Effect of Moisture on the Stability of a Lyophilized Humanized Monoclonal Antibody Formulation

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Purpose. To determine the effect of moisture and the role of the glass transition temperature (T_g) on the stability of a high concentration, lyophilized, monoclonal antibody.

Methods. A humanized monoclonal antibody was lyophilized in a sucrose/histidine/polysorbate 20 formulation. Residual moistures were from 1 to 8%. T_g values were measured by modulated DSC. Vials were stored at temperatures from 5 to 50°C for 6 or 12 months. Aggregation was monitored by size exclusion chromatography and Asp isomerization by hydrophobic interaction chromatography. Changes in secondary structure were monitored by Fourier transform infrared (FTIR).

Results. T_g values varied from 80°C at 1% moisture to 25°C at 8% moisture. There was no cake collapse and were no differences in the secondary structure by FTIR. All formulations were stable at 5°C. High moisture cakes had higher aggregation rates than drier samples if stored above their T_g values. Intermediate moisture vials were more stable to aggregation than dry vials. High moisture samples had increased rates of Asp isomerization at elevated temperatures both above and below their T_g values. Chemical and physical degradation pathways followed Arrhenius kinetics during storage in the glassy state. Only Asp isomerization followed the Arrhenius model above the T_g value. Both chemical and physical stability at $T \geq T_g$ were fitted to Williams-Landel-Ferry (WLF) kinetics. The WLF constants were dependent on the nature of the degradation system and were not characteristic of the solid system.

Conclusion. High moisture levels decreased chemical stability of the formulation regardless of whether the protein was in a glassy or rubbery state. In contrast, physical stability was not compromised, and may even be enhanced, by increasing residual moisture if storage is below the T_g value.

KEY WORDS: protein stability; glass transition temperature; modulated DSC; aggregation; Asp isomerization.

INTRODUCTION

Lyophilization is commonly used to provide long-term storage stability to protein pharmaceuticals. Stability has been attributed to the fact that many lyophilized proteins exist in highly viscous amorphous glassy states with low molecular mobilities and low reactivities. According to this "vitrification hypothesis," stability is highly dependent on storage temperature. If the lyophilized material is stored at a temperature greater than its glass transition temperature (T_g) the viscous glass will be transformed to a less viscous "rubbery" state with increased heat capacity, molecular mobility, and, thus, decreased stability (1). Crystallization of the glass-forming excipients can occur above the T_g value, further decreasing stability (2). Stabilization has also been described by the "water substitution" hypothesis. Excipients such as sugars are thought to hydrogen bond with the protein in the same manner as water, conserving native structure by replacing water lost on drying and stabilizing the formulation during storage (3). Recent studies have shown that optimal stabilization is provided by glass-forming excipients that hydrogen bond to the protein molecule, thus preserving its native structure during lyophilization and storage (2,4).

The increase in heat capacity on transformation from the glassy to the rubbery state can be measured by differential scanning calorimetry (DSC), and the midpoint of the step change in heat capacity is generally taken as the T_g value. Knowledge of the T_g value is important, as a lyophilized product must be stored below this value to ensure long-term stability. Accelerated stability data generated above the T_g value will not be predictive of behavior at lower storage temperatures and cannot be used to estimate shelf life (5). As limited molecular mobility does occur below the T_g, some degradation can occur in the glassy state (6,7). It has been suggested that, if possible, samples should be stored at 50°C below their T_g values to minimize molecular mobility and achieve stability (8). The fragility of the amorphous material describes the temperature dependence of molecular mobility in the glass transition region, and this can determine how the chemical, physical, or mechanical stability changes with changing temperature (9). Strong glasses show a small change in heat capacity (C_p) at their T_g and temperature dependence of their relaxation time follows Arrhenius kinetics. Fragile glasses show large increases in C_p at the T_g with increased changes in molecular mobility and stability as the storage temperature nears the T_g (10). Relaxation time constants are used to quantify molecular mobility and may be useful in predicting physical and chemical stability in the amorphous state (11).

A number of authors have investigated the effect of moisture, excipients, and the role of the T_g value on the chemical and physical stability of proteins. The T_g value is a function of the relative proportions of the glass-forming components in the formulation and the residual moisture content (12). Increasing moisture decreases the T_g value, thus compromising stability. "Dry" is often defined as being less than 1% residual moisture (1) but overdrying of proteins is also detrimental to stability (13,14). Greater rates of deamidation and aggregation were seen in IL-2 stored above its T_g value (15). Aggregation rates increased in insulin as the storage temperature increased to exceed the T_g value, but deamidation occurred even in the glassy state (16). Other authors have reported no large effect of T_g on stability monitored by enzyme activity (17). Little work has been done on the effect of T_g on the stability of monoclonal antibodies. In one study, monoclonal antibodies had increased aggregation when stored above their T_g value (5). Studies with an antibody-vinca conjugate (18) also showed increasing rates of aggregation and conjugate decomposition with increasing moisture due to a glass transition effect.

This study investigates the effect of moisture on the stability of a high concentration lyophilized monoclonal antibody formulation with low levels of excipients. Such formu-

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lations are necessary to deliver the high doses often necessary for efficacy of these molecules. Physical and chemical stabilities were monitored. Physical instability refers to changes in the three-dimensional conformational integrity of the protein and does not necessarily involve covalent modification. Physical phenomena include denaturation, aggregation, precipitation, and adsorption. Chemical instability involves covalent modification of a protein or amino acid to produce a new molecule via bond cleavage, bond formation, rearrangement, or substitution. Examples of chemical reactions are deamidation, oxidation, sulfydrol-disulfide interchanges, and hydrolysis (19). The different roles of moisture and the T_g value on both physical and chemical stabilities are illustrated.

MATERIALS

rhuMAb was produced by Genentech, Inc., South San Francisco, CA. Sterile water for injection (SWFI) and 0.9% NaCl, were obtained from Abbott Laboratories. Wheaton glass vials (5 cc) and West 890 gray butyl stoppers (20 mm) were obtained from Genentech Clinical Manufacturing.

Formulated bulks (40 mg/mL protein, 85 mM sucrose, 5 mM histidine, pH 6.0, 0.01% polysorbate 20) were provided by Genentech Clinical Manufacturing. The 5 cc vials were filled at 4.8 ml of formulated bulk and lyophilized by a one-step 25°C primary drying cycle in a GT20 lyophilizer. Three sets of samples were prepared to different moisture contents. In the first study, vials were exposed to a 30°C, 64% relative humidity (RH) atmosphere for 3.5 h or to a 22°C, 52% RH atmosphere for 24 h. Control vials were unopened. In study 2, high moisture vials were prepared by reopening stoppered vials in the cold lyophilizer and restoppering. Control vials were not opened. In study 3, high moisture vials were prepared by a shortened lyophilization cycle, whereas control vials were dried by the regular lyophilization cycle.

METHODS

Moisture

Residual moisture was measured by the Karl Fisher titration method using a Metrohm KF apparatus. At least 75 mg of pulverized cake were dispersed in a methanol bath and titrated with Riedel-deHaen Hydranal-Composite 2 reagent (Hoechst Celanese Corp., Germany) until the end point was reached as determined by the KF processor.

DSC Measurements

DSC measurements were performed on a Perkin Elmer (Shelton, CT) DSC 7, a Seiko DSC 120, and a TA Instruments (New Castle, DE) MDSC 2920. For the Perkin Elmer DSC, ~8 mg samples were scanned from 5 to 200°C at 10°C/min. Seiko (Torrence, CA) DSC runs (5 to 150°C) were performed at scan rates of 5°C/min and samples were from 6 to 22 mg. All samples were encapsulated in standard sealed aluminum DSC pans. Modulated DSC runs were performed on 9 to 13 mg samples at a scan rate of 2°C/min with a modulation period of 40 s and a modulation amplitude of ±0.5°C. Scans were from 5 to 180°C. Samples were encapsulated in hermetically sealed aluminum DSC pans.

Vial Storage

Samples were placed at six temperatures (–70, 5, 15, 30, 40, and 50°C) and sampled over 6 or 12 months. Study 2 vials were not placed at 50°C. In the first study, vials were sampled singly, whereas in studies 2 and 3 vials were sampled in duplicate.

Color, Clarity, and Appearance

Visual inspection was performed prereconstitution to assess cake appearance and postreconstitution to assess the clarity of the reconstituted liquid and to provide a qualitative assessment of particulate matter.

Turbidity Measurement

Optical density (OD) measurements of undiluted samples were taken on a Hewlett-Packard (Wilmington, DE) Vectra XM Series 4 spectrophotometer, with a 1 cm cuvette, using Milli-Q water as a reference. The OD values from 360 nm to 340 nm were obtained, averaged, and reported as an average OD at 350 nm (20). Samples were centrifuged at 10,000 rpm for 10 min using an Eppendorf 5415 C centrifuge and the supernatants remeasured.

Native Size Exclusion Chromatography

Size exclusion chromatography (SEC) was performed with an HP 1090 liquid chromatography system equipped with a diode array detector and a TSKG3000SWXL, 7.8 &mult; 300 mm column (TosoHaas, Montgomeryville, PA). A 200 μg load was eluted with a 100 mM potassium phosphate, pH 6.8, mobile phase. Detection was at 280 nm. A BioRad (Richmond, CA) gel filtration standard (catalog no. 151-1901) containing proteins from 670 KDa (thyrogloulin) to 1.35 KDa (vitamin B12) was included in each run.

SDS PAGE

SDS PAGE was carried out on 4–15% gradient Tris glycine gels on a Bio-Rad mini protean II electrophoresis system. Sample preparation, application, and electrophoresis were carried out according to the manufacturer's instructions. Samples were diluted to 1.0 mg/mL in reducing or nonreducing sample buffer, heated to 90°C for 5 min and 10 μg of sample loaded onto the gel. Electrophoresis was at 200 volts for ~40 min. The gels were stained with coomaisse blue for 1 h and destained until the backgrounds were clear. A broad range molecular weight standard (Bio-Rad, catalog no. 161-0317) containing proteins from 6.5 KDa (aprotonin) to 200 KDa (myosin) was included on each gel. The gels were visually inspected to evaluate intact antibody, heavy and light chains, nondissociated aggregates and fragments.

Hydrophobic Interaction Chromatography (HIC) of a Pepsin Digest

Formulations were diluted to 1 mg/mL with 0.1 M sodium citrate, pH 3.5, and freshly prepared 1 mg/mL pepsin (Boehringer Mannheim, Indianapolis, IN) added at 10 μl/ml. Samples were incubated at 37°C for 2 h. Ten micrograms of digested protein were analyzed by HIC using a TSK-Butyl NPR 3.5 cm × 4.6 mm column (TosoHaas) on a HP1090 chro-

matography system. Detection was at 214 nm. The column was held at 10% mobile phase B for 1 min after sample application. A 10 to 62% mobile phase B gradient was run over 20 min at a flow rate of 1 ml/min and a column temperature of 40°C. Mobile phase A was 2 M ammonium sulfate in mobile phase B (20 mM Tris in 20% glycerol, pH 8.0). The column was washed with 100% B for 10 min after each sample application and equilibrated at the initial gradient conditions for 10 min before the next injection.

HIC of a Papain Digest

Samples were diluted to 1 mg/mL in a final volume of 250 μL L-cysteine buffer. The L-cysteine buffer was 0.024 g of L-cysteine qs to 10 mL with EDTA buffer (0.1 M Tris HCl pH 7, 0.004 M EDTA). Five microliters of papain, freshly prepared at 0.25 mg/ml with this buffer was added and samples were incubated at 37°C for 2 h. Samples of digested protein (25 μg) were analyzed on a TSK-Phenyl 5PW, 7.5 cm × 75 mm column (TosoHaas) on a HP 1090 HPLC system. Detection was at 214 nm. The column was held for 1 min at 40% mobile phase B, followed by a 40–80% gradient over 40 min (mobile phase A was 2 M ammonium sulfate in mobile phase B, which was 20 mM Tris, pH 7.5), at a flow rate of 1 mL/min and a column temperature of 40°C. The column was washed with 100% mobile phase B for 5 min following each analysis and equilibrated at the initial gradient conditions 10 min prior to injection.

Fourier Transform-Infrared (FTIR) Measurements

Absorbance of aqueous protein was measured using a liquid cell holder, CaF_2 windows, and a 6 μm mylar spacer (Spectra Tech, Inc., Shelton, CT). The absorbance of water was subtracted from that of protein using the criteria of a straight baseline from 2,000 to 1,720 cm^{-1} and no negative absorbance peaks. Measurements on solid samples were performed using 13 mm KBr discs prepared by mixing 400 mg of KBr and 0.8 mg (0.2% w/w) freeze-dried protein. Discs were formed under vacuum in a KBr Die (Spectra Tech, Inc.) with a Carver Laboratory Press (Fred S. Carver, Inc., Vabash, IN) at 2,600 psi for 4 min. The die press was rotated 90° half-way through the pressing process. Discs were placed in a Magnetic KBr pellet holder (Spectra Tech, Inc.) and spectra obtained immediately using a Galaxy 5000 FT-IR spectrophotometer (ATI Mattson, Inc., Madison, WI) equipped with a narrow range mercury cadmium telluride detector. The resolution was 4 cm^{-1} with 256 scans coadded, using a forward mirror velocity of 50 kHz, no zero filling, and no apodization. The instrument was purged with nitrogen gas, and a purge time of 8 min was employed each time the chamber was opened. Data collection and analysis (smoothing, calculation of second order spectra) was carried out using WinFIRST software (ATI Mattson, Inc.)

RESULTS AND DISCUSSION

Cake Appearance and Moisture Content

All cakes appeared similar on preparation at T_0 with no visible collapse. In study 1, unopened vials were 2.3 ± 0.4% moisture. Vials incubated at 30°C, 64% RH were 5.4 ± 0.4% moisture. Vials at atmospheric conditions for 24 h were 8.5 ± 0.5% moisture. In study 2 controls were 1.4 ± 0.2% moisture, whereas vials exposed to condensation during stoppering were 3.0 ± 0.4% moisture. In study 3 controls were 1.1+ 0.1% moisture, and vials produced by the shortened lyophilization cycle were 2.4 + 0.4% moisture.

DSC

T_g values were not detectable by conventional DSC instrumentation. Previous authors have ascribed the difficulty in T_g detection for proteins to the large internal heterogeneity of the molecule, which results in a gradual increase in heat capacity rather than a sharp transition as seen in homogenous polymers (12). Small changes in heat capacity at the T_g are characteristic of strong glasses and are typical of globular protein systems (21). T_g values were detectable by conventional DSC in placebo vials and in active vials >1:500 molar ratio of protein:sugar. A 1:500 molar ratio protein:sugar formulation had a T_g value of 59°C at 1% moisture. A second endotherm was detected at around 140°C with a melting endotherm at 180 to 190°C. It was possible to scan in and out of the first endotherm but not the higher endotherms, indicating that the first endotherm was indeed a T_g.

The bulk of the formulations in this study had a 1:310 molar ratio protein:sugar and T_g values were detectable only by modulated DSC (MDSC). Increasing residual moisture in the cake decreased the T_g value from 80°C at 1% moisture to 25°C at 8% moisture. A similar trend was reported for amorphous sucrose (22) but the antibody formulation showed higher T_g values than sucrose alone and a slower decrease in the T_g value with increasing moisture (Fig. 1).

We are unsure as to whether the formulation excipients crystallized when stored above the T_g value. Although sucrose crystallizes at temperatures above its T_g (22), no recrystallization peaks were visible in any of the preparations by MDSC. Crystallization of sucrose would increase the moisture content of the remaining amorphous phase, further compromising stability. The high ratio of protein to sucrose in our formulation may prevent sucrose crystallization during storage above the T_g value (23), allowing the excipients to remain in an amorphous state.

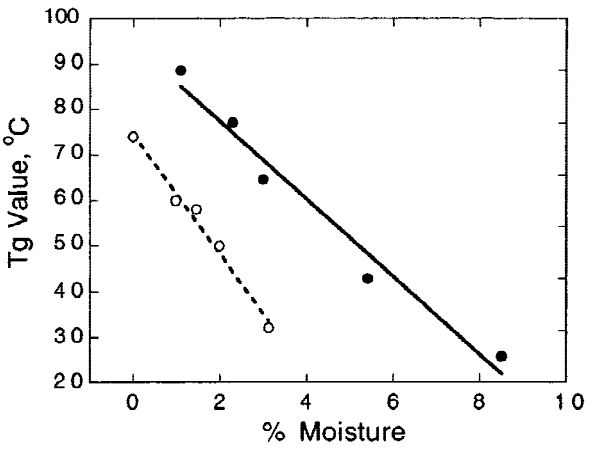

Fig. 1. T_g values vs. residual moisture in amorphous sucrose (----) (22) and 40 mg/mL protein, 85 mM sucrose, lyophilized monoclonal antibody formulations (——). Linear regression analysis yields intercepts of 74.7 and 88.1, slopes of –13.2 and –7.5 and R values of 0.992 and 0.984 for amorphous sucrose and 40 mg/ml protein formulation, respectively.

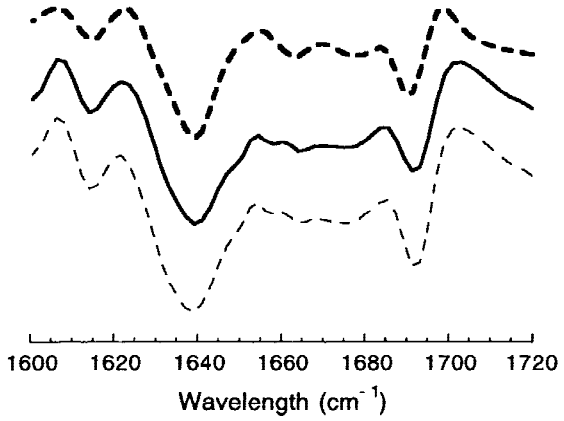

Fig. 2. FTIR of 40 mg/mL protein, 85 mM sucrose, monoclonal antibody pre-lyophilization (----), lyophilized monoclonal antibody formulations at 2% residual moisture stored for 9 months at 5°C (····) and at 8% residual moisture stored for 9 months at 50°C (——). All second derivative curves were offset for comparison.

FTIR

FTIR spectrometry has been widely used to study protein secondary structure. Previous authors have demonstrated its usefulness in investigating lyophilization and temperature-dependent protein aggregation (24). Changes in FTIR spectra following irreversible or partially irreversible DSC transitions have been reported (25,26). Residual moisture contents of up to 10% do not interfere with the amide I second derivative spectrum (24). This amide I region (1720 to 1600 cm^{-1}), primarily due to the C=O stretching vibrations in the polypeptide structure, is commonly used to characterize protein structure, and various structural components have been assigned to its absorption maxima. The amide I region contains a complex mixture of overlapping bands that are resolved to qualitatively assign secondary structure types. Here we used the second derivative analysis method with a seven-point data smoothing function. We did not quantify the various structural components but analyzed them by pattern comparison.

The amide I spectra of 2, 5, and 8% moisture cakes were compared after 6 and 9 months storage at 5, 40, and 50°C. All samples had similar FTIR spectra. Spectra were also similar to that of an aqueous protein preparation, indicating that little change occurred in the secondary structure during the initial lyophilization (Fig. 2). The high moisture samples stored at high temperatures were stored above their T_g values and were very degraded as assessed by our other assays. This did not appear to effect the secondary structure of the protein (Fig. 2).

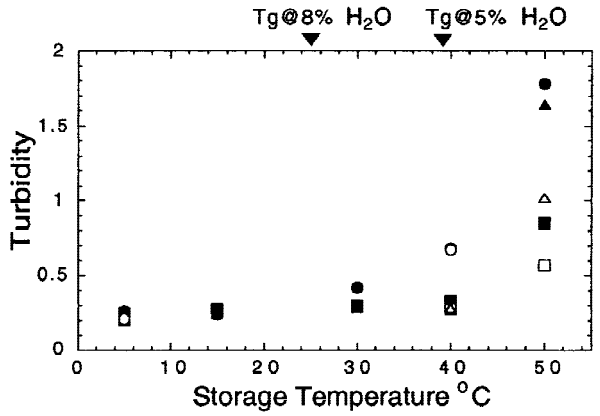

Fig. 3. Turbidities (mean OD 340–360 nm) pre- (closed symbols) and postcentrifugation (open symbols) of 2% (■, □), 5% (◆, ▲, △), and 8% (●, ○) residual moisture formulations stored for 6 months from 5 to 50°C and reconstituted to 125 mg/mL.

Stability Assessment

No cake collapse was seen in active vials even during prolonged storage at up to 25°C above their T_g values. Pla-

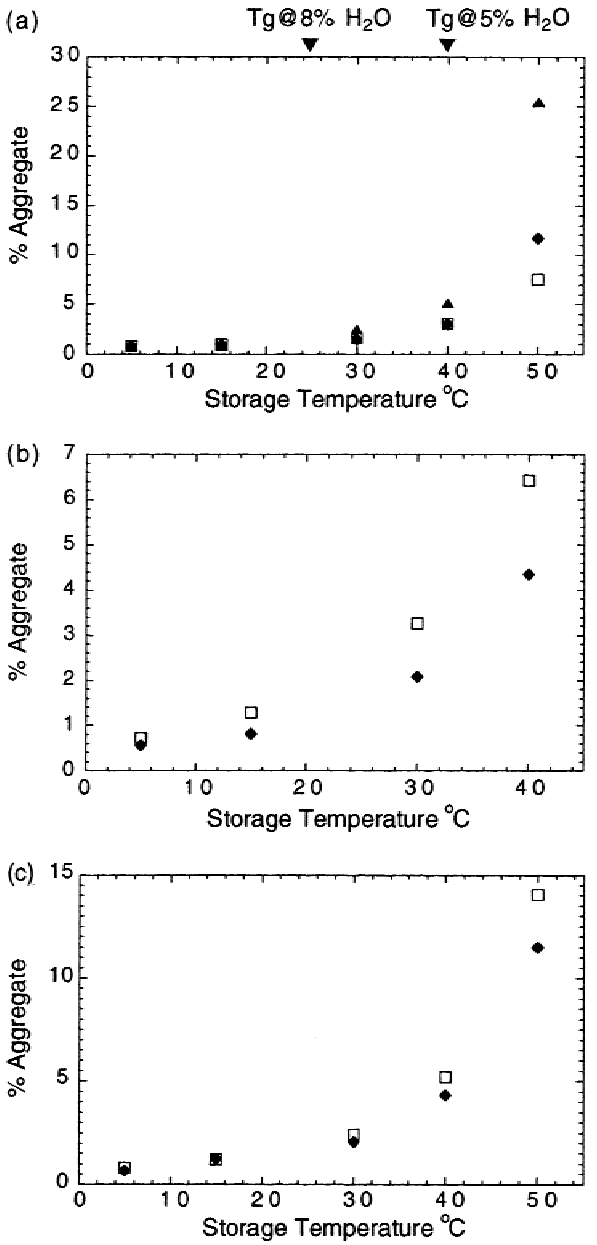

Fig. 4. Soluble aggregate levels in (a) study 1 cakes at 6 months at 2.3% (□), 5.4% (◆), and 8.5% (▲) moisture; (b) study 2 cakes at 12 months at 1.4% (□) and 3.0% (◆) moisture; (c) study 3 cakes at 12 months at 1.1% (□) and 2.4% (◆) moisture. Study 1 samples were stored above and below their T_g values, all other samples were stored below their T_g values.

cebo vials readily collapsed during storage at 40°C. The structural stability of the cakes may be due to cake microheterogeneity. Sucrose dominant regions may collapse above the T_g value on the microscopic scale, whereas protein dominant regions, with a higher T_g value, retain their rigid structure.

Moisture and Physical Stability

The physical stability of the lyophilized protein was monitored by SEC, SDS-PAGE, and turbidity (mean OD 340–360 nm). SEC was used to measure soluble aggregate and low molecular weight species formation. SDS-PAGE indicated whether aggregates were covalent or noncovalent, reducible or nonreducible. The OD 350 value measures turbidity due to soluble and insoluble aggregates. Insoluble aggregates can be removed by centrifuging at 10,000 g for 10 min and the samples reread. Solutions with turbidities of 0.08 to 0.131 are termed very opalescent, from 0.131 to 0.183 slightly cloudy, and from 0.183 to 0.498 cloudy (20).

Immediately after lyophilization at T_0 all samples were cloudy after reconstitution with turbidities of >0.2. This cloudiness is an inherent quality of the preparations due to their high protein concentrations. Particulate contents of similar preparations have been measured and are far below the current USP limits. Turbidity did not increase during 5°C storage of any of the samples. Turbidities increased during high-temperature storage, particularly for the higher moisture samples stored above their T_g values (Fig. 3). The 8% moisture formulation, with a T_g value of 25°C, had a higher turbidity than 2 and 5% moisture samples stored at ≤30°C. The turbidity of the 5% moisture sample was increased relative to the 2% moisture sample when stored above its T_g value of 40°C. Differences in turbidities between centrifuged and uncentrifuged samples were relatively small during storage at ≤40°C, even for samples stored in the rubbery state. All samples contained insoluble aggregates after 6 months storage at 50°C, regardless of whether storage was above or below the T_g value.

A comparable result was achieved by the SEC assay. Moisture formulations of 2, 5, and 8% all had approximately 1% aggregate after 6 months at 5 and 15°C. At 30°C and higher, the 8% moisture formulation, stored above its T_g value, had a higher aggregate content than the drier formulations (Fig. 4a). This sample also had ~1% lower molecular weight species after 6 months at 50°C. No lower molecular weight species occurred in any other samples. The 5% moisture sample had a T_g value of 40°C. Moisture formulations of 2 and 5% both had 3% aggregate after 6 months at 40°C, but at 50°C the 2% moisture sample was 7% aggregate, whereas the 5% moisture sample was 12% aggregate. Therefore, differences in physical stability occurred only if the T_g value was exceeded. Covalent reducible aggregates were visible in degraded samples by SDS-PAGE and the intensity of the aggregate bands increased with increasing soluble aggregate (data not shown).

Pseudo first order rate constants for percent monomer remaining by SEC were calculated at each moisture level (Table I). Figure 5a shows the rate plots for the 5% moisture formulation at the various storage temperatures. The natural log of (percentage monomer remaining) versus storage time is plotted and the straight lines are the best-fit linear regression lines. Samples had similar aggregation rates when stored below their T_g values. The 8% moisture formulation had increased rates of aggregate formation at 30°C and above whereas the 2% and 5% formulations had similar aggregation rates except at 50°C.

A different, though not contradictory, result was observed in the other two moisture-stability studies. All samples were stored below their T_g values. Controls and test preparation were similar at the lower temperatures but 2–3% moisture formulations appeared to be more stable than 1% moisture controls by SEC under accelerated conditions (Fig. 4b, 4c). In study 2, 1% moisture cakes were 6% aggregate after 12 months at 40°C whereas 3% moisture cakes were only 4% aggregate. Similarly, 1% moisture controls in study 3 were 14% aggregate after 12 months at 50°C whereas 2% moisture cakes were 11% aggregate.

Soluble aggregate formation is the main degradation route for our antibody. Increasing sugar to give a protein: sugar ratio to 1:500 greatly enhances stability. Further increases in excipient levels do not have a large effect on aggregation rates. Interestingly, 500 is also the theoretical number of water binding sites on the molecule (27). If we are to accept the water substitution hypothesis of excipient stabilization, then at a lower than optimal level of an excipient, residual moisture may substitute for the excipient, increasing protein stability in the 2 to 3% moisture cakes. Adding addi-

Table I. Stability of a High-Concentration Lyophilized Monoclonal Antibody Formulation at Various Residual Moisture Levels: Pseudo First Order Rate Constants for Percentage Monomer Decrease by SEC

Storage	Rate constant, K ($\times 10^{-4}$ days^{-1})						
	Study 1[a]			Study 2[b]		Study 3[b]	
Tg value	70°C	40°C	25°C	≥70°C	59°C	80°C	70°C
Residual moisture	2.3%	5.4%	8.4%	1.4%	3.0%	1.1%	2.4%
5°C	0.17 ± 0.09	0.14 ± 0.14	0.11 ± 0.2	0.05 ± 0.01	0.03 ± 0.02	0.003 ± 0.06	0.07 ± 0.03
15°C	0.26 ± 0.12	0.26 ± 0.12	0.12 ± 0.18	0.21 ± 0.02	0.10 ± 0.01	0.07 ± 0.07	0.09 ± 0.06
30°C	0.58 ± 0.23	0.57 ± 0.17	1.02 ± 0.37	0.74 ± 0.15	0.42 ± 0.07	0.45 ± 0.07	0.32 ± 0.10
40°C	1.41 ± 0.30	1.27 ± 0.35	2.43 ± 0.47	1.59 ± 0.25	1.07 ± 0.13	1.14 ± 0.16	0.86 ± 0.18
50°C	3.90 ± 0.60	6.63 ± 0.76	16.97 ± 2.54	nd	nd	3.73 ± 0.43	2.98 ± 0.63

[a] Rates calculated on 6 months data, four data points.
[b] Rates calculated on 12 months data, five data points.
[c] nd = not determined.

tional moisture would not further increase stability as the optimal ratio of protein to "excipients" has been reached. Thus, the 5 and 8% moisture formulations do not have very different aggregation rates compared with the 2.4% formulation during storage in the glassy state. Very high levels of residual moisture decrease stability by lowering the T_g values. Although the 8% moisture formulation is stable when stored at 5°C, storage at or above its T_g value of ~25°C would lower shelf life considerably. Two to three percent moisture vials retain high T_g values and would remain in the glassy state during refrigerated and room temperature storage apparently with enhanced physical stabilities.

Moisture and Chemical Stability

The antibody contains an aspartate (Asp) residue at position 32 on the light chain that is susceptible to isomerization to iso-aspartate (iso-Asp) via a cyclic imide intermediate. Pepsin or papain digestion of the antibody followed by HIC allows for the separation of the Asp, iso-Asp, and cyclic imide species. Digestion of the antibody with pepsin results in an Fc portion and a bivalent $F(ab')_2$. The HIC main peak contains the $F(ab')_2$ with the Asp-32 species on both light chains. Other peaks are also $F(ab')_2$ but with various combinations of Asp, iso-Asp, and cyclic imide (28). With papain digestion, an Fc portion and two monovalent F(ab)s are produced. The papain digest main peak contains the Asp-32 species, peak 2 is also an Asp-32 form of the F(ab) but with a free thiol, whereas the other peaks contain the various isomers.

Stability samples were digested with either pepsin or papain as indicated (Table II, Fig. 6) and the isomers separated by HIC. The pseudo first order rate constants for decrease in percentage main peak were calculated (Table II). Although two different digest enzymes were used, rates for high moisture samples can be compared with controls in each study. Rates for 5 and 8% moisture samples stored at 50°C were not calculated, as the HIC main peak could not be resolved at the 6 month time point.

All samples were stable at 5°C and comparable to reference samples stored at −70°C. The calculated rates of Asp isomerization at 5°C are very small with large error values (Table II), and the differences in the rates reflect only the variation in the assay. For the study 1 samples, an average of the 5°C rates for the 2, 5, and 8% moisture samples was used to construct the Arrhenius plot for the HIC data (Fig. 5b), which is discussed in the next section. The 5 and 8% moisture samples from study 1were less stable than the 2% moisture control during high temperature storage above or below their T_g values (Fig. 5b, 6a). It appears that being in the glassy state is not enough to protect the protein against increased rates of the hydrolysis reaction in high moisture cakes. There was little difference in the chemical stability of samples with 1, 2, and 3% moisture at the various storage temperatures (Fig. 6b, 6c). The study 2 samples were digested with pepsin and therefore have a lower percentage main peak at T0 (Fig. 6b). Some Asp isomerization did occur in lower moisture cakes at the higher storage temperatures but at slower rates than seen in the 5 and 8% moisture vials (Table II). Thus, moderate increases in residual moisture had little or no effect on chemical stability, whereas high moisture samples were chemically unstable.

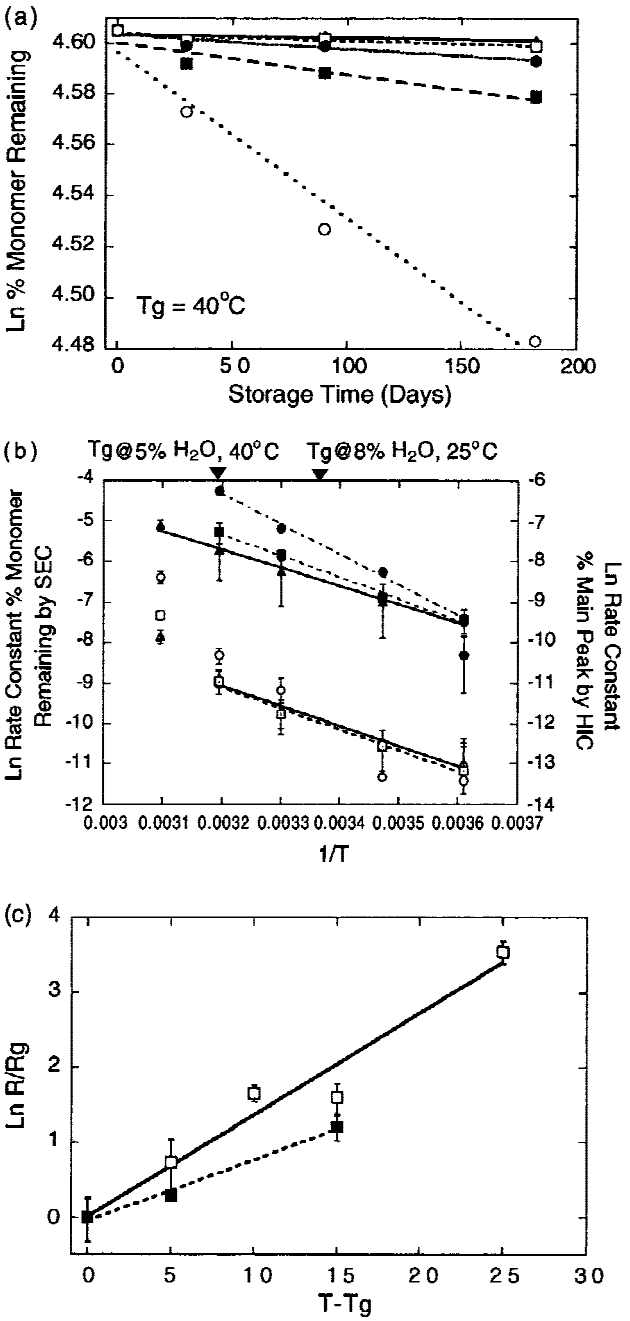

Fig. 5. Kinetics of antibody degradation: (**a**) Pseudo first order rate plot for percentage monomer remaining by SEC in a 5.4% moisture formulation stored for 6 months at 5°C (▲), 15°C (□), 30°C (•), 40°C (■), and 50°C (○). (**b**) Arrhennius plot of SEC (open symbols) and HIC (closed symbols) degradation rates for 2.3% (▲) and 5.4% (■), and 8.3% (•) moisture samples. (**c**) WLF kinetics for SEC (□) and HIC (■) data at $T > T_g$.

Stability, Arrhenius, and WLF Kinetics

The Arrhenius model is commonly used to describe the kinetics of chemical and biological reactions in the glassy state. At or above the T_g value, reactions do not necessarily obey the Arrhenius model (5). Williams-Landel-Ferry (WLF) kinetics, originally derived to describe relaxation processes in amorphous materials (29), have been used to model reactions at or above the T_g value. Roy *et al.* reported that the stability

Table II. Stability of a High-Concentration Lyophilized Monoclonal Antibody Formulation at Various Residual Moisture Levels: Pseudo First Order Rate Constants for Percentage Main Peak Decrease by HIC

Storage	Rate constant, K ($\times 10^{-4}$ days^{-1})						
	Study 1[a] HIC papain assay			Study 2[b,c] HIC pepsin assay		Study 3[b] HIC pepsin assay	
Tg value	70°C	40°C	25°C	≥70°C	59°C	80°C	70°C
Residual moisture	2.3%	5.4%	8.4%	1.4%	3.0%	1.1%	2.4%
5°C	1.17 ± 0.16	0.88 ± 0.26	0.33 ± 0.45	0.39 ± 0.74	0.06 ± 0.67	2.11 ± 0.45	2.50 ± 1.44
15°C	1.22 ± 0.71	1.39 ± 0.03	2.61 ± 0.29	1.61 ± 0.25	1.22 ± 1.09	0.67 ± 2.43	2.94 ± 0.48
30°C	2.61 ± 1.51	3.94 ± 0.55	7.67 ± 0.19	2.83 ± 0.25	2.72 ± 0.22	3.17 ± 2.28	6.94 ± 2.53
40°C	4.39 ± 2.28	6.94 ± 1.76	19.06 ± 3.24	6.78 ± 0.13	5.61 ± 0.67	5.17 ± 2.92	4.50 ± 0.48
50°C	8.33 ± 0.96	nd	nd	nd	nd	21.11 ± 4.04	26.22 ± 3.72

[a] Rates calculated on 6 months data, four data points.
[b] Rates calculated on 12 months data, five data points.
[c] nd = not determined.

of their lyophilized MAb-vinca conjugate was consistent with the WLF theory of rate processes in amorphous systems near their T_g value (18).

According to WLF kinetics the stability of a material at temperatures between T_g and $T_g + 100°C$ is best described by the equation: $R = (R_g \exp [\, C_1(T-T_g)])/\, C_2+(T-T_g)$, where R is the rate of the process, R_g is the corresponding rate at the T_g, T is the storage temperature, and C_1 and C_2 are constants. In an experiment where both T_g and temperature are varied, stability is a function of one variable, $T-T_g$ (1). Universal values have been derived for C_1 and C_2 (29), but this approach has been criticized and most authors allow the equation constants to vary (30) as we have done in this analysis. It has also been shown that a plot of $\ln(R/R_g)$ vs. $(T-T_g)$ can be analyzed by linear regression since the constant $C_2 > (T-T_g)$.

In our studies, except at 5 and 8% moisture, samples were stored below their T_g values and appeared to follow Arrhenius kinetics (Fig 5b). At 5% moisture the T_g value was 40°C and rates of aggregation and Asp isomerization followed Arrhenius kinetics up to and including this temperature. After 6 months at 50°C, the HIC main peak could not be resolved and the rate was not calculated. The aggregation rate was also higher than would be predicted by the Arrhenius plot. Asp isomerization in the 2% moisture sample followed the Arrhenius model up to 50°C but again the aggregation rate at 50°C was higher than that predicted by the Arrhenius model. This may have been linked to the formation of insoluble aggregates that occurred only during 50°C storage (Fig. 3). Soluble aggregate formation in the 8% moisture formulation, with a T_g value of 25°C, did not follow Arrhenius kinetics, indicating that passing through the glass transition had a major impact on physical stability. Asp isomerization rates did appear to follow the Arrhenius model, through the glass transition, up to 40°C. Again, at 50°C the HIC main peak could not be integrated. The energies of activation (Ea) were calculated from the Arrhenius plots. The Ea values for the aggregation reaction were 9.9 and 10.3 Kcal/mol at 2 and 5% moisture, respectively, indicating that increasing moisture did not increase the temperature dependence of the aggregation reaction during storage below the T_g value. For the HIC reaction, Ea values increased with increasing moisture content and were 9.0, 10.6, and 14.8 Kcal/mol at 2, 5, and 8% moisture.

Reaction rates at $T \geqslant T_g$ were plotted according to the WLF equation for 5 and 8% moisture samples (Fig. 5c). The 5% moisture sample was stored at its T_g value of 40°C and therefore R_g was determined directly. The 8% moisture formulation was not stored at its T_g and R_g values were extrapolated from the other data. The aggregation rate was assumed to be equal to the rate for the 2 and 5% moisture samples at 25°C, and the Asp isomerization rate was read from the Arrhenius plot of the 8% moisture data. Although the data available at $T \geqslant T_g$ is limited, it appears that both aggregate formation and Asp isomerization follow WLF kinetics, suggesting that storage stability is associated with the glass transition. Roy *et al.* (18) reported that a single constant, k = 0.134, described three degradation pathways (aggregation, hydrolysis of hydrazone linkage, and vinca decomposition) in their stability assessment of an antibody-vinca conjugate. This k is the slope of the WLF best-fit straight line and equals C_1/C_2. In our study, chemical and physical stability were described by two separate equations when fitted to the WLF model. For aggregate formation, k = 0.14, close to the value reported by Roy *et al.,* whereas for Asp isomerization, k = 0.08. This indicates that the WLF constants were not characteristic of the solid system but rather were dependent on the nature of the degradation pathway and that physical stability had a stronger dependence on $(T-T_g)$ than did chemical stability.

CONCLUSIONS

Increasing moisture in the lyophilized cakes decreased T_g values and MDSC was necessary to measure the T_g values of the formulations. The small changes in heat capacity at the T_g are indicative of a strong glass and may account for the absence of product shrinkage during prolonged storage above the T_g value. Soluble aggregate formation and isomerization of aspartic acid were the two main degradation routes for the antibody. Insoluble aggregates and low molecular weight species formed at 50°C only. All samples were stable at 5°C. Small increases in residual moisture appeared to increase the physical stability of the protein during high temperature storage with little effect on chemical stability. Larger amounts of residual moisture decreased physical stability during high temperature storage only by lowering the T_g value of the

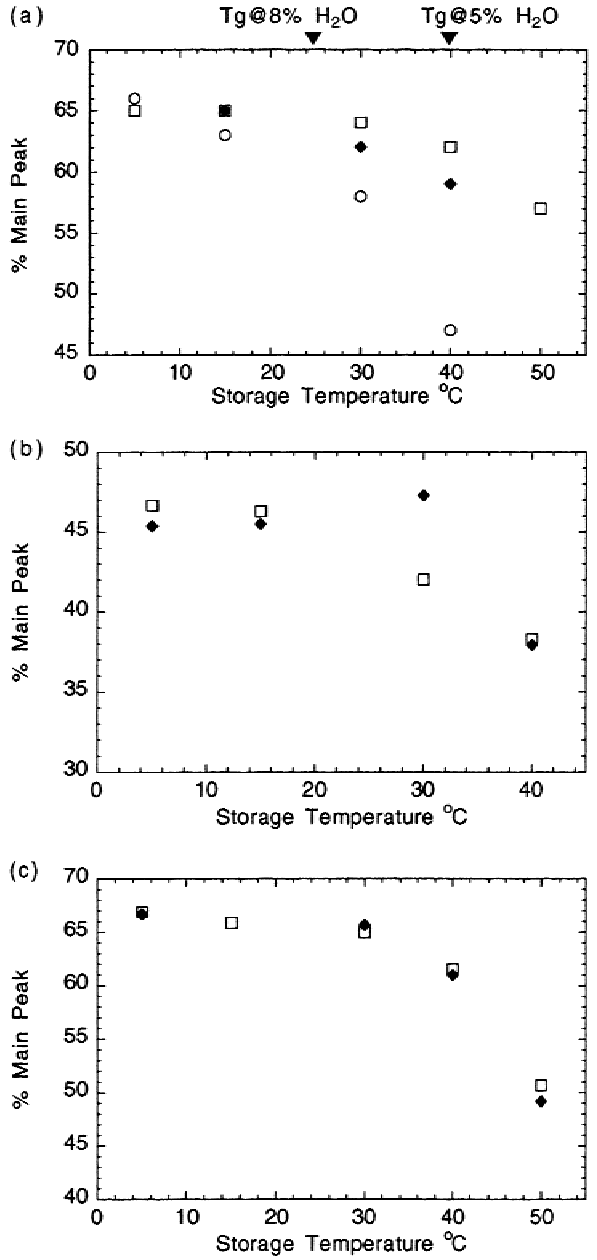

Fig. 6. Percentage HIC main peak in (a) papain digest of study 1 cakes at 6 months at 2.3% (□), 5.4% (◆), and 8.5% moisture (○); (b) pepsin digest of study 2 cakes at 12 months at 1.4% (□) and 3.0% (◆) moisture; and (c) papain digest of study 3 cakes at 12 months at 1.1% (□) and 2.4% (◆) moisture. Study 1 samples were stored above and below their T_g values, all other samples were stored below their T_g values.

formulation. Below their T_g values, high moisture levels did not have a detrimental effect on physical stability. In contrast, high residual moisture levels decreased the chemical stability of the protein at elevated temperatures both above and below the T_g values. Asp isomerization is a hydrolytic reaction, and therefore it is not surprising that the rate of the reaction increased with increasing residual moisture content even in the glassy state.

This study indicates that both the water substitution and the vitrification hypotheses play a role in protein stability in the solid state and that stability is not only dependent on the T_g value. Residual moisture should be optimized during formulation development and a careful balance achieved between drying sufficiently to maintain the formulation in a glassy state while not overdrying to the detriment of protein stability. Both physical and chemical pathways of degradation should be considered when determining the optimum moisture level. It may be beneficial to consider residual moisture a formulation excipient rather than as something that simply has to be minimized.

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