption with coverage of a heterogeneous mixture of high-affinity binding sites and does not actually refer to monolayer surface coverage on the solid particles (Hageman, 1988, 1992). This is further pointed out in comparisons with nitrogen adsorption below.

Assuming that rbSt is a spherical molecule of 20-Å radius (Havel et al., 1989), a reasonable estimate of surface area per molecule might be ≈5000 $Å^2$. The average monolayer value of 80 mol of H_2O/mol of protein would only be ≈860 $Å^2$ or 17% of the estimated molecular surface area. This is consistent with observations of others that the monolayer does not represent coverage of the molecular surface, but only some portion of it, i.e., the highly polar functionalities (Kuntz and Kauzmann, 1974; Rupley et al., 1983). Therefore, this monolayer value has little to do with coverage of the solid particle or molecular surface but is more appropriately related to coverage of highly polar amino acids. Regardless of the actual origin, the importance of determining the BET monolayer lies in some of the potential changes in physical properties of the solid, mobility of water, and internal flexibility of the protein which can occur at this point (Parak, 1986; Hageman, 1988, 1992; Pikal, 1990a).

Analysis of gas adsorption via BET treatment has commonly been used in conjunction with the cross-sectional surface area of adsorbate molecules to estimate the surface areas of materials (Lowell and Shields, 1984). Comparisons of surface areas for lyophilized and spray-

dried rbSt, determined by N_2 and water sorption, differed drastically (Table II). Even though the much smaller particles generated by spray-drying have much larger N_2 measured surface areas, the quantity of water sorbed was quite similar to that of the lyophilized. The large H_2O/N_2 surface area ratios result from the actual penetration of moisture into the protein powder, typical of many amorphous polymers (Levine and Slade, 1987; Zografi, 1988; Hageman, 1992). This penetration of water, i.e., dissolution of water in the polymer, can have a significant impact on the physicomechanical properties of the protein or polymer (Kakivaya and Hoeve, 1975; Morozov and Morozova, 1988; Slade et al., 1989; Oksanen and Zografi, 1990). Such physicomechanical changes may greatly influence protein stability (Sanches et al., 1986; Ahlneck and Zografi, 1990; Franks, 1990; Pikal, 1990a; Hageman, 1992).

The hysteresis observed in the sorption/desorption profiles for proteins and other amorphous polymers indicates that the system was at quasi-equilibrium during the sorption process (Figure 4) (Kapsalis, 1981; Franks, 1982; Morozov and Morozova, 1988; Slade et al., 1989). Therefore, the protein solid is perceived to be in a kinetically metastable state which is dynamically constrained by high activation barriers between states. These nonequilibrium conditions limit the use of thermodynamic parameters obtained from various sorption models in the description of molecular level interactions of water and protein. Likewise, the activity of water in the vapor phase cannot rigorously be assumed to be equal to its activity in the hydrated solid (Franks, 1982; Slade et al., 1989). Regardless, the mathematical descriptions above can still be quite useful in a practical sense for purposes of prediction and qualitative understanding throughout the formulation process.

A potentially important issue, especially when small quantities such as reference standards are weighed, was the very rapid rate at which the hygroscopic protein samples can sorb or desorb water (Figure 5). Therefore, samples should be pre-equilibrated to a given rvp, where the water content has been established, and then rapidly weighed at a very similar vapor pressure or, ideally, in a controlled atmosphere. The hysteresis can become problematic if the prior history of a sample is unknown and a precise amount of protein is desired. As shown in Table III, it was possible to minimize the hysteresis problem before samples were weighed by exposing them to P_2O_5 prior to equilibration at a selected constant rvp for actual weighing (i.e., 31% rvp). Closure of the hysteresis loop at low rvp provides a means to minimize the uncertainties of prior storage. Depending on the protein, dehydration at very low rvp of water may lead to alterations in protein structure, which are usually reversible upon rehydration (Poole and Finney, 1984; Colombo and Sanches, 1990).

The importance of residual water levels in the stability of lyophilized drugs has long been recognized, although the need for optimal moisture levels with proteins continues to be discussed (Pristoupil et al., 1985; Franks, 1990; Pikal, 1990a; Hageman, 1992; Hsu et al., 1991; Pikal et al., 1991; Roy et al., 1991). The stability of a lyophilized rbSt formulation for reconstitution was clearly impacted by its prior exposure to varying rvp and the resulting water content (Figure 6). Insufficient data were available to determine whether the apparent increase in the rate of decomposition between 0.028 and 0.036 g of H_2O/g of dry weight of formulation was real, but certainly moisture had a significant impact on the rate of loss. The decomposition products were typical of what has been observed with the bulk drug, i.e., cleavage and deamidation at position 99 and covalent dimerization (Hageman et al., 1992). Also, similar to previous solid-state stability on bulk drug (Hageman et al., 1992), dimerization accounts for greater than 80% of the loss (i.e., loss by SE-HPLC/loss by RP-HPLC).

The effect of water content on the stability of proteins in the solid state has been described in terms of the following: (1) changes in the dynamic flexibility of the protein segments and functional groups; (2) participation of water in reactions; or (3) action of water as a medium for mobilization of reactants (Hageman, 1988, 1992; Pikal, 1990a). The first effect is a function of the impact of water on the flexibility of the protein, whereas the latter two are functions of the mobility of the water and its freedom to solubilize/mobilize reactants.

Interactions of water, temperature, and composition of the solid all play critical roles in defining the dynamic mobility within an amorphous polymer system (Slade et al., 1989; Pikal, 1990a; Hageman, 1992). The enhanced mobility of water and protein that is encountered upon hydration of a protein solid may be attributed to the action of water as a plasticizer and its subsequent effect when the glass transition temperature, T_g, is lowered. T_g is the temperature at which the physical properties of a solid change drastically from a rigid "glassy" character to a much more fluid "rubbery" character. Consequently, the polymer fluidity is similarly impacted by either the sorption of the plasticizer water or an increase in temperature. Provided a sufficient quantitative understanding of water sorption and its impact on stability can be obtained, an interesting alternative to the typical Arrhenius-based accelerated stability studies may be possible (Slade et al., 1989).

The impact of both hydration and temperature on the flexibility of protein structure and mobility of water have been demonstrated (Frauenfelder and Gratton, 1986; Parak, 1986). Furthermore, the mobilities of water and protein are mutually limited below the BET monolayer level of hydration, equivalent to stage 1 of the isotherm (Parak, 1986). Increased levels of water above the BET monolayer coincide with an onset of protein flexibility (Rupley et al., 1983; Poole and Finney, 1984). At much higher levels of water, near the point at which stage 3 begins in the isotherm, increases in heat capacity, enzymatic activity, and mobility of probes indicate that the dynamics of the system are increasing (Rupley et al., 1983). The beginning of stage 3 has been correlated to a point where sufficient water has been sorbed so that the T_g has been decreased to a point where it is below the temperature used for the isotherm (Oksanen and Zografi, 1990).

Although it may be appealing to attribute the apparent discontinuity in rbSt stability between 0.028 and 0.036 g of H_2O/g of dry formulation weight (Figure 6) to a glass transition phenomenon, no such phase transition was detected when investigated by differential scanning calorimetry. This lack of phase transition was also noted by Pikal and co-workers (Pikal et al., 1991) for lyophilized preparations of human somatotropin, where significant increases in decomposition with increasing water content were also observed.

The importance of moisture levels in the stability of proteins in the solid state warrants a careful characterization of the sorption properties of the protein. Limited success at predicting sorption isotherms can be achieved at low relative vapor pressures if some of the potential pitfalls are recognized. The use of equations from models such as the GAB model is generally able to describe the data over the entire range of rvp and provide an element

of prediction for subsequent work in the formulation stages of the project.

ACKNOWLEDGMENT

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