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Mechanisms of Aggregate Formation and Carbohydrate Excipient Stabilization of Lyophilized Humanized Monoclonal Antibody Formulations

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ABSTRACT

The purpose of this study was to evaluate the mechanisms of aggregate formation and excipient stabilization in freeze-dried formulations of a recombinant humanized monoclonal antibody. Protein degradation was measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and native size exclusion chromatography, and protein structure was studied using Fourier transform-infrared spectrometry and circular dichroism. The results showed that protein aggregates present following reconstitution were composed of native antibody structure and a reduced amount of free thiol when compared to protein monomer, which implied that intermolecular disulfides were involved in the aggregation mechanism. An excipient-free formulation resulted in reversible solid-state protein structural alteration and increased aggregation during storage. This correlated with dehydration to an extent that the amount of water was less than the estimated number of surface-accessible hydrogen-bonding sites on the protein. Improved native-like solid-state protein structure and reduced aggregation were obtained by formulation with enough carbohydrate to fulfill the hydrogen-bonding sites on the surface of the protein. Carbohydrate in excess of this concentration has less of an influence on protein aggregation. Reduced aggregation during storage was obtained by the addition of sufficient excipient to both stabilize solid-state protein structure and provide an environment that consisted of an amorphous glassy state matrix.

KEYWORDS: protein formulation, protein stability, recombinant humanized monoclonal antibody, aggregation, FT-IR spectrometry

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INTRODUCTION

Proteins are subject to structural modification by a variety of degradation mechanisms that have been classified in terms of chemical and physical instability.[1,2] The presence of water either as a reactant or as a solvent that facilitates the transfer of reactants is critical to many routes of chemical degradation that lead to protein instability. Therefore, the exclusion of water from the environment surrounding a protein may provide resistance to chemical modification of protein structure during storage, and processes such as freeze- and spray-drying have been employed to prepare solid-state dosage forms of pharmaceutical proteins that have vastly reduced amounts of water.[3,4] However, water also plays a critical role in the formation of native protein structure. For instance, in an aqueous environment the forces of hydrophobic interactions and hydrogen bonding determine protein folding.[5,6] The dominant force is the hydrophobic effect—the fact that during folding, nonpolar amino acids are driven to the core of the molecule to avoid solvent contact and, conversely, residues that contain polar side chains are predominately found on the surface. Hydrogen bonds of water and these surface-accessible polar sites is a significant additional cumulative force that contributes to the stabilization of native protein structure. Hence a solid-state formulation with a vastly reduced amount of water may be an environment that promotes protein structural alteration. This can lead to physical instability that is often manifested as the formation of insoluble or soluble protein aggregates.[7]

Formulation with carbohydrate excipients, such as sucrose and trehalose, has proven to be effective in the stabilization of freeze-dried proteins.[8,9] Under conditions of dehydration these excipients result in a solid-state environment that, below the glass transition temperature (T_g), is both amorphous and characterized by extremely high viscosity.[10] Dilution and limited molecular mobility of proteins suspended in this glassy state matrix reduce protein-protein contacts that may initiate aggregation. Carbohydrate also contains multiple hydroxyl groups that may in an amorphous solid-state environment form hydrogen bonds with polar groups on a protein surface.

Therefore, when water is removed during dehydration, these excipients may substitute for water to maintain hydrogen bonds with surface-accessible polar sites on the protein.[11] This interaction facilitates the preservation of native-like solid-state protein structure and may result in a reduced propensity of the protein molecules to form aggregates during storage.[12]

Recombinant humanized monoclonal antibodies (rhuMAb) prepared by grafting antigen-specific binding residues (i.e., complementarity-determining regions) into the framework residues of a human IgG1-class antibody have emerged as important therapeutic proteins.[13-15] Previous investigations of the stability of solid-state rhuMAb dosage forms have shown that soluble protein aggregation is a significant degradation that may be effectively minimized by formulation with carbohydrates such as sucrose and trehalose.[16-18] Recently, the stabilization influence of sucrose in freeze-dried rhuMAb formulations has been thoroughly evaluated in terms of residual moisture, chemical stability, and T_g.[19] However, we were also interested in exploring the physical stability of the protein and the relationship between solid-state protein structure and mechanisms of both aggregate formation and excipient stabilization in freeze-dried rhuMAb formulations prepared with sucrose and trehalose.

MATERIALS AND METHODS

Materials

Purified rhuMAb was produced by Genentech, Inc. (South San Francisco, CA). Formulation buffers and chromatography mobile phase were prepared by mixing the appropriate amount of buffer and excipient with Milli-Q (Milli-Por, Billerica, MA) water in a volumetric flask.

Protein Concentration

The concentration of rhuMAb was determined by optical density (OD) measured from 240 to 400 nm relative to a water blank in a 1-cm cuvette, as described previously.[17]

Formulation

Formulations of rhuMAb were prepared by dialysis into either water (excipient-free) or buffer at pH 6 containing sucrose or trehalose. Samples were diluted to the proper protein concentration and sterile-filtered with a 0.22-μm filter prior to filling into glass vials.

Freeze-Drying

Vials were loaded into a GT-20D lyophilizer (Leybold-Heraeus Vacuum Products, Monroeville, PA) at a shelf temperature of 5°C and frozen to -55°C by reducing the temperature at a rate of 10°C/hour. The vial contents were kept frozen at a temperature of -55°C under ambient pressure for 4 hours. Primary drying was then carried out in 3 temperature steps. The first step was incubation for 40 hours at a shelf temperature of -30°C and a chamber vacuum pressure of 100 mTorr. The second and third steps were conducted at a shelf temperature of -20 and -10°C, respectively, for 10 hours each under a chamber vacuum pressure of 100 mTorr. Secondary drying was carried out for 10 hours at 5°C under a chamber vacuum pressure of 100 mTorr. The vials were sealed under 760 ± 50 mbar of nitrogen at the end of the cycle.

Residual Moisture

Residual moisture content of the lyophilized cakes was measured using the Karl Fisher titration method.[20] A minimum of 75 mg of pulverized cake was dispersed into an anhydrous methanol bath and titrated with Riedel-deHaen Hydranal-Composite 2 reagent (Hoechst Celanese, Sumerville, NJ).

Chromatography

Soluble protein aggregate was determined by analytical native size exclusion chromatography (SEC) performed with a model 1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA) using a TSK-gel G3000SWXL column (TosoHass, Montgomeryville, PA) run in phosphate buffered saline (PBS) at a flow rate of 0.5 mL/min. The column was loaded with 50 μg of antibody, and protein was detected by monitoring the OD at 280 nm.

Protein aggregates were also fractionated by preparative SEC using Sephacryl S-200 High Resolution resin (Amersham Biosciences, Piscataway, NJ) packed in a 2.5 cm (diameter) x 60 cm (length) column run in PBS at a flow rate of 0.3 mL/min. The column was loaded with 50 mg of antibody, and protein was detected by monitoring the OD at 280 nm.

Light Scattering

The solution weight average molecular weight (MW_{AV}) was determined by size exclusion chromatography-light scattering (SEC-LS) using on-line MiniDAWN LS and

OptiLab DSP refractive index detectors (Wyatt Technology, Santa Barbara, CA) as described previously.[17]

Protein Conformation

Circular dichroism was measured using a model AVIV 62 DS spectropolarimeter (Protein Solutions, Lakewood, NJ). Buffer and protein samples were analyzed at 20°C with an interval of 0.5 nm, a bandwidth of 1 nm, and an averaging time of 5 seconds. Measurements were obtained in the far- and near-ultraviolet (UV) regions using 0.01- and 1.0-cm path-length quartz cuvettes, respectively. Background spectra were subtracted from that of protein, and the mean residual ellipticity was calculated using a mean residual weight for the antibody of 110.6.

Fourier transform-infrared (FT-IR) spectrometry spectra were collected using a Galaxy 5000 Series FT-IR spectrometer (Thermo Nicolet, Madison, WI) equipped with a narrow-range mercury cadmium telluride quantum detector. The instrument was purged with nitrogen, and single-sided interferograms were measured without apodization. Absorbance was measured at a resolution of 4 cm^{-1}, and a total of 1000 scans were co-added. Spectral noise was removed by smoothing using a 7-point function, and the absorbance was deconvoluted by the calculation of the second derivative using Grams/32 Spectral Notebase software (Thermo Galactic, Salem, NH). Absorbance of aqueous protein was measured using a liquid cell holder, CaF_2 windows, and a 6-µm Mylar spacer (Thermo Nicolet, Madison, WI). The absorbance of water was subtracted from that of protein using the criteria of a straight baseline from 2000 to 1720 cm^{-1} and no negative absorbance peaks.[21] Solid-state protein spectra were measured using pressed potassium bromide discs containing 0.2% protein as described previously.[19]

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was run under reducing and nonreducing conditions on 4% to 15% gradient tris glycine gels as previously described.[17]

Free Thiol Assay

Antibody samples adjusted to pH 8 by dilution with 0.1M phosphate, 1mM EDTA, pH 8 buffer, were mixed with Ellman's reagent and incubated at room temperature for 15 minutes. The absorbance at 412 nm was determined and the concentration of free thiols was calculated using an absorptivity of 14150 as described previously.[22]

T_g

T_g of the carbohydrate-containing freeze-dried antibody formulations was measured by heating approximately 5 mg of freeze-dried powder in a sealed sample tray using a DSC120 differential scanning calorimeter (SII Seiko Instruments, Austin, TX). Samples were equilibrated to 10°C and heated to 150°C at a rate of 1°C/min, and the transition temperatures were determined using the instrument software as previously described.[19]

RESULTS

T_g and Residual Moisture

In this study, freeze-dried rhuMAb formulations prepared both excipient-free and with increasing concentrations of the disaccharides sucrose and trehalose were evaluated. The residual moisture and T_g of the various formulations tested are shown in **Table 1**. Excipient-free rhuMAb contained a residual moisture level of about 5%, whereas the residual moisture of each of the carbohydrate-containing formulations was about 3%. Using conventional DSC, the measured T_g of the sucrose and trehalose formulations was approximately 60 and 80°C, respectively. As previously reported, the T_g could not be measured in formulations prepared excipient-free or with an amount of carbohydrate that was less than the molar ratio of 500:1 excipient:protein.[19]

Characterization of rhuMAb Aggregate

The formation of soluble protein aggregates during storage at a temperature below the T_g of the formulations was evaluated by SEC-LS and SDS PAGE. The chromatography of freeze-dried rhuMAb that was formulated excipient-free and stored for 1 year at 30°C is shown in **Figure 1**. Three peaks were resolved, and the solution MW_{AV} was determined by on-line LS (**Table 2**). The MW_{AV} of peak 1 was approximately 150 kd, which corresponded to the MW of antibody monomer that was calculated from the amino acid sequence and carbohydrate composition.[17] Using a similar comparison, peaks 2 and 3 were identified as rhuMAb dimers and trimers. Furthermore, an SEC-LS analysis of freeze-dried rhuMAb that was formulated with sucrose at a molar concentration of 500:1 excipient:protein and stored for 1 year at 30°C also resulted in the resolution of peaks that corre-

Table 1. The Residual Moisture and T_g of Freeze-Dried rhuMAb Formulations*

Formulation	Excipient Concentration (mol:mol)†	Residual Moisture (%), n = 3	T_g (°C)
Excipient-free	—	5.1 ± 0.1	—
Sucrose	260:1	3.2 ± 0.2	—
Sucrose	380:1	3.1 ± 0.2	—
Sucrose	500:1	3.4 ± 0.1	58
Sucrose	1000:1	2.9 ± 0.1	58
Sucrose	1500:1	3.0 ± 0.1	59
Sucrose	2000:1	3.1 ± 0.1	62
Trehalose	260:1	3.2 ± 0.2	—
Trehalose	380:1	3.2 ± 0.2	—
Trehalose	500:1	3.5 ± 0.4	78
Trehalose	1000:1	2.9 ± 0.5	78
Trehalose	1500:1	3.2 ± 0.1	82
Trehalose	2000:1	3.1 ± 0.1	81

*T_g indicates glass transition temperature; rhuMAb, recombinant humanized monoclonal antibodies.
†Molar ratio of carbohydrate to protein.

sponded to antibody monomer and dimer (**Table 2**). Similarly characterized aggregates were observed during storage of the antibody formulated with trehalose and in samples stored at lower temperatures (data not shown).

An IgG1-class antibody is a complex protein that is composed of 2 heavy and 2 light chains and contains 12 intra- and 4 interchain disulfides. The involvement of intermolecular disulfides in the linkage of protein aggregates was studied by SDS PAGE and measurement of protein free thiols. For example, an SDS PAGE analysis of the excipient-free and sucrose-containing freeze-dried rhuMAb samples that had been stored for 1 year at 30°C is shown in **Figure 2**. Aggregates corresponding to protein dimer and trimer in the excipient-free formulation and dimer in the sucrose-containing formulation were observed in the gel run under nonreducing conditions. However, the same samples run under reducing conditions resulted in bands that corresponded to antibody heavy and light chains. Therefore, the protein aggregates, like the heavy and light chains of an intact rhuMAb, were linked by intermolecular disulfides. Further evidence of this conclusion was obtained by an analysis of free thiol following the separation of rhuMAb aggregate and monomer using preparative SEC. As shown in **Table 2**, protein monomer contained free thiol at a molar ratio of 1:1 free thiol:protein, which was in agreement with the amount of free thiol measured in aqueous protein that had not undergone lyophilization. However, the measured amount of free thiol decreased significantly in the rhuMAb aggregate peaks, which was consistent with intermolecular thiol linkage as the mechanism of protein aggregation.

Aggregation Kinetics

Soluble protein aggregation observed during storage at 30°C of the excipient-free and carbohydrate-containing formulations was modeled as a pseudo first-order reaction, and rate constants for the loss in rhuMAb monomer were calculated as previously described.[17] The effect of carbohydrate excipient concentration on soluble protein

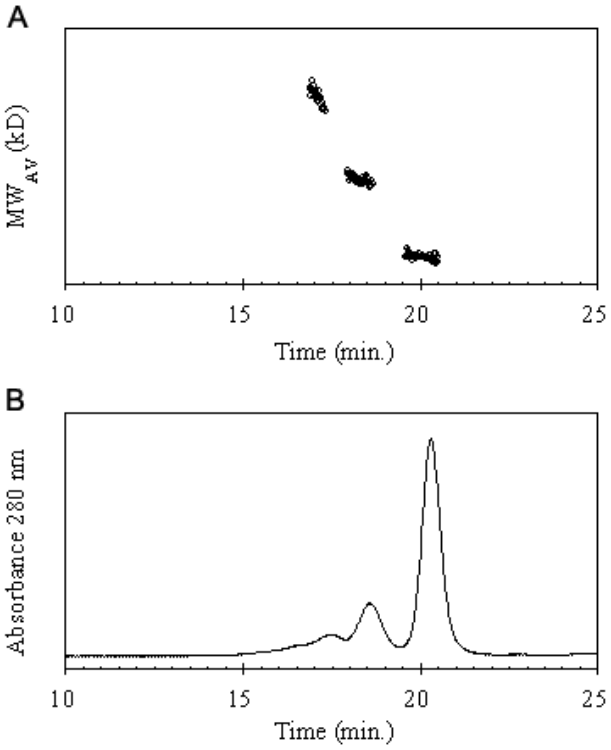

Figure 1. SEC-LS of excipient-free freeze-dried rhuMAb that had been stored for 1 year at 30°C: (A) MW_{AV}; (B) column elution monitored by the absorbance at 280 nm.

aggregation kinetics after storage for 1 year at 30°C is shown in **Figure 3**. A similar trend of reduced aggregation was observed as the amount of either sucrose or trehalose was increased from 0 to a molar ratio of approximately 500:1 excipient:protein. Formulation with carbohydrate in excess of this concentration had less of an influence on antibody aggregation, as evidenced by the reduced slope in the plot of observed rate constant versus excipient concentration. Moreover, in all the formulations, reduced rates of aggregation were obtained by reduced storage temperature, but a similar trend of the effect of carbohydrate on aggregation was observed (data not shown).

Solid-State Protein Structure

Aqueous and solid-state protein secondary structure was evaluated using FT-IR spectrometry. In this analysis IR absorbance in the amide I region was assessed qualitatively following deconvolution using the second derivative, an analysis technique that has the advantage of being nonsubjective.[7] A representation of native antibody structure was obtained from the absorbance of excipient-free aqueous protein that had not undergone lyophilization (**Figure 4A**). IR bands were assigned to protein secondary structure by comparison to previously published IgG antibody spectra.[23,24] For example, bands that were characteristic of beta-sheet structure occurred at frequencies of 1692 and 1640 cm^{-1}, and turn structure was represented by the well-resolved bands at frequencies of 1676 and 1662 cm^{-1}. The IR band at 1618 cm^{-1} was indicative of more beta-sheet structure or was from the contribution of amino acid side chains.

A comparison of the FT-IR absorbance of excipient-free solid-state and aqueous rhuMAb showed the occurrence of protein secondary structural alteration following freeze-drying (**Figure 4A**). In the solid-state formulation, modification of beta-sheet structure was observed as a decreased intensity in the IR band at 1640 cm^{-1} and a broadening and shifting of the band at 1690 cm^{-1} to a frequency of 1694 cm^{-1}. Other structural changes included the broadening of the bands that represented rhuMAb turn structure and the appearance of a broad band in the range of 1650 cm^{-1} that may indicate protein unfolding to unordered structure.[25]

A more native-like solid-state rhuMAb structure was obtained by formulation with sucrose or trehalose (**Figure 4B**, **4C**). A comparison of solid-state rhuMAb structure in the carbohydrate-containing formulations to that of aqueous protein showed similar beta-sheet structure and no evidence of unordered structure following freeze-drying. Moreover, native-like turn structure was obtained depending on the formulation. For example, formulation with sucrose or trehalose at a concentration that was equal to or greater than a molar ratio of 500:1 excipient:protein resulted in improved resolution of the 2 peaks that were indicative of turn structure.

The effect of storage on solid-state rhuMAb conformation was determined with protein formulated excipient-free and with sucrose at a molar ratio of 500:1 excipient:protein. FT-IR absorbance shows that the solid-state rhuMAb secondary structure of both formulations did not change following storage at 30°C for 1 year, a condition where, in the case of excipient-free formulation, considerable soluble aggregate occurred (**Figure 5**).

Reconstituted Protein Structure

The effect of reconstitution on protein conformation was determined by both FT-IR absorbance and circular dichroism of rhuMAb formulated excipient-free and with sucrose at a molar ratio of 500:1 excipient:protein. The FT-IR absorbance of the samples following reconstitution is shown in **Figure 6**. The spectra of both formula-

Table 2. Characterization of Excipient-Free and Sucrose-Containing Freeze-Dried rhuMAb That Has Been Stored at 30°C for 1 Year*

Formulation	SEC Peak†	MW_{AV}† (kd)	Identification‡	Free Thiol:Protein§ (mol:mol)
Excipient-free	1	151 ± 20	Monomer	1
	2	297 ± 33	Dimer	< 0.1
	3	469 ± 49	Trimer	< 0.1
Sucrose‖	1	151 ± 20	Monomer	1
	2	290 ± 31	Dimer	< 0.1

*rhuMAb indicates recombinant humanized monoclonal antibodies; SEC, size exclusion chromatography; MW_{AV} = average molecular weight; SEC-LS, size exclusion chromatography–light scattering.

†Peak number and MW_{AV} determined by SEC-LS (Figure 1).

‡Protein identification by comparing the MW_{AV} with the MW calculated from the amino acid sequence.

§Free thiol concentration measured from protein that eluted from the SEC column. Each sample contained greater than 90% of the identified peak.

‖Formulation prepared with sucrose at a molar concentration of 500:1 excipient:protein.

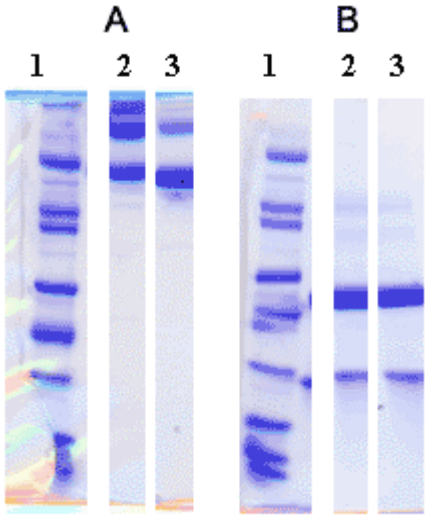

Figure 2. SDS PAGE of freeze-dried rhuMAb run under (A) nonreducing and (B) reducing conditions. Samples consists of (Lane 1) molecular weight standards and freeze-dried rhuMAb formulated (Lane 2) excipient-free and (Lane 3) with sucrose at a molar ratio of 500:1 excipient:protein. Freeze-dried rhuMAb samples were stored at 30°C for 1 year prior to the analysis.

tions were similar to that of aqueous protein that had not undergone lyophilization (**Figure 6A**). A comparison of the same samples by far- and near-UV circular dichroism also resulted in similar spectra (**Figure 6B**, **6C**). This analysis suggested that rhuMAb secondary structure was well conserved following reconstitution and that the previously observed structural alteration of solid-state excipient-free protein was reversible following rehydration.

Although the combination of FT-IR spectrometry and circular dichroism spectroscopy improves the level of confidence in an analysis of protein structure, these techniques can offer only a global estimation of conformation.[26] This limitation makes analysis of a large protein, such as a rhuMAb, especially difficult, as small changes in structure in a subpopulation of molecules may influence stability and yet remain undetected. Improved sensitivity may be obtained by a direct analysis of degraded protein. Therefore, preparative SEC was used to isolate soluble protein aggregates that were formed during storage of the excipient-free freeze-dried rhuMAb. An analysis of the SEC fractions that contained increased amounts of aggregate by far- and near-UV circular dichroism still showed no evidence of protein structural alteration (**Figure 7**). Similar results were observed following a collection of aggregate generated during storage of freeze-dried rhuMAb formulated with sucrose at a molar ratio of 500:1 excipient:protein (data not shown). Therefore, it was likely that in all the formulations tested, pro-

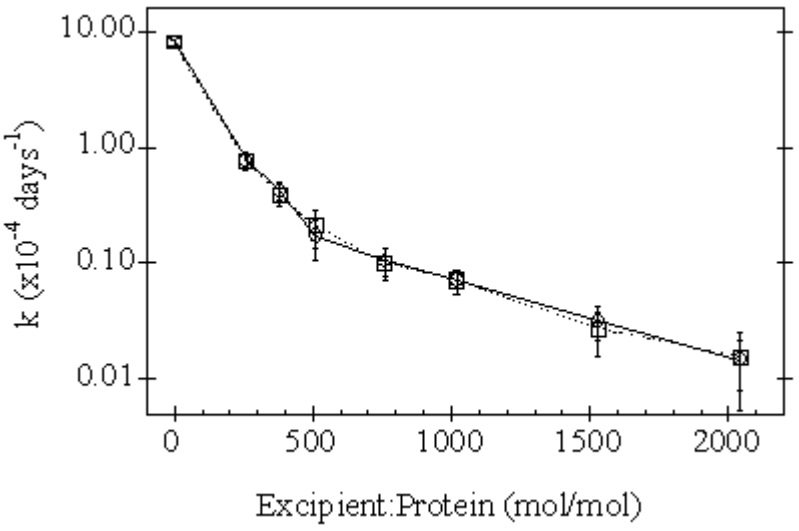

Figure 3. Soluble aggregation kinetics of freeze-dried rhuMAb formulated excipient-free and with various concentrations of (open circles) sucrose and (open squares) trehalose. Pseudo first-order rate constants were determined from samples stored at 30°C for up to 1 year.

tein structure was well conserved following lyophilization, storage, and reconstitution.

DISCUSSION

Water titration of dry protein has shown that at the initial stage of hydration a protein surface contains from 0% to 7% water that is preferentially bound to charged sites on the surface.[27] Solid-state excipient-free freeze-dried antibody contained residual water in this range, and in this environment water-protein interaction probably also occurred with surface-accessible charged groups (**Table 1**). Based on Green and Pauling's hypothesis, these water-binding sites on a protein consist of the side chains of polar amino acids, the C terminus, and carbohydrate.[28]

By simply using the protein primary structure and carbohydrate composition, one can calculate the number of water-binding sites. Moreover, this estimate may be refined by taking into account residue side chain solvent exposure and the number of hydrogen-bonding sites. For example, an estimate of the solvent exposure of the polar residues in protein structure has been determined from the application of a theoretical solvent probe to the highly resolved x-ray crystallography structure of 50 different water-soluble proteins.[29] Furthermore, the polar side chains of Asp, Asn, Glu, Gln, and His amino acids contain 2 hydrogen-bonding sites, whereas the side chains of Thr, Ser, Tyr, Lys, and Arg residues contain a single site.[6] The application of these data to rhuMAb primary structure and carbohydrate composition resulted in an estimation of approximately 550 water-binding sites on the surface of the protein. The total amount of water required to interact with these sites was 6.7%; this result was in agreement with the water monolayer (ie, tightly bound water) of excipient-free rhuMAb measured by sorption isotherms and calculated using the Brunauer-Emmett-Teller equation.[30]

A comparison of the estimated water monolayer with the measured residual moisture showed that excipient-free freeze-dried rhuMAb contained approximately 25% fewer water molecules than required to interact with surface-accessible protein hydrogen-binding sites. As previously discussed, the uncovering of charged sites by dehydration was likely responsible for the observed reversible alteration in solid-state protein structure and the related instability during storage of this sample.[11] Similar results were also observed when freeze-dried excipient-free tissue type plasminogen activator was prepared at a residual moisture level that was below the estimated water monolayer.[3]

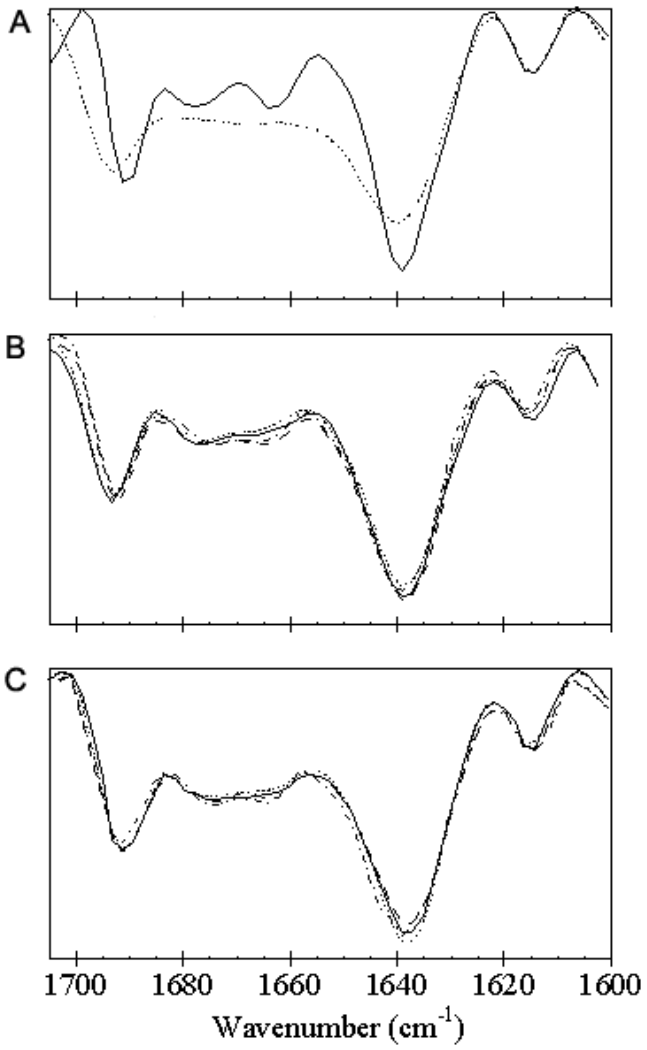

Figure 4. The effect of freeze-drying on rhuMAb secondary structure. Second-derivative FT-IR absorbance spectra are shown from an analysis of (a) excipient-free (——) aqueous and (-----) freeze-dried rhuMAb as well as freeze-dried protein formulated with (b) sucrose and (c) trehalose at a molar ratio of (——) 260:1, (-----) 384:1, ( ) 500:1, and (— - - - —) 1000:1 excipient:protein.

The water substitution hypothesis suggests that, in an amorphous solid-state formulation, protein water-binding sites may hydrogen-bond with either excipient or water.[11] The addition of carbohydrate to the rhuMAb formulation prior to freeze-drying resulted in reduced residual moisture when compared to the excipient-free formulation, which was evidence of the displacement of protein-bound water by excipient (**Table 1**). Furthermore, in each of the formulations prepared with sucrose or trehalose, the combined molar amount of hydrogen-bonding excipients (i.e., water or carbohydrate) exceeded the estimated number of rhuMAb surface-accessible hydrogen-bonding sites, and this correlated with improved native-like solid-state protein conformation. Therefore, as predicted by the water substitution hypothesis, maintaining a critical amount of excipient for interaction with surface-accessible charged sites was a key factor in the stabilization of solid-state rhuMAb structure.

Soluble aggregates may be formed by mechanisms that involve either covalent or non-covalent linkage of protein monomers. Because denaturation can expose hydrophobic regions that more readily associate following protein-protein contact, the formation of non-covalently linked aggregate is often the result of protein structural alteration.[31] Conversely, the formation of covalently linked aggregates may or may not involve a change in protein structure. For instance, a properly folded protein that contains a noncoupled thiol may form aggregates by intermolecular thiol exchange.[8] On the other hand, protein structural alteration may result in disulfide bond breakage, and the resulting free thiol may participate in the disulfide exchange reaction. In the case of freeze-dried rhuMAb, the aggregates that occurred during storage were characterized as native protein molecules that were linked by intermolecular disulfides. As discussed above, the preparation of excipient-free formulation caused significant structural alteration in the solid state. This structural change probably facilitated the association of conformationally altered protein during storage. Although reconstitution resulted in the protein reassuming native structure, the aggregates were likely stabilized during rehydration by the intermolecular exchange of disulfides that were placed into close proximity in the solid state. Formulation with carbohydrate resulted in improved native-like solid-state protein structure, which may have reduced the propensity of antibody monomers to self-associate and form disulfide-linked aggregates during storage.

Improved solid-state protein structure was not the only factor involved in the stabilization of the antibody. This was evidenced by the fact that formulation with excipient at a concentration that exceeded that required to stabilize protein structure still resulted in reduced aggregation during storage (**Figure 1**). In these formulations carbohydrate that was not involved in interaction with the protein probably contributed to the further dilution of protein in the amorphous glassy state matrix that is characterized by high viscosity and restricted molecular mobility. This environment would reduce protein-protein contacts, which could lead to the formation of a disulfide-linked aggregate. A more complete analysis of the influence of T_g on rhuMAb stability is presented elsewhere.[19]

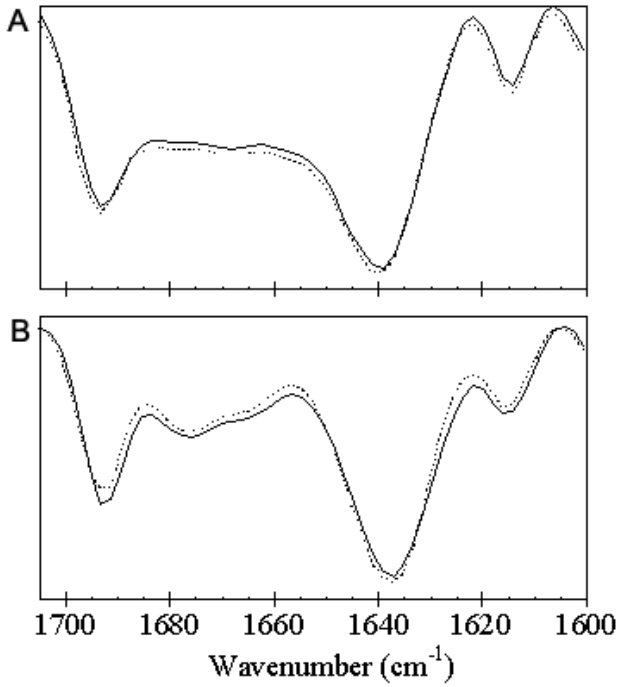

Figure 5. The effect of storage on solid-state rhuMAb structure. Second-derivative FT-IR absorbance spectra of freeze-dried protein formulated (a) excipient-free and (b) with sucrose at a molar ratio of 500:1 excipient:protein were analyzed (——) after freeze-drying and (-----) following storage at 30°C for 1 year.

CONCLUSION

Protein aggregates observed following the storage and reconstitution of freeze-dried rhuMAb formulations consisted of antibody dimers and trimers that were covalently linked by intermolecular disulfides. The preparation of freeze-dried rhuMAb formulated without excipients resulted in reversible solid-state protein structural alteration. This was likely caused by the removal of tightly bound water from the protein surface by excessive dehydration and correlated with increased rates of formation of the protein aggregates during storage. The addition of the carbohydrate excipients sucrose or trehalose to the formulation provided a solid-state environment where complete coverage of protein surface-accessible hydrogen binding sites was achieved. This correlated with improved native-like solid-state protein structure and reduced protein aggregation during storage.

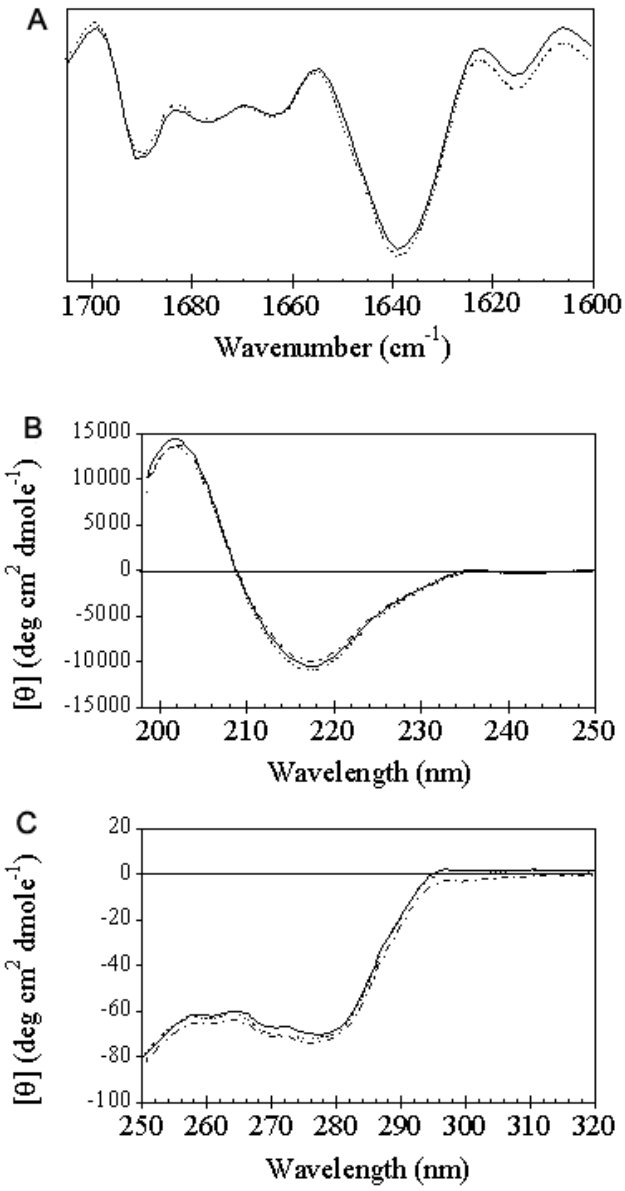

Figure 6. The effect of reconstitution on rhuMAb structure as determined by (a) second-derivative FT-IR absorbance and circular dichroism in the (b) far- and (c) near-UV regions. Samples analyzed include (— - - - - —) excipient-free antibody that had not undergone lyophilization, and freeze-dried and reconstituted rhuMAb formulated (——) excipient-free and (-----) with sucrose at a molar ratio of 500:1 excipient:protein.

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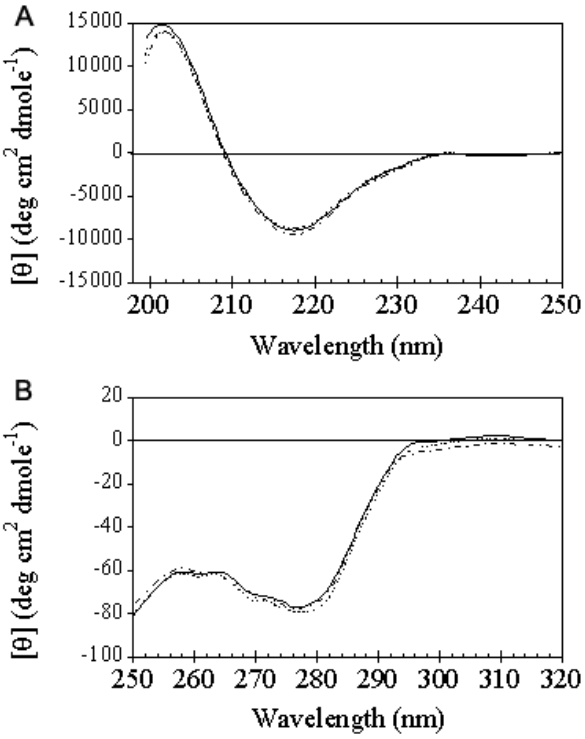

Figure 7. Circular dichroism of freeze-dried excipient-free antibody that has been stored at 30°C for 1 year and reconstituted. The rhuMAb sample was fractionated by SEC (Figure 1), and circular dichroism spectra in the (a) far- and (b) near-UV region are shown from an analysis of rhuMAb monomer (——) peak 1 as well as soluble aggregate samples (-----) peak 2 and (— - —) peak 3.

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