Solid State Chemistry of Proteins: II. The Correlation of Storage Stability of Freeze-Dried Human Growth Hormone (hGH) with Structure and Dynamics in the Glassy Solid

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Received 27 August 2007; revised 12 December 2007; accepted 1 February 2008

Published online 19 March 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.21374

ABSTRACT: This research presents storage stability of human growth hormone, hGH, in lyophilized di-saccharide formulations. Stability *via* HPLC assay was assessed at 40 and 50°C. Structure of the protein in the solids was assessed by infrared spectroscopy. Molecular mobility was characterized by structural relaxation times estimated from DSC data and by measurement of atomic motion on a nanosecond time scale by neutron scattering. Very large stability differences were observed among the various formulations, with both chemical and aggregation stability showing the same qualitative trends with formulation. Near the T_g, T_g appeared to be a relevant stability parameter, but for storage well below T_g, stability seems unrelated to T_g. Stability (chemical and aggregation) was weakly correlated with secondary structure of the protein, and there was a partial quantitative correlation between degradation rate and the structural relaxation time. However, at equivalent levels of disaccharide relative to protein, sucrose systems were about a factor of two more stable than trehalose formulations, but yet had greater mobility as measured by structural relaxation time. Secondary structure was equivalent in both formulations. Neutron scattering results documented greater suppression of fast dynamics by sucrose than by trehalose, suggesting that well below T_g, fast dynamics are important to stability. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:5106–5121, 2008

Keywords: freeze drying; human growth hormone; protein stability; glass transition temperature; protein stability and secondary structure; structural relaxation time; fast dynamics

INTRODUCTION

Therapeutic proteins and vaccines are important classes of health care products that often have inadequate stability in aqueous solution to allow distribution and use. Thus, such products are commonly dried to increase stability, with freeze-drying being the drying method of choice for most products.[1,2] However, due to thermodynamic destabilization during the drying process, in-process degradation may occur, and even if a dry product can be produced without significant degradation, satisfactory stability during storage

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Journal of Pharmaceutical Sciences, Vol. 97, 5106–5121 (2008)
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is not assured.[3,4] Sugars and polyols are commonly added to formulations to protect proteins against degradation during processing and storage.[3–5] Vaccines appear to respond to many of the formulation and process variations as do proteins,[6] but vaccines also have some unique stability issues. Although the general understanding of the key stability variables has improved significantly over the past 15 years, and general guidelines for successful formulation exist,[2,3,7] a number of apparent violations of the general rules have been noted.[7–11] Moreover, it should be noted that essentially all protein products must be stored under refrigeration (i.e., 2–8°C) to maintain product quality, even those product forms manufactured as freeze-dried formulations. Clearly, stabilization technology does not yet meet the requirements for long-term stability, particularly for third world or military applications where ambient temperature storage is often critical. It should be noted that ambient temperature (e.g., in a warehouse) in tropical and subtropical regions far exceed so-called "controlled room temperature" (ca. 15–30°C).

Both the "water substitute hypothesis" and the "vitrification hypothesis" are commonly used to rationalize stabilization of a solid by added formulation components, and the salient features and limitations of these concepts have been reviewed.[3,7,8] The key difference between the two mechanisms is that the "water substitute hypothesis" argues for stabilization by thermodynamics; that is, addition of the stabilizer increases a key free energy change associated with the degradation process to a point where the key process (i.e., partial unfolding) is no longer spontaneous (i.e., ΔG is no longer negative) and does not occur, regardless of the time scale. On the other hand, the vitrification hypothesis would apply whenever the free energy change for the process of interest is negative and therefore, spontaneous, but the kinetics are so slow that the process cannot occur on the time scale of relevance.

Briefly, the water substitute hypothesis states that an additive, such as sucrose or trehalose, operates by replacing water lost during secondary drying, thereby stabilizing the native conformation of the protein during drying. That is, the thermodynamic destabilization of the protein caused by water removal is ameliorated by the "water substitute," which hydrogen bonds to the protein and thermodynamically stabilizes the native conformation as did the water. This hypothesis qualitatively accounts for increased storage stability in that the rate for any degradation process facilitated by a non-native structure (e.g., oxidation of a methionine residue that becomes exposed to environment upon protein unfolding) will be reduced for proteins in a native or near-native conformation. The water replacement hypothesis also provides a rational explanation for the fact that protein aggregation, which is often noted upon rehydration of unprotected lyophilized proteins, can be inhibited if a hydrogen bonding excipient is added to the formulation.

The vitrification hypothesis essentially states that since all degradation processes require some level of molecular mobility, a system that transforms into the glassy state will be much more stable since the glassy state is characterized by very limited molecular mobility. Below the glass transition, the system will behave essentially as a solid, with a much reduced degradation rate. Thus, under the assumption that "$T - T_g$" is a reliable metric of molecular mobility, it becomes a critical stability parameter.[12–15] The function of the excipient is to form a glass at a reasonably high temperature in which the protein is molecularly dispersed in the host glass and adopts the rigid dynamics of the glassy host system. Here, it is implicitly assumed that the dynamics of the protein molecule effectively couples with the dynamics of the host, at least to the point where large-scale motion, such as whole molecule diffusion of the protein molecules, is nearly eliminated. It is argued that coupling internal motion of the protein to the rigid glassy matrix is also likely important to stability.[16]

Empirically, it is found that low molecular weight nonreducing saccharides stabilize effectively, and largely because of prior use in marketed products, sucrose and trehalose are obvious choices for commercial products.[2,3] Both sucrose and trehalose easily form glasses with relatively high glass transition temperatures; 75°C for sucrose and 118°C for trehalose in the absence of residual water,[2] as well as effectively hydrogen bond to proteins, thus satisfying the minimum necessary criteria for stabilization as required by both the water substitute and vitrification mechanistic concepts. Consistent with a physical chemical analysis[17] and based upon a series of stability experiments and general observations,[18] trehalose has been promoted as the superior stabilizer.[18] Although this view was challenged,[19]

there remain periodic references to the superior stabilization potential for trehalose. Certainly, if "$T - T_g$" were a critical stability indicating parameter, a trehalose formulation would be superior to sucrose since T_g for trehalose is much higher than T_g for sucrose. However, results are mixed, with sucrose and trehalose formulations often showing very similar storage stability.[8,20,21]

Human growth hormone shows little or no degradation during freeze drying, even when stabilizers are not added, but storage stability is problematic.[4] Thus, the present work is an investigation of the storage stability of human growth hormone, hGH, in a variety of formulations, including sucrose and trehalose. This study is an extension of previous work with hGH[4,22–24] to include a direct comparison between sucrose and trehalose with a focus on analysis of stability trends in terms of both structure and molecular mobility. Further, both aggregation and chemical degradation (methionine oxidation and asparagine deamidation) are evaluated. We also investigate the effects of variation in stabilizer level, water content and presence of oxygen in the vial headspace.

The presentation of results begins with an analysis of the time dependence of degradation, which while consistent with previous studies on glassy systems, is not typical of what is found in equilibrium systems. A phenomenological comparison of stability trends with formulation follows, where it is demonstrated that formulation variations may produce stability variations of more than an order of magnitude. Next, the effects of moisture content and headspace on stability are presented where the effects of moisture are interpreted in terms of the impact of water content on the glass transition temperature. The balance of the manuscript is focused on attempts to understand the underlying mechanisms responsible for the stability variations observed in the dry formulations. Infrared spectroscopy is used to show a qualitative, but imperfect, correlation between secondary structure and stability. Comparisons of stability with glass transition temperatures (T_g) show that well below T_g, T_g is a poor indicator of stability. Structural relaxation time is shown to be a far better predictor of stability, but the only property measured that is consistent with the superior stability of sucrose systems is the amplitude of fast dynamics as measured by neutron scattering. The implications of this observation are discussed.

MATERIALS AND METHODS

Sample Preparation

Origin of materials, freeze-drying procedures and residual water content measurement were as previously described.[25] Briefly, all lots formulated with sugars were prepared from the same lot of bulk hGH obtained from Eli Lilly. Sucrose was Analytical Reagent grade obtained from Baker, and sodium phosphate was Analytical Reagent grade obtained from Mallinckrodt. Trehalose was obtained from Sigma and was "reduced metal ion content" material. Nominal 5 mL tubing vials were used along with 13 mm finish West gray butyl rubber stoppers (#1816 rubber stock) for most of the samples. The samples used for the residual water variation study used Daikyo FluroTec stoppers (low moisture release stoppers). Freeze dried samples for the residual water study were prepared from the corresponding "dry" samples by equilibrating overnight with 11%, 22%, and 33% relative humidity in vacuum desiccators. All samples were confirmed 100% amorphous by noting the absence of birefringence by polarized light microscopy. Note that all samples contained $\approx$6 mM sodium phosphate buffer (15 mass % of the protein content), which remained amorphous. Each sample contained 5 mg hGH in 1 mL fill volume. All formulations were freeze-dried at least 3°C below their collapse temperatures, and visual inspection confirmed the absence of collapse. Water content varied from 1.0% to 0.4%, depending upon formulation. All samples were stored at −20°C until use. Water contents were determined by Coulometric Karl Fischer procedures and sample handling procedures that avoided exposure of the samples to ambient humidity.

Assay for Chemical Degradation and Protein Aggregation

Assay protocols were essentially the same as previously described.[4] Assay for chemical degradation (i.e., methionine oxidation and asparagine deamidation) was by reversed phase HPLC, and assay for aggregation used size exclusion HPLC under non-denaturing conditions. All assay values represent the mean of assays made on two independent vials. Agreement between the two independent (nominally equivalent) samples was usually in the range of 0.05–0.1% for aggregation

results and within the range 0.1–0.2% for chemical degradation results.

Infrared Spectroscopy

Infrared spectra were obtained using a Nicolet Magna 560 spectrometer (thermoNicolet, Madison, WI) and conventional techniques.[26,27] A total of 256 scans and a resolution of 4 cm^{-1} were used for each spectrum. Spectra of solids were obtained using KBr pellets (0.3 mass % hGH in KBr). The spectrum for native, aqueous hGH (used as a control) was obtained by placing a 15 mg/mL solution between 2 CaF_2 windows separated by a 6 μm Mylar spacer. The spectrum of hGH in solution was corrected by subtracting the spectra of liquid water and water vapor as described elsewhere.[26] Effects of water vapor and carbon dioxide in the chamber were eliminated by subtracting the background spectrum. All spectra are presented in the conformationally sensitive amide I region, as second derivatives that have been smoothed, baseline corrected and area normalized.[26,27]

Characterization of Glass Dynamics

Structural relaxation time, τ, characterizing "global" long time scale and long length scale mobility, was estimated by calculation using the relationship between structural relaxation time, glass transition temperature, and glass transition temperature width, ΔT_g established previously,[28]

$$\ln \tau^{\beta} \cong (\ln \tau^{\beta})_{T_g} + \left\{ \frac{C}{(\Delta T_g / T_g)} \left[1 - \gamma \left(1 - \frac{36.9\beta}{C} \frac{\Delta T_g}{T_g} \right) \right] \right\} \times \left(1 - \frac{T}{T_g} \right) \quad (1)$$

where τ is the structural relaxation time, β is the "stretch constant", $0 < \beta < 1$, of the Kohlrausch–Williams–Watts expression describing the kinetics of the relaxation process. The quantity in brackets, {}, is the value of the temperature dependence of the relaxation time constant, $d\ln(\tau^{\beta})/dT$, which is directly related to glass fragility, and is evaluated here from the width of the glass transition region, ΔT_g, and the values of C, γ, and β. The quantity, C, represents a constant[28] dependent on the DSC protocol used to generate the T_g data. With modest variations in β, the corresponding variation in calculated relaxation time constant from Eq. (1) is minimal.[28] Normally, β is found to be in the range (0.3–0.5) for pharmaceutical systems, and the value of $\ln(\tau^{\beta})T_g \approx -1.6$, with τ in hours; here, we used $\beta = 0.3$.[28] The symbol, γ, represents the thermal history of the glass and is calculated from the heat capacity of the amorphous system above T_g, defined by Eq. (2),

$$\gamma = \frac{C_p^l - C_p^g}{C_p^l - C_p^{xstal}} = \frac{\Delta C_p}{C_p^l - C_p^{xstal}} \quad (2)$$

The use of Eq. (2), however, requires accurate values of the heat capacity of the liquid, C_p^l, heat capacity of the glass, C_p^g, and heat capacity of the crystal, C_p^{xstal}. Approximate values of γ may be evaluated from the empirical relationship,[28]

$$\gamma \cong \frac{\Delta C_p}{\Delta C_p + 0.00331 \exp(6.63 \Delta C_p)} \quad (3)$$

where ΔC_p is the heat capacity change at the glass transition, which is much easier to obtain. Here, values of γ were obtained from Eq. (3). While relaxation times evaluated from Eqs. (1 to 3) are certainly not exact, they seem to provide results with semi-quantitative accuracy, at least for the systems studied.[28]

Fast dynamics were characterized by neutron scattering, which measures the mean square amplitude of hydrogen motion occurring over a nanosecond time scale. Data were accumulated over a wide temperature range from above T_g to roughly 50 K. Samples were quenched from room temperature quickly to 50 K and warmed at 0.5 K/min to 350 K during the scattering experiment. The techniques used were described in detail previously.[10]

Stability Protocols and Definition of Empirical Rate Constant

Samples were stored in a temperature controlled (±1°C) room and samples taken at pre-determined times for assay. We represent the chemical stability and aggregation data as a percentage purity (P), where $P = 100 \times$ (mass of the non-degraded protein/total mass of protein), and mass of degraded and non-degraded protein are deduced from HPLC or reverse-phase chromatograms. Purity values were determined at 0, 1, 3,

and 6 months storage at 40°C and at 1 month at 50°C. In most cases, the amount of degradation was very small, and P was $\geq$90. Because of this fact, and our level of precision in determining P, we cannot distinguish between zero, first, or second-order reaction kinetics directly. The degradation rate, dP/dt should be essentially constant, and P should appear as linear in time. Thus, under these conditions, one would expect to obtain a pseudo zero order rate constant. For a unimolecular reaction, the rate constant would be a true constant, independent of both time and initial concentration, but for a bimolecular reaction, this pseudo first order rate constant would be proportional to the initial concentration of reactant.

As demonstrated below, and contrary to expectations based on classical kinetics, the degradation rates we observe in this study often decreased significantly as time progressed, even though the total level of degradation was very small and concentration of the reactant (hGH) remained essentially constant. Although four time points are not sufficient to uniquely determine the reaction kinetics, even from a phenomenological viewpoint, the data do suggest that that appearance of degradation product or loss of "parent" followed "square root of time" kinetics as a good first approximation. That is, P varies with square root of time according to the simple expression,

$$P = P(t = 0) - k\sqrt{t}, \qquad (4)$$

where $P(t=0)$ is the percentage purity at time zero (i.e., initial value) and "k" is an empirical rate constant. The implication of Eq. (4) is that the formal pseudo zero order rate constant is inversely proportional to the square root of time for $t > 0$. The kinetic behavior is discussed in the next section in more detail.

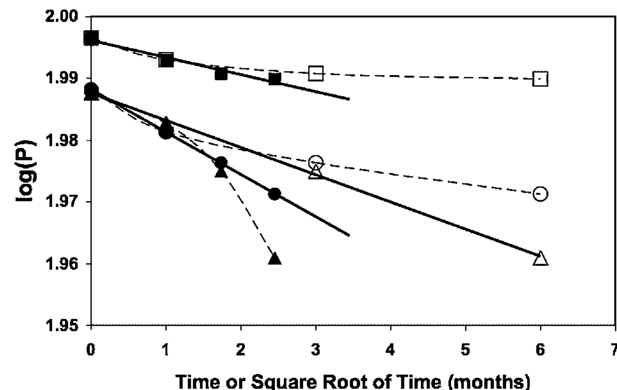

Figure 1. Examples of the kinetics of degradation of hGH in solid formulations at 40°C: comparison of square root of time with zero order kinetics. Open symbols are plots versus time, and filled symbols are plots versus square root of time. Squares are aggregation in "dry" 1:1 hGH/Trehalose, circles are chemical decomposition in "dry" 1:3 hGH/Trehalose, and triangles are chemical degradation in 22% relative humidity exposed 1:6 hGH/Trehalose.

RESULTS AND DISCUSSION

Kinetics and Time Dependence of Degradation

Figure 1 shows representative chemical degradation and aggregation profiles for hGH in trehalose glass under "dry" conditions, and equilibrated at 22% relative humidity. We note that the plots use a $\log P$ versus t or $\sqrt{t}$ plotting format, mostly since a "first order" plot is linear with $\log P$ plotted versus t. However, with only a small amount of degradation, as for the data in Figure 1, plots of P versus t or P versus $\sqrt{t}$ would appear qualitatively the same as shown in Figure 1 for the $\log P$ format. As previously noted,[29–31] degradation in glassy systems often follow "stretched time kinetics" with degradation level increasing linearly with square root of time. Such kinetics are also characteristic of all "dry" saccharide formulations studied in this research, as illustrated by Figure 1. Note that the data shown as filled symbols (vs. $\sqrt{\text{time}}$) exhibit better linearity than the corresponding data shown as open symbols (vs. time) for the two "dry" samples, but better linearity is found with log(P) vs. time for the sample equilibrated with 22% relative humidity. These results are typical of the data obtained in this research in that samples of low moisture content that are being studied well below their T_g fit a square root of time degradation model better than a first order degradation model. On the other hand, samples of high moisture content typically fit a first order degradation model better.

If we naively calculate a reaction order as the slope of log[dP/dt] versus log(P) from the time course of the degradation data plotted in Figure 1, we obtain a reaction order of 236 for aggregation in 1:1 hGH/Trehalose. We arrive at similar large values for all formulations that follow square root of time kinetics. In our view, such an observation is not surprising and might even be expected. Classical concepts of reaction order apply to solution systems, either gas or condensed state, that are in thermal and structural equilibrium. In

 DOI 10.1002/jps

an equilibrium system, as a "high energy molecule" reacts, the distribution of energies and configurations in the system is immediately reestablished. Amorphous solids are not in configurational equilibrium. With reactions in the amorphous solid state (i.e., glass), the "stretched time kinetics" likely arises from the fact that a glassy system is composed of a number of subsystems, of different local structure and dynamics, and thus of different reactivity, which are not in internal equilibrium.[29,31] As the degradation study begins, the more reactive configurations degrade quickly, leaving the more stable configurations, and since the system does not instantly shift the distribution of configurations to again populate the more reactive states, the reaction rate now is characteristic of the more stable states, and the degradation rate slows. Thus, the overall kinetics is a superposition of reactions from a number of sub-states, each with its own rate constant, which leads to stretched time kinetics as described by Eq. (4),[29,31] with the value of the exponent being smaller the larger the spread of component system rate constants. Such a mechanism could lead to powers other than 0.5 in the time dependence of purity, but 0.5 seems to typically be a good approximation for degradation in most glassy systems used for protein preservation. Thus, a degradation rate, which in an equilibrium system (i.e., a solution) would be effectively constant (i.e., zero order in time dependence) at the low levels of degradation we typically study, now becomes highly time dependent. While such "stretched time" kinetics does indicate a complex overall degradation pathway, rate constants between different samples are directly comparable as least as long as the stretch powers are the same.

Although the time dependence of degradation is clearly not well described by the concept of reaction order, a dilution effect should be still be observed, as classically expected, for second order reactions, such as a bimolecular aggregation. As an inert component is added (i.e., disaccharide stabilizer), the probability of protein–protein contact decreases roughly in proportion to the protein volume fraction,[8] and the observed degradation rate will depend on the initial concentration. Thus, one might expect the empirical rate constant, k, as defined by Eq. (4), might be proportional to the volume fraction of protein (or mass fraction since densities do not differ greatly). Such a dilution effect would not, of course, apply for degradation processes that are likely to be unimolecular in the rate determining step, such as chemical degradation.

Stability Trends with Formulation

Table 1 summarizes the thermal properties and degradation rate constants, k (i.e., see Eq. 4) of the nominally dry formulations studied. Since the widths of the glass transition regions are typically about 10°C (Tab. 1), onset T_g values are about 5°C lower than the mid-point values given in Table 1. We note that chemical degradation in the pure hGH formulation actually obeyed first order degradation kinetics as well as or better than square root of time kinetics.[4] However, to allow a systematic comparison of stability *via* rate constants that includes the pure hGH system, it was necessary to use square root of time kinetics for the pure hGH system as well. The rate constants for 40°C were, in general, evaluated from an initial and three time points, but the rate constants for 50°C were evaluated from only the initial assay and the 1 month time point. Thus, linearity of degradation on a square root of time plot was assumed for the 50°C data. The water content and T_g reported are the means of the initial and final 40°C time point materials to better represent average values during the study. Due to sorption of water from the stopper, the moisture content increases during the stability study, and therefore, the T_g decreases during the stability study. As will be discussed later, this modest variation in water content appears not to have a significant impact on stability.

Except for pure protein and stachyose, the values of $\Delta T_g/T_g$ and ΔC_p are values for the dry samples taken from Table 1 in Ref. 25 which have been corrected to the water content given in Table 1 of the present work, using the water content dependence as determined experimentally, $d(\Delta T_g/T_g)/d(\text{mass \% } H_2O) = -0.0012$; $d(\Delta C_p)/d(\text{mass \% } H_2O) = -0.015$. The stachyose ΔT_g and ΔC_p data were taken as the mean of the experimental values for the initial and 40°C end point materials. For pure protein, the value of $\Delta T_g/T_g$ in the dry state was determined as the mean of data for sucrose and trehalose containing systems linearly extrapolated to zero sugar content.[25] The quantity, γ, representing some aspects of the thermal history of the initial system, was evaluated from Eq. (1), except for the pure protein. For pure protein, an accurate value of ΔC_p could not be obtained, so the value of γ was taken as unity.

Table 1. Thermal Analysis and Stability Parameters for Human Growth Hormone Formulations

Sugar	Sugar/hGH	$<H_2O>$ (Mass %)	T_g (°C), Midpt	$\Delta T_g/T_g$ at $<H_2O>$	ΔC_p (J/gK)	γ	$k(\sqrt{t})$ 40°C, RP	$k(\sqrt{t})$ 40°C, SEC	$k(\sqrt{t})$ 50°C, RP	$k(\sqrt{t})$ 50°C, SEC
None	0	2.6	92.0	0.0559	—	1.00	8.2	3.4	—	—
Stachyose	1	2.8	122.8	0.0335	0.289	0.93	3.2	1.2	—	—
Trehalose	1	2.3	103.2	0.0383	0.237	0.94	2.9	0.97	4.3	1.41
Trehalose	3	1.4	103.0	0.0227	0.481	0.86	1.5	0.25	2.7	0.51
Trehalose	6	0.7	112.6	0.0183	0.524	0.83	0.92	0.15	1.9	0.33
Sucrose	1	1.9	78.3	0.0343	0.304	0.92	1.4	0.50	3.3	1.14
Sucrose	3	1.2	71.8	0.0264	0.480	0.86	0.92	0.10	1.6	0.31
Sucrose	6	0.7	67.8	0.0244	0.532	0.83	0.38	0.07	0.9	0.19

The rate constants, $k(\sqrt{t})$ are rate constants for square root of time degradation kinetics. Stoppers are West gray butyl (#1816). RP means chemical degradation *via* reverse phase HPLC assay, and SEC means aggregation *via* size-exclusion HPLC assay.

Aggregation rate data for trehalose formulations of hGH were previously reported,[24] and although the compositions of formulations studied were not identical, the qualitative trend of decreasing degradation rate with increasing trehalose content is the same, and when the sensitivity of the assay[24] permitted a quantitative comparison, the results are roughly comparable. For example, we find $1.4 \pm 0.1\%$ increase in aggregation after one month at 50°C for the 1:1 hGH trehalose formulation while the literature study[24] gives $1.2 \pm 0.6\%$ aggregation for a system of essentially the same composition.

Rate constants for degradation, from Table 1, are compared in Figure 2, where rate constants are given on a log scale. Error bars refer to standard errors in the rate constants as given by the regression analysis. Light shading represents chemical degradation, which is the sum of methionine oxidation and asparagine deamidation, while the dark bars represent aggregation, which is primarily non-covalent dimer formation.[4] In addition to data from Table 1, we provide data for a common commercial formulation of 1:1:5 weight ratio of hGH/glycine/mannitol,[4] and a 1:1 hGH/HES formulation, where HES refers to hydroxyethylstarch. These formulations are not represented in Table 1; we could not detect a glass transition for these formulations, presumably because the systems behaved as strong glasses.[25] A glass transition for the hGH-only formulation could not be observed with DSC for the same reason, but here glass transition information was obtained indirectly by extrapolation from hGH/disaccharide mixtures.[25]

Note that there is an enormous range of stability, with the rate constants for chemical degradation and aggregation varying by (roughly) factors of 20 and 50, respectively. Calculations using the data in Table 1 suggests less degradation at the end of 2 years at 25°C for the more stable disaccharide formulations than in 2 years at 5°C for the common commercial formulation (glycine/mannitol). The stability trends are not correlated with residual moisture in the moisture content range represented (Tab. 1); variation with moisture is discussed later. Rate constants for both aggregation and chemical degradation generally decrease from left to right from pure hGH (stabilizer = none) to 6:1 Trehalose (6:1 trehalose/hGH). Comparison of the trehalose systems with the sucrose systems of the same disaccharide level indicate superior stabilization is obtained with sucrose. At 40°C, the rate

 DOI 10.1002/jps

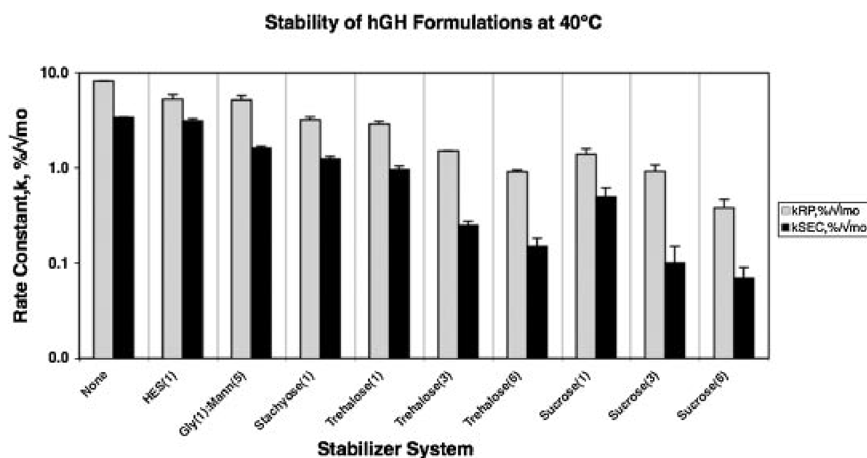

Figure 2. Comparison of stability of freeze dried hGH formulations at 40°C. The light shaded bars are rate constants, based on square root of time kinetics, for chemical degradation as measured by reverse phase HPLC assay, and the dark shaded bars represent rate constants, based upon square root of time kinetics, for aggregation as measured by size exclusion HPLC assay. All formulations are nominally "dry." Error bars are standard errors as given by the regression analysis.

constants for sucrose systems are about a factor of two less than for the corresponding trehalose systems.

The log of the rate constant is approximately linear in weight fraction protein for both trehalose and sucrose systems, with the slopes for aggregation at 40°C being 0.046 (sucrose) and 0.036 (trehalose) and slopes for chemical degradation at 40°C being 0.033 (sucrose) and 0.024 (trehalose). In short, stabilization does not saturate, although as disaccharide levels increase stability improves to the point where high assay sensitivity is needed to detect degradation. Assuming a temperature dependence of rate constant according to absolute reaction rate theory, a plot of $\ln(k)$ versus composition at constant temperature is equivalent to a plot of activation free energy versus composition. Thus, the implication of this log-linear trend is that the activation free energy increases linearly with weight fraction of sugar, but more so with sucrose than with trehalose. Also, it is significant to note that although the formulation variations are larger for aggregation, the qualitative stability trends with formulation are the same for both chemical decomposition and aggregation. Since the "transition state" for non-covalent aggregation is most likely different than for either methionine oxidation or asparagine deamidation, this similarity in trend suggest the controlling factor(s) are not specific to the chemistry of the process but rather is more likely related to the dynamics of the systems.

Variation of Stability with Water Content and Headspace Oxygen

Table 2 summarizes the stability variations with water content and gas composition (i.e., oxygen) in the vial headspace. Since linearity on a plot of degradation versus time or square root of time was not consistent for this entire series of samples (i.e., sometimes linear in square root of time and sometimes linear in time), we report changes in aggregation and chemical purity (*via* Reverse Phase assay) at selected time points. Note that low moisture release stoppers were used in this particular study, so water content increases during the stability studies were minimal. Actually, the highest moisture samples lost 1–2 mass % water by the end of the stability study; that is, moisture transferred from the product to the stopper. As discussed in more detail later, these large increases in degradation between "intermediate" and "high" water content are believed directly related to the proximity of the glass transition temperature to the storage temperature.

As expected, oxygen (i.e., air) in the vial headspace has no impact on aggregation, but contrary to observations with the glycine/mannitol formulation,[23]

Table 2. Degradation of 6:1 Saccharide/hGH Systems as a Function of Water Content and Vial Headspace

Sample Condition	Initial Mass % H_2O	Initial T_g (°C)	Initial % Aggregation	Δ%Agg 1m at 50°C	Δ% Agg 6m at 40°C	Initial Chemical (RP) Purity (%)	Decrease in % RP Purity 1m at 50°C	Decrease in % RP Purity 6m at 40°C
Sucrose								
Dry/N_2	0.5	80.7	1.05	0.04	0.26	97.4	0.9	0.7
Dry/Air	0.5	80.7	1.05	0.07	0.21	97.4	0.9	0.9
11% RH/N_2	3.1	49.0	1.12	0.21	0.35	97.4	3.1	2.3
22% RH/N_2	4.9	36.4	1.16	3.37	1.11	97.4	17.3	12.2
Trehalose								
Dry/N_2	0.3	121.1	1.05	0.21	0.50	97.4	1.7	1.9
Dry/air	0.3	121.1	1.05	0.21	0.39	97.4	1.6	2.2
11%RH/N_2	3.3	69.2	1.15	0.17	0.28	97.4	1.7	1.7
22%RH/N_2	7.0	49.9	1.18	0.50	0.54	97.2	5.7	5.8

% aggregation is mass % of soluble aggregate by size exclusion HPLC assay and chemical purity is % purity by reverse phase (RP) HPLC. For nominally "dry" samples, headspace was either nitrogen (N_2) or air. Stoppers are Daikyo Fluro Tec.

chemical degradation is insensitive to oxygen in the vial headspace for the disaccharide formulations (Table 2). Whether the difference in oxygen sensitivity between the glycine/mannitol formulation and the disaccharide formulations is due to some catalytic effect from formulation components, or has its origin in differences in diffusion coefficient of reactive oxygen species in the different formulations, is unknown. However, it is clear that the disaccharide formulations provide excellent stabilization regardless of the presence of oxygen in the vial headspace or presence of moderate levels of residual water.

Correlation of Stability with Protein Secondary Structure in the Solid State

The 2nd derivative infrared band at 1658 cm^{-1} represents the alpha helix structure in hGH and is the "marker band" for native-like structure.[24] Thus, a more intense 1658 cm^{-1} band means a higher degree of native structure in the sample. Consistent with the expectation that storage stability improves as the degree of "native structure" increases,[2,3,7,8,11,20,24] stability increases with increase of the intensity and area of the alpha helix band at 1658 cm^{-1} (Fig. 3). Spectrum #1 is for an hGH samples that was intentionally denatured in solution. This spectrum was obtained from an aqueous sample of hGH without excipients (except for buffer) that was heated to the peak of the thermal denaturation peak (75°C), cooled, and then freeze dried. Spectrum 2 is for a freeze dried sample of hGH without excipients (except for buffer), and spectra 3, 4, 5, and 6 represent, respectively: HES/hGH(1:1), stachyose/hGH(1:1), trehalose/hGH (1:1), trehalose/hGH(3:1), and trehalose/hGH (6:1). Note that the rate constants for aggregation at 40°C show a monotonic decrease from spectra 2–6 in qualitative agreement with the increase in intensity of the alpha helix band. Rate constants for chemical degradation show a similar correlation with the intensity of the 1658 cm^{-1} marker band. Thus, stability correlates with retention of native protein secondary structure in the solid state, at least in a qualitative sense. Note, however, that such an observation does not necessarily mean that the sugar acts as a water substitute; it does mean that structure in the dried solid correlates with stability, regardless of the mechanism critical to maintaining the structure throughout the freeze drying process. Certainly, the water substitute concept provides a plausible explanation for the stabilization, but variations in glass dynamics and/or other effects might also be important in determining the variations in protein structure that occur during processing.

It was noted earlier that the rate constants for degradation in sucrose systems are about a factor of two smaller than those in the corresponding trehalose systems. Thus, if structural retention in the dried solid is the single critical factor governing stability in the dried solid, the intensity of the alpha helix band for sucrose formulations should be more intense than for the corresponding trehalose formulation. However, this is not the

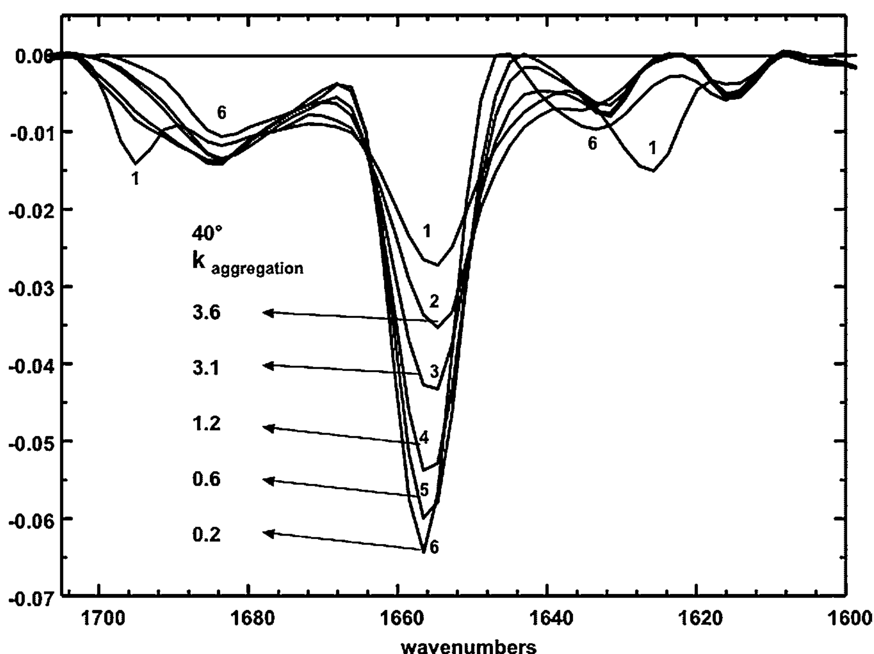

Figure 3. The correlation between secondary structure of the hGH in the freeze dried solid as measured by FTIR and rate constants for aggregation at 40°C. The band at 1658 cm^{-1} represents the alpha helix band and is the "marker band" for native structure. Curve #1 represents material that was 50% denatured by heating the solution to 75°C, cooling and then freeze drying. The other curves represent different formulations that were freshly freeze dried: #2 = no excipient; #3 = hydroxyethylstarch (1:1), #4 = stachyose (1:1), # 5 = trehalose (1:1), #6 = trehalose (1:6 protein/saccharide). Rate constants (square root of time) for aggregation at 40°C are given beside each FTIR curve. Vertical axis units are arbitrary units for second derivative of absorption data.

observation, as illustrated in Figure 4. Figure 4 compares the 2nd derivative FTIR spectra of the two 6:1 disaccharide formulations studied and shows clearly that no difference exists. In fact, the spectra are indistinguishable, and are very close to the spectra obtained for the aqueous solution (data not shown). It is possible that infrared spectroscopy is not measuring all relevant structural differences. After all, infrared spectroscopy measures only secondary structure, and it is certainly possible that differences in tertiary structure not directly coupled with secondary structure could have a significant impact on stability. It is also possible that other general physical factors are controlling stability in this series of samples.

Correlation of Protein Stability with the Glass Transition Temperature and Relaxation Dynamics

The sensitivity of stability to T_g, is illustrated in Figure 5 where stability against aggregation of 6:1 disaccharide/hGH systems at 50°C are compared for samples exposed to different relative humidities, using data from Table 2. This plot gives a clear picture of the impact of moisture content and T_g on stability. That is, when samples are "dry," aggregation is greater in the trehalose formulation. But when they are "wet" (i.e., exposed to 22% relative humidity), the comparison reverses, and the trehalose formulation is more stable. This trend is likely a direct result of the fact that, at 22% relative humidity storage, the sucrose formulation T_g is 36°C (Tab. 2), well below the storage temperature of 50°C, but the T_g of the trehalose formulation is essentially equal to the storage temperature. Thus, although the aggregation rate of the trehalose formulation does increase significantly from the rates at lower humidities, the increase is small in comparison to the increase observed for the sucrose formulation. Moreover, the sucrose formulation was severely collapsed after one month storage at 50°C, a direct result of storage well above its T_g. Thus, for hGH, sucrose is a better stabilizer for samples kept

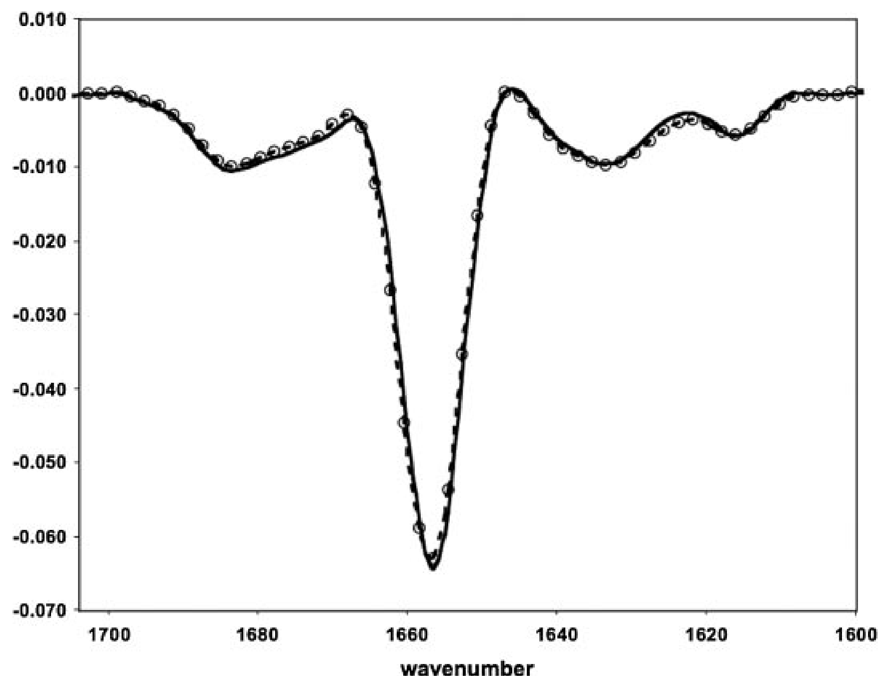

Figure 4. Comparison of FTIR structure in freeze dried sucrose and trehalose formulations of hGH. Formulations are 1:6 protein/disaccharide and are "dry" formulations. The solid line represents the trehalose formulation while the dashed line and open circles represents the sucrose formulation. Vertical axis units are arbitrary units for second derivative of absorption data. The band at 1658 cm^{-1} represents the alpha helix band and is the "marker band" for native structure.

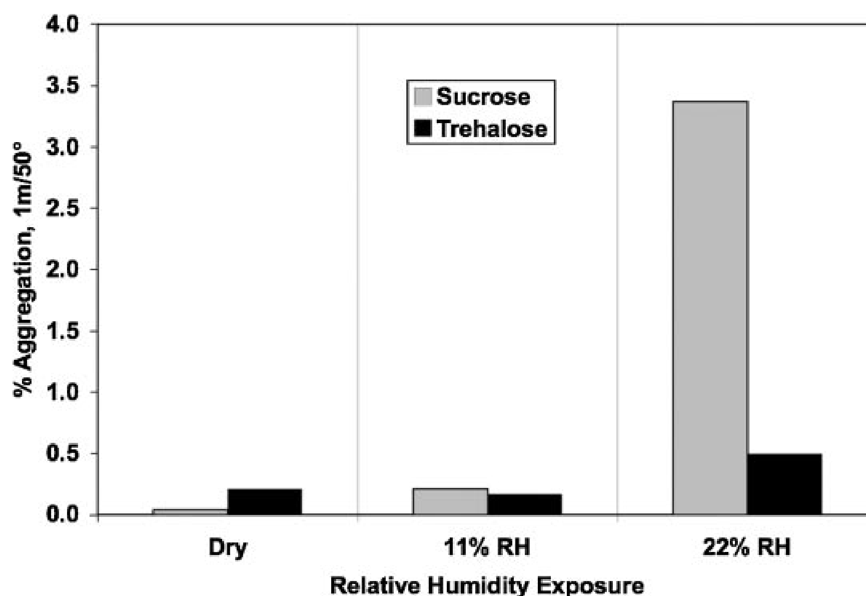

Figure 5. The effect of moisture on aggregation stability in 1:6 (w/w) hGH/disaccharide formulations: the Role of T_g in Determining Stability. The light shaded bars represent the sucrose formulation and the dark shading represents the trehalose formulation.

relatively dry, but if circumstances allow sorption of significant quantities of water, trehalose would be the stabilizer of choice. Such an effect was likely responsible for much of the superior stabilizing performance of trehalose described earlier.[18] The observations also emphasize the fact that formulation comparison without control of water content can be misleading.

If T_g is an important stability variable, as suggested by the above analysis results presented in Figure 5 and discussions in the literature,[12,13,15,21] one might expect stability to improve as the quantity $(T - T_g)$ decreases. That is, as T_g becomes large relative to the storage temperature, T, due to either water content differences or differences in formulation, stability should monotonically increase. This concept is based on the idea that the further a system is below its T_g, the lower the mobility, and therefore, the more stable it should be. Figure 6 indicates this concept is flawed, at least for the hGH systems studied. The plot is divided into two sections, all systems except sucrose formulations and systems containing sucrose. With the non-sucrose systems, there is obviously no correlation, but it should be noted that all these systems have large negative values of $T - T_g$; they are systems at temperatures very much below T_g. At least for the non-sucrose formulations, the generalization might be that for systems being studied well below T_g, other characteristics of the glass dominate mobility

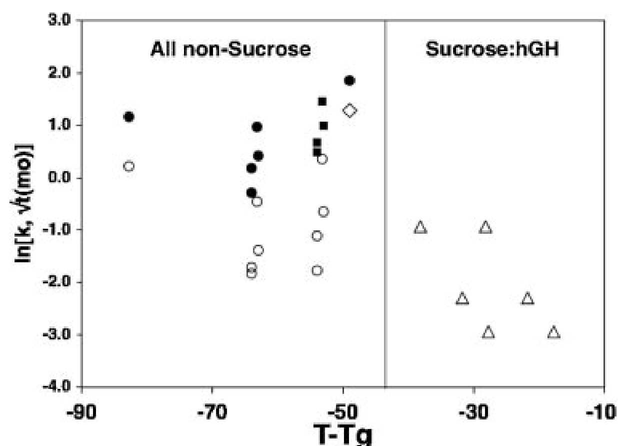

Figure 6. Correlation of stability with the difference between storage temperature, T, and the glass transition temperature of the formulation, T_g. Open symbols are aggregation, filled symbols are chemical degradation, diamonds are pure protein, and circles are disaccharide systems other than sucrose. Sucrose data are triangles.

differences in the solid.[7] The sucrose systems do show a correlation, but the correlation is nonsense in terms of dynamics measured by $T - T_g$ and is an example of a correlation not representing a cause/effect relationship. The trend is increasing stability with increasing $T - T_g$, which is simply a reflection of the reduction of the T_g of a protein system (T_g, dry $\approx$136°C) by addition of sucrose (T_g, dry $\approx$75°C)[25] and the increasing stabilization offered by increasing levels of sucrose.

Relaxation dynamics in a series of materials does not always correlates with $T - T_g$ since in addition to $T - T_g$, both fragility and thermal history impact relaxation time,[7] and if one expects a correlation between stability and glass dynamics for a wide range of materials, potential differences in fragility and thermal history cannot be ignored. Thus, the failure to observe a sensible correlation between stability and $T - T_g$ for systems well below T_g is not surprising; one should examine the correlation between stability and structural relaxation time to better characterize the impact of glass dynamics on stability. Although structural relaxation times were not measured for the samples studied in this research, we did evaluate approximate values of the structural relaxation time from the width of the glass transition (reflecting fragility), from heat capacity data (reflecting thermal history), and from $T - T_g$, as described in detail in the experimental section. The comparison of these calculated values of relaxation time, as τ^{β}, with stability is shown in Figure 7. Ignoring the sucrose data for the moment, it is clear that there is indeed a correlation between stability and structural relaxation time, but the correlation line representing aggregation (i.e., open symbols) has a larger slope ($\approx$0.5) than the correlation line representing chemical degradation ($\approx$0.3). This qualitative difference might be anticipated. The slope is a measure of the coupling coefficient, that is, the degree of coupling between degradation rate and structural relaxation rate,[7] with a larger value indicating stronger coupling. Note that the coupling coefficient for a diffusion controlled reaction where the inverse relationship between diffusion coefficient and viscosity is valid (i.e., Stokes–Einstein equation) would be unity. The slopes observed are significantly less than unity, but given the approximations inherent in this analysis, the slopes may only have qualitative significance. What is important is that the coupling coefficient for aggregation is significantly greater than that for chemical degradation.

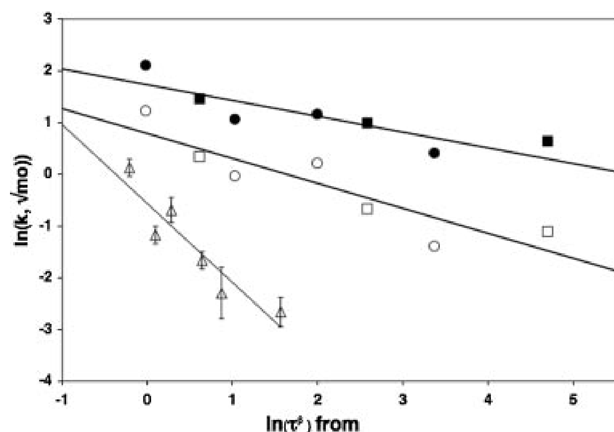

Figure 7. Correlation between stability and structural relaxation time constant, τ^{β}, as estimated from the width of the glass transition, ΔT_g. Circles and squares are pure hGH, stachyose (1:1), and trehalose (1:1, 1:3, 1:6 weight ratios of protein/saccharide). The triangles represent aggregation data in sucrose formulations. The rate constant is from square root of time (months) kinetics, and τ is in hours. All input data for τ is for mean moisture samples. Error bars (standard errors) for the non-sucrose formulations are smaller than the symbol size. Note that sucrose formulations are more stable than expected based upon the structural relaxation time constant.

Aggregation is believed to involve partial unfolding and perhaps some limited translational motion, mobility likely close to the type of mobility involved in viscous flow and segmental diffusion. That is, one might expect both viscous flow and partial protein unfolding to involve more extensive disruption of the surrounding matrix than chemical degradation pathways would require. We note that aggregation itself may not be manifested until rehydration, even though it is dependent on time dependent alterations in the protein occurring during storage in the dried solid. However, even minor perturbations in protein tertiary structure in the dried solid would most likely require substantially more mobility than that required for chemical degradation. Oxidation involves mobility of small reactive oxygen species and deamidation requires intramolecular motion of only small segments of the molecule, with likely less disruption of the matrix. Thus, one might guess that the coupling coefficient for aggregation would be greater than that for chemical degradation, as observed.

In the above analysis, we have ignored the dilution effect discussed earlier. If aggregation is controlled by a bimolecular process in the solid state, and it need not be, we argued earlier that the empirical rate constant, k, should be roughly proportional to the mass fraction of hGH. If we instead evaluate the ratio of k to the mass fraction of hGH, denoted k_2, and analyze for correlation with τ^{β} as we did above for k, we find a correlation between k_2 and τ^{β} similar to that shown by Figure 7, but the coupling coefficient (i.e., slope) is less than half of that found in the correlation with k, and indeed is less than the coupling coefficient found for chemical degradation. Also, because much of the variation in k was removed by calculating k_2, the correlation coefficient is only about 0.5, as contrasted with the value of 0.87 for the analysis using k. In short, much but not all, of the variation of k might possibly be attributed to the dilution effect. However, unless chemical decomposition would also be subject to a dilution effect, we are then faced with the unsatisfying result that the coupling coefficient is higher for chemical degradation than for aggregation, contrary to our previous argument. A possible solution to the apparent dilemma is if the rate determining step in aggregation is, at least in part, a unimolecular unfolding, with the actual bimolecular process occurring during reconstitution. Although observations consistent with this postulate can be found,[32,33] we have no direct evidence to support this speculation for the systems studied in this research.

The Superior Stability of Sucrose Formulations: The Role of Fast Dynamics

Sucrose formulations clearly do not fit the correlation established by the other formulations. That is, sucrose formulations are much more stable than the estimated relaxation times would suggest, an observation similar to that made a decade ago with a monoclonal antibody.[34] The sucrose systems have much smaller relaxation times (i.e., higher molecular mobility) than the other stabilizer systems at equivalent stabilizer/hGH ratios, largely because of the much smaller T_g's of the sucrose systems. In fact, the T_g's of formulations not containing sucrose have T_g values in a relatively narrow range, and the trends in relaxation time are dominated by the differences in fragility, as reflected in the widths of the glass transition regions (Tab. 1). Fragilities for the sucrose formulations are nearly identical to the corresponding trehalose formulations, and

the much lower relaxation times are entirely due to the lower T_g's.

To address possible reasons for the superior stability of sucrose based formulations, fast dynamics of the two 6:1 disaccharide/hGH formulations were characterized using neutron scattering (Fig. 8). Previous results[10,35] suggest that well below T_g, it may be dynamics on a nanosecond time scale that are primarily responsible for stability differences in glassy protein systems. Results for mean square amplitude as a function of temperature show significantly less amplitude of motion for the 6:1 sucrose/hGH formulation (solid line) than for the 6:1 trehalose/hGH formulation (broken line). Note also that the differences are a maximum around 40°C, and the two curves cross at a temperature higher than the T_g of the sucrose formulation but lower than the T_g of the trehalose formulation. That is, at temperatures above its T_g, but below the trehalose T_g, the sucrose system becomes more mobile than the trehalose system, indicating that fast dynamics does respond to the glass transition. Thus, at least for these two formulations, stability does correlate with dynamics in the glass, but it is dynamics on a nanosecond timescale that seems predictive of stability rather than dynamics on the much longer time scale of global or structural relaxation.

This conclusion presents a dilemma. Why is stability, which is on a time scale of months, better

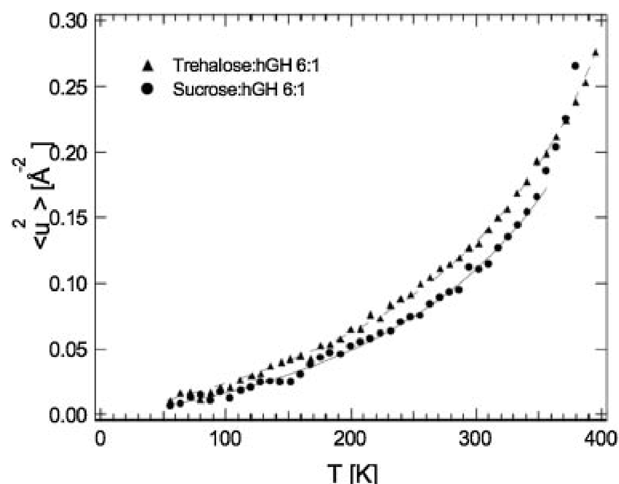

Figure 8. Amplitude of fast dynamics as measured by neutron scattering for 1:6 hGH/disaccharide trehalose and sucrose formulations of hGH. The mean square amplitude of hydrogen motion (Å^2) as a function of temperature is plotted vs. temperature. The T_g values for the sucrose and trehalose formulations are 348 and 386 K, respectively. Uncertainties (standard error) are roughly the size of the plotting symbol.

correlated with motion on a nanosecond time scale than with structural relaxation, which is on a times scale similar to stability? We do not have a rigorous explanation, but consider the following hypothesis. For the change from reactants to product, there may be (at least) two possible parallel pathways. One pathway, dominant at higher temperature, might involve the concerted motion of a large number of matrix molecules to generate the "free volume" needed to proceed along the reaction pathway. This would be a highly cooperative process with high activation energy, that is, the α-process described by the Adam-Gibb theory,[36] but it would be reasonably fast near and above T_g. However, this process would become exceedingly slow at temperatures well below T_g because of the high activation energy, that is, lack of free volume (and configurational entropy) deep in the glassy state.[7] An alternate pathway, consisting of a great many very small steps (i.e., small length scale) on a fast (nanosecond) time scale, each requiring low cooperativity and low activation energy, would now become the fastest pathway to product. That is, due to the many steps involved, this pathway is slower than the α-process mediated pathway near and above T_g, but due to much smaller activation energy than the α-process, becomes the fastest pathway at temperatures well below T_g. Thus, the rate determining pathway changes from the highly cooperative processes correlated with viscous flow and structural relaxation to one more closely related to the small length scale, small time scale process measured by the fast dynamics of neutron scattering.

Finally, we need to acknowledge that while fast dynamics is qualitatively consistent with the difference in stability between the sucrose and trehalose formulations, the relationship between our measure of fast dynamics and stability is not a simple proportion, and it is certainly possible β-process dynamics is not the only contributing factor. If the mean square amplitude for β motion were directly proportional to the rate constants for degradation, the activation energies for degradation and fast dynamics would be essentially the same. However, the activation energies for both the sucrose and trehalose systems (6:1 disaccharide/hGH) are ≈18 kcal/mol for aggregation (from Tab. 1)and ≈1.4 kcal/mol for $<u^2>$ (from Fig. 8), clearly a huge difference in temperature dependence between degradation and $<u^2>$. Furthermore, the ratio of $<u^2>$ for trehalose/sucrose is 1.2 at 40°C while the

corresponding ratio for rate constants is 2.1. Obviously, the relationship between fast dynamics and stability is more complex than a direct proportion. Finally, we note that while it is clear that differences in protein secondary structure cannot explain the stability differences between the sucrose and trehalose formulations, subtle changes in tertiary structure might impact stability and yet not impact secondary structure. Unfortunately, we have no reliable measure of tertiary structure in the glassy solid state.

CONCLUSIONS

Both chemical stability and aggregation stability in hGH formulations are extremely sensitive to formulation, with differences of nearly two orders of magnitude in degradation rates between the least and most stable formulations. Trends in stability are the same for chemical degradation and for aggregation, although the differences are larger for aggregation. Although stability seems sensitive to the difference between storage temperature and the formulation glass transition temperature, T_g, when the system is near or above the T_g, for storage well below T_g, stability seems unrelated to T_g. Both aggregation and chemical degradation are qualitatively correlated with secondary structure of the protein in the solid state, as measured by infrared spectroscopy, and there is a partial quantitative correlation between rate constant for degradation, both chemical and aggregation, and the estimated structural relaxation time. Here, differences in relaxation time between formulations are dominated by differences in glass fragility. However, sucrose systems appear anomalous. At equivalent levels of disaccharide relative to protein, sucrose systems are about a factor of two more stable than trehalose systems. Yet, sucrose systems have much greater mobility as measured by structural relaxation time and identical secondary structure as viewed by infrared. However, neutron scattering studies show greater suppression of fast dynamics by sucrose than by trehalose, supporting the observation that sucrose formulations are more stable, and suggesting that well below T_g, fast dynamics may be controlling stability differences between formulations.

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