The Effect of Formulation Excipients on Protein Stability and Aerosol Performance of Spray-Dried Powders of a Recombinant Humanized Anti-IgE Monoclonal Antibody[1]

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Received November 19, 1998; accepted December 9, 1998

Purpose. To study the effect of trehalose, lactose, and mannitol on the biochemical stability and aerosol performance of spray-dried powders of an anti-IgE humanized monoclonal antibody.

Methods. Protein aggregation of spray-dried powders stored at various temperature and relative humidity conditions was assayed by size exclusion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein glycation was determined by isoelectric focusing and affinity chromatography. Crystallization was examined by X-ray powder diffraction. Aerosol performance was assessed as the fine particle fraction (FPF) of the powders blended with coarse carrier lactose, and was determined using a multiple stage liquid impinger.

Results. Soluble protein aggregation consisting of non-covalent and disulfide-linked covalent dimers and trimers occurred during storage. Aggregate was minimized by formulation with trehalose at or above a molar ratio in the range of 300:1 to 500:1 (excipient:protein). However, the powders were excessively cohesive and unsuitable for aerosol administration. Lactose had a similar stabilizing effect, and the powders exhibited acceptable aerosol performance, but protein glycation was observed during storage. The addition of mannitol also reduced aggregation, while maintaining the FPF, but only up to a molar ratio of 200:1. Further increased mannitol resulted in crystallization, which had a detrimental effect on protein stability and aerosol performance.

Conclusions. Protein stability was improved by formulation with carbohydrate. However, a balance must be achieved between the addition of enough stabilizer to improve protein biochemical stability without compromising blended powder aerosol performance.

KEY WORDS: aggregation; glycation; fine particle fraction; protein formulation; protein stability; spray drying.

INTRODUCTION

The recombinant humanized monoclonal antibody anti-IgE (rhuMAbE25) is targeted to a region in the F_c portion of human IgE (1). Binding blocks IgE interaction with high-affinity F_c receptors located on the surface of mast cells and basophils, thus removing IgE as a mediator of allergic disease (2). When delivered systemically, rhuMAbE25 has therapeutic value for the treatment of a subgroup of patients with allergic asthma (3,4). However, because asthma is a pulmonary disease, we explored direct airway administration via a dry powder inhaler.

Spray drying is a manufacturing process that yields powders that are small enough for aerosol delivery to the lower airways of the lung (5). This process consists of liquid atomization into a fine mist followed by rapid hot air drying (6). Therefore, the stability of proteins prepared by spray drying may be influenced by denaturation at the gas-liquid interface, thermal stress, and the stress of long-term storage in the dried state. For instance, spray drying of a model protein, β-galactosidase, resulted in soluble and insoluble protein aggregation following spray drying and storage of the powder (7). The application of spray drying to the production of protein pharmaceuticals has been reported with recombinant tissue-type plasminogen activator, human growth hormone, and human deoxyribonuclease (8–10). In each case, soluble or insoluble protein aggregation was observed. While formulation additives such as carbohydrates may be employed to improve both the thermal and dehydration stability of proteins (11), such excipients must not interfere with the aerosol performance of a powder intended for pulmonary delivery.

The cohesive nature of small inhalation powders may result in poor flow characteristics, which are problematic in powder manufacturing and filling processes. To improve handling, powders are typically non-covalently attached to a larger carrier powder (usually lactose) by a blending process, and for efficient lung deposition the small powders must be dispersed from the carrier by shear forces during inhalation (9). Aerosol performance may be evaluated by dispersion of the powders using a commercially available inhalation device and measurement of the resultant particle size distribution by a multiple stage liquid impinger (MSLI). Although other methods such as laser diffraction and dynamic light scattering are available to analyze particle size, the MSLI offers the advantage of determining the aerodynamic particle size, which is directly relevant to the behavior of particles during inhalation. Furthermore, the MSLI results in a quantitative measurement of the amount of drug associated with particles that fall within a specific size range, which is of primary importance in dose estimation. It has been established that powders with a size of less than 7 μm have the best potential to deposit in the deep regions of the lung, and this size range is typically referred to as the fine particle fraction (FPF, (9)). For anti-IgE, the FPF was defined as the amount of protein associated with aerosolized particles within the size range of 1 to 6 μm as a fraction of the total protein loaded into the inhalation device, and represents the amount of drug deposited in the deep regions of the lung. The specification of a pharmaceutically acceptable level of FPF depends on many factors such as the manufacturing cost, required dose and bioavailability of the drug, drug loading in the powder formulation, and administration frequency. In consideration of these factors, a FPF of 20 to 30% was acceptable for pulmonary delivered anti-IgE.

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0724-8741/99/0300-0350$16.00/0

Here we report on the effect of various sugar and polyol excipients on the protein stability of spray-dried rhuMAbE25 and aerosol performance of powders blended with coarse carrier lactose. Soluble aggregation was the primary protein degradation pathway observed during powder storage, and protein stability was ameliorated by the addition of excipients to the formulation. However, in some cases increased excipient concentration adversely affected the FPF of the blended powders. Therefore, a balance must be achieved between the addition of sufficient stabilizer to obtain improved protein stability, while maintaining the aerosol performance of the blended powder (12).

MATERIALS AND METHODS

Materials

A recombinant humanized anti-IgE monoclonal antibody, rhuMAbE25, was produced by Genentech Inc. (South San Francisco, CA) as described previously (2). Analytical grade lactose was obtained from Mallinckrodt (Paris, KY). Trehalose and D-mannitol were obtained from Sigma (St. Louis, MO).

Powder Preparation

Formulation

Formulations were prepared by exhaustive dialysis of rhuMAbE25 into water at a protein concentration of greater than 10 mg/mL. Carbohydrate was added, and a final protein concentration of 10 mg/mL was obtained by dilution. Formulations were sterile filtered with a 0.22 μm filter unit (Nalgene) prior to spray drying.

Spray-Drying

Spray-drying was performed using a model 190 spray-dryer (Büchi) as described previously (10). The operating condition was an inlet temperature of 105°C, a drying air flow rate of 1.1 m^3/min, an atomizing air flow rate of 1.1 m^3/h, and a liquid feed rate of 15 mL/min. This resulted in an outlet temperature of 50–55°C.

Protein Stability Assessment

Powder Storage

Spray-dried powders were stored for up to 1 year at temperatures that included 5, 30, and 40°C in chambers equilibrated with saturated lithium chloride (LiCl) and calcium chloride ($CaCl_2$) salt solutions (13). Saturated LiCl maintained an approximate 11% RH environment regardless of storage temperature, whereas saturated $CaCl_2$ maintained about 38% RH environment with storage at 5°C and about 23% RH environment with storage at 30 and 40°C.

Protein Concentration

Optical density (OD) measured from 240 to 400 nm relative to a water blank in a 1-cm cuvette was used to determine rhuMAbE25 concentration using a model 8453 spectrophotometer (Hewlett Packard). Spectra was corrected for light scattering by fitting the OD from 340 to 360 nm using an equation, $OD = a\lambda^n$, where λ is the wavelength, n is the order of the polynomial, and a is a constant. The corrected OD at 280 nm was used to determine rhuMAbE25 concentration using an absorptivity of 1.6 $(mg/mL)^{-1}cm^{-1}$ (14).

Soluble Protein Aggregation

The total amount of soluble aggregate was determined by native size exclusion chromatography (SEC) performed with a model 1090L liquid chromatography system (Hewlett Packard) using a TSK-gel G4000SWXL column (TosoHass) run in phosphate buffered saline (PBS) at a flow rate of 0.5 mL/min. The column was loaded with 100 μg of rhuMAbE25, and protein was detected by monitoring the OD at 280 nm.

Covalently-linked soluble aggregate was determined by sodium dodecyl sulfate (SDS) SEC performed on a model 1090L liquid chromatography system using a TSK-gel G4000SWXL column run in PBS with 0.5% SDS at a flow rate of 0.5 mL/min. Samples were incubated with 0.5% SDS for 1 hour at 50°C prior to analysis. The column was loaded with 100 μg of rhuMAbE25, and protein was detected by monitoring the OD at 280 nm.

The weight average molecular weight (MW_{Av}) was determined by light scattering using on-line miniDAWN light scattering and OptiLab DSP refractive index detectors (Wyatt Technology). The detectors were connected directly following the SEC column, and light scattering data was analyzed using ASTRA software (Wyatt Technology) as described previously (15).

SDS polyacrylamide gel electrophoresis (PAGE) was performed on 4–15% gradient trisglycine gels (Bio-Rad). Samples prepared in either reducing (25 mM dithiothreitol) or non-reducing sample buffer were heated at 90°C for 5 min prior to electrophoresis. The gel was loaded with 10 μg of rhuMAbE25, and following electrophoresis proteins were visualized by coomassie stain. Molecular weight standards (Bio-Rad) myosin (200 kDa), β-galactosidase (116.3 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa), and aprotinin (6.5 kDa) were used to estimate molecular weights.

Protein Conformation

Circular dichroism (CD) spectra in the far ultraviolet region was obtained using a model 62 DS spectropolarimeter (AVIV Associates). Measurements of buffer and protein samples were made at 20°C using a 0.5 cm pathlength quartz cuvette. The average of three spectra was obtained from 200 to 250 nm using an interval of 1 nm, a bandwidth of 1.5 nm, and an averaging time of 5 sec. Samples were prepared by dialysis, and were diluted to a concentration of 0.1 mg/mL. Spectra of the buffer was subtracted from that of the protein, and the mean residual ellipticity (MRE, degree cm^2 $decimole^{-1}$) was calculated using a mean residual weight for rhuMAbE25 of 110.6.

Protein Glycation

Isoelectric focusing (IEF) was run on Ampholine PAGEplate pH 5.5–8.5 gels (Pharmacia Biotech). Samples were

diluted to a protein concentration of 1 mg/mL in water. The gel was loaded with 10 μg of rhuMAbE25, and focusing was carried out using running conditions of 1.6 kV, 50 mA, and 25 W. Following IEF proteins were visualized by coomassie stain. The protein isoelectric point (pI) was estimated using IEF standards (Pharmacia, Biotech) consisting of myoglobin-acidic band (pI 6.9), myoglobin-basic band (pI 7.4), lentil lectin-acidic band (pI 8.2), lentil lectin-middle band (pI 8.5), and lentil lectin-basic band (pI 8.7).

Affinity chromatography was carried out using a TSK-gel boronate-5PW column (TosoHass) and a model 1090L liquid chromatography system. The column was equilibrated and run in 100 mM HEPES at pH 8.0. Sample load was 75 μg of rhuMAbE25, and protein was eluted by a linear gradient of 0 to 700 mM sorbitol in equilibration buffer applied over 15 min. Protein was detected by monitoring the OD at 280 nm.

Physical Stability and Aerosol Performance of the Powder

Crystallization

X-ray powder diffraction (XRD) was conducted using a 35 kV × 15 mA Rigaku (*D*/max-β, Cu K_α radiation) X-ray diffractometer operated at room temperature and humidity as described previously (10).

Blending of the Powders with Coarse Carrier

Blend doses were prepared by mixing 1 mg of spray-dried powder and 9 mg of coarse carrier lactose (200M Pharmatose, DMV International). Blends were sieved using a stainless steel 250 μm sieve and mixed by a tumbling blender (Turbula, Glen Mill) for 5 min. The sieving and tumbling procedure was repeated.

FPF Assay

The size distribution of blended powder aerosols generated by a Dryhaler (Dura Pharmaceuticals) powder inhaler was determined by a MSLI (Astra), a four stage inertial impactor that incorporates both an inlet bend designed to represent the mouth and oropharynx and a terminal filter to capture all material passing the last impaction stage. Ten individual doses of blended powder were discharged into the MSLI at an air flow rate of 60 L/min. Residual rhuMAbE25 in the device as well as protein in the MSLI throat, stages, and terminal filter was measured by UV absorption spectroscopy after a quantitative wash recovery. The FPF was calculated as the percentage of rhuMAbE25 collected on MSLI stages 3 and 4 as well as the terminal filter. This corresponded to the amount of rhuMAbE25 delivered by powders of ≤6.4 μm mass median aerodynamic diameter, a term that incorporates powder size and density.

RESULTS AND DISCUSSION

Soluble Aggregation and Aerosol Performance Following Spray-Drying of rhuMAbE25

Biochemical stability and aerosol performance are important parameters of a dry powder formulation for pulmonary delivery of a protein. We have recently reported on the effect of temperature and storage RH on the biochemical stability and aerosol performance of spray-dried rhuMAbE25 formulated with 20% dry weight mannitol (excipient to protein molar ratio of 200:1, (16)). During storage of the powder biochemical instability manifested as soluble protein aggregation was observed by native SEC, and the amount of aggregate was dependent upon the humidity of the storage environment. In contrast, the FPF of the powder blended with lactose coarse carrier was maintained during storage, even when the storage RH was increased to as high as 50%. These results suggest that optimization of the formulation for protein stability may be more problematic than aerosol performance, and to improve the formulation, various excipients were screened for enhanced protein stability. At the same time, the aerosol performance of the spray-dried powders was measured initially to determine the suitability of a formulation for pulmonary delivery of rhuMAbE25.

Formulations of rhuMAbE25 containing the disaccharides trehalose and lactose, the polyol mannitol, and excipient-free were evaluated following spray drying. Trehalose and lactose are excipients that when amorphous and devoid of residual water have a glass transition temperature (T_g) of 115 and 108°C respectively (17). The high Tg of these excipients may provide an advantage in the stabilization of dried proteins due to a better maintained glassy state in solid-state formulations with increased amount of moisture (18). Furthermore, lactose was also a logical candidate as an excipient because it is already employed as a carrier in the blending process. Finally, although the T_g of mannitol is much lower (4°C, (19)), the excipient was tested due to previous use in both freeze-dried (20) and dry powder (8,10) protein formulations. To determine the effect of concentration, the excipients were added to the aqueous solution prior to spray-drying at molar ratios ranging from approximately 100:1 to 900:1 excipient:protein.

The effect of formulation excipients on protein quality following spray-drying was determined by native SEC. Aggregation in excipient-free rhuMAbE25 powders increased to approximately 5–6%, compared to ≤1% aggregates in the trehalose- and lactose-containing formulations. (Fig. 1a,b). The mannitol-containing formulations resulted in less of an improvement in protein stability, as aggregation following spray-drying was 1–3% depending on the amount of mannitol in the formulation (Fig. 1c).

To evaluate aerosol performance, spray-dried powders were blended with coarse carrier lactose. The FPF of excipient-free rhuMAbE25 was approximately 30% (Fig. 1), and no change in aerosol performance occurred following the addition of trehalose up to a molar ratio of about 200:1 excipient:protein (Fig 1a). However, further increased trehalose in the spray-dried formulation resulted in decreased FPF. This correlated with increased cohesiveness of the powders with increased excipient in the formulation. This tendency was also observed by scanning electron microscopy (SEM), which showed that powders prepared with 50% dry weight trehalose (sugar to protein molar ratio of about 430:1) resulted in particles with a morphology that suggested that powder agglomeration had occurred (10). It follows that increased powder cohesiveness resulted in larger particles, poor dispersion, and reduced aerosol performance.

In contrast to trehalose, lactose had little effect on the FPF of rhuMAbE25 powders blended and dispersed following spray-

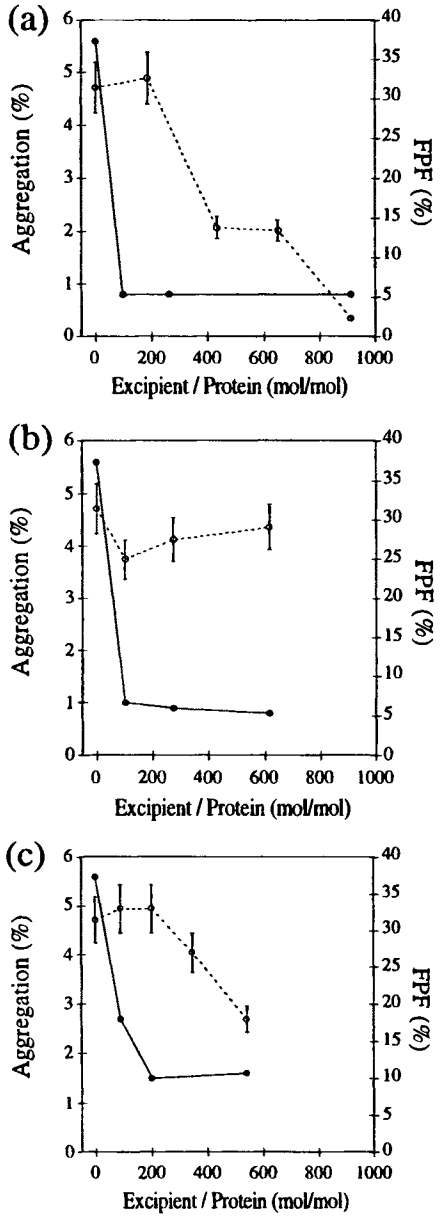

Fig. 1. The effect of excipient concentration on protein soluble aggregation and aerosol performance immediately following spray-drying of rhuMAbE25. Formulation excipients consists of (a) trehalose, (b) lactose, and (c) mannitol. In addition, results from excipient-free rhuMAbE25 are included in each plot. The increase in soluble aggregation is plotted on the Y1 axis as (-●-), whereas FPF of the blended powder is plotted on the Y2 axis as (--○--).

drying (Fig. 1b). The FPF of powders prepared with lactose at a molar ratio of up to 600:1 excipient:protein was in the range of 25–30%. These findings are in agreement with SEM and particle-size data that showed no evidence of powder agglomeration with increased amount of lactose in the formulation (10).

For powders prepared in mannitol, the FPF of formulations that contained a molar ratio of 200:1 excipient:protein was similar to that of excipient-free powders (Fig. 1c). However, increased mannitol resulted in significantly decreased FPF. As discussed below, reduced aerosol performance correlated with crystallization of the spray-dried powders that occurred with increased concentration of mannitol in the formulation.

Aggregation Kinetics of Excipient-Free Spray-Dried rhuMAbE25

To further assess protein stability, excipient-free spray-dried rhuMAbE25 powders were stored for 1 year under two RH conditions. Aggregation measured by native SEC was successfully modeled by pseudo first-order kinetics, as evidenced by a linear regression of a plot of natural log (% monomer) *vs.* storage time (Fig. 2a). The first-order rate constants showed that aggregation was more pronounced during storage at the higher temperatures (Fig. 2b). This is expected, based on the well-established effect of temperature on protein unfolding, which may lead to increased aggregation in either liquid or solid state formulations (21). Furthermore, at each storage temperature aggregation occurred more readily at the higher relative humidity condition of approximately 38% RH (chamber equilibrated with saturated $CaCl_2$) *vs.* approximately 11% RH (chamber equilibrated with saturated LiCl). We have previously shown that spray-dried rhuMAbE25 powders will sorb more moisture in a higher humidity storage environment (16). The destabilizing influence of moisture is presumably due to its ability to increase the mobility and flexibility of a solid state protein, which would promote aggregation (22).

Aggregate Characterization

Chromatograms obtained from native SEC of spray-dried rhuMAbE25 powders that were stored for 1 year at the high

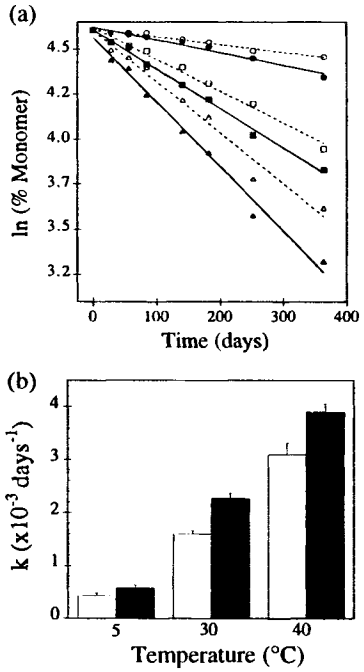

Fig. 2. Soluble aggregation during storage of spray-dried excipient-free rhuMAbE25. Aggregation kinetics are shown in (a) for powders stored in chambers equilibrated with saturated LiCl and temperatures of (--○--) 5, (--□--) 30, and (--△--) 40 °C and saturated $CaCl_2$ with temperatures of (-●-) 5, (-■-) 30, and (-▲-) 40 °C. The obtained first-order rate constants are shown in (b) for powders stored in chambers equilibrated with saturated (□) LiCl and (■) $CaCl_2$.

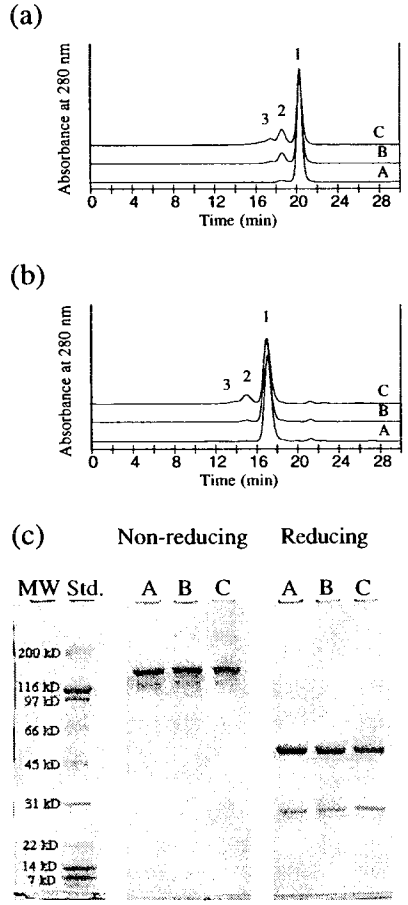

Fig. 3. Spray-dried rhuMAbE25 analyzed by (a) native SEC, (b) SDS-SEC, (c) SDS-PAGE. Powders were stored prior to assay at temperatures of (A) 5, (B) 30, and (C) 40 °C for 1 year in chambers equilibrated with saturated $CaCl_2$. SDS-PAGE was run on samples prepared under both non-reducing and reducing conditions. SEC peaks 1, 2, and 3 are defined in Table I.

RH condition (approximately 38% RH) are shown in Fig. 3a. Three peaks were resolved by the chromatography, and the MW_{AV} of each peak was determined by on-line laser light scattering of the sample stored at 40°C (Table I). Chromatography peaks were identified by comparison to the molecular weight (MW) of rhuMAbE25 calculated from the amino acid and carbohydrate composition (2). Peak 1 was composed of protein with a MW_{AV} of 154 ± 20 kD, which corresponded to that of the monomer (i.e., MW of 148 kD). Protein that eluted in peaks 2 and 3 had a MW_{AV} of 310 ± 31 and 467 ± 47 kD respectively, which matched the MW of rhuMAbE25 dimer (296 kD) and trimer (444 kD). Furthermore, native SEC showed that soluble aggregate formed during storage of spray-dried rhuMAbE25 at 5°C was primarily dimer, and the trimer species only appeared with storage at higher temperature (Fig. 3a).

Spray-dried rhuMAbE25 was also characterized by SDS-SEC and SDS-PAGE (Fig. 3b,c). In these assays, test samples were heated with SDS prior to analysis, which dissociates non-covalent aggregate. The elution profile obtained in SDS-SEC was similar to that of native SEC, except for decreased retention time of the three peaks that eluted from the column (Fig 3b). Using on-line laser light scattering, we determined that SDS-protein interaction resulted in an approximate 1.7 fold increase in the MW_{AV} of rhuMAbE25 monomer and aggregate (Table I), which would lead to decreased column retention time relative to native SEC. Protein elution in SEC is also dependent upon molecular conformation, and far ultraviolet CD spectra of rhuMAbE25 in SDS showed modification of protein secondary structure (i.e., unfolding) occurred (Fig. 4a). Protein unfolding results in increased hydrodynamic radius, which would further contribute to the observed decreased column retention time. Table I shows the total percent monomer, dimer, and trimer determined by native and SDS-SEC in a sample of spray-dried rhuMAbE25 that was stored at 40°C for 1 year. Less aggregate is observed in the SDS-SEC assay, and because this assay measures only covalent aggregate, soluble aggregate formed during storage was a mixture of both non-covalent and covalent aggregate.

Monoclonal antibodies are composed of 2 heavy and 2 light chains with inter-chain disulfides existing both between the light and heavy chains and between the two heavy chains. To examine the role of disulfides in rhuMAbE25 aggregation, SDS-PAGE was conduced under both reducing and non-reducing conditions (Fig. 3c). An analysis of spray-dried rhuMAbE25 powders that were stored for 1 year by SDS-PAGE under non-reducing conditions yielded results similar to that of SDS-SEC. However, SDS-PAGE of the samples run under reducing conditions resulted in only heavy and light chains of rhuMAbE25. This indicated the covalent aggregates were disulfide linked. Inter-molecular thiol-disulfide exchange is an aggregation mechanism previously observed with solid state pharmaceutical proteins (22). In the case of rhuMAbE25, covalent aggregates were either formed independently, or non-covalent

Table I. SEC Characterization of Spray-Dried Excipient-Free rhuMAbE25[a]

Peak[b]	Identity	MW[c]	Native SEC		SDS-SEC	
			MW_{AV} (LS)[d]	Area %	MW_{AV} (LS)[d]	Area %
1	Monomer	148,000	154,000 ± 20,000	67	277,000 ± 37,000	83
2	Dimer	296,000	310,000 ± 31,000	25	531,000 ± 72,000	14
3	Trimer	444,000	467,000 ± 47,000	8	690,000 ± 110,000	3

[a] Powder stored for 1 year in chambers equilibrated with a saturated $CaCl_2$ solution (approximately 38% RH).
[b] Peaks resolved by SEC (Fig 3a,b).
[c] MW of rhuMAbE25 calculated by amino acid and carbohydrate composition (2).
[d] MW_{AV} determined by on-line laser light scattering.

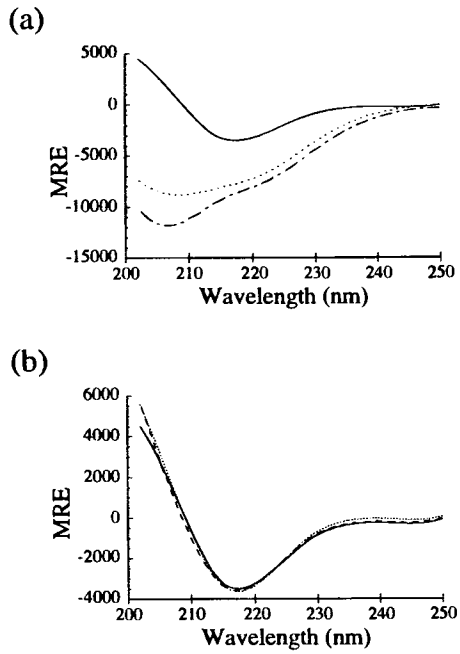

Fig. 4. Far ultraviolet CD spectra of rhuMAbE25. The effect of SDS is shown in (a), and samples consist of rhuMAbE25 (——) in PBS, (-----) PBS with 0.05% SDS, and (— - ---) PBS with 0.5% SDS. The effect of spray drying and storage of the powders on the CD spectra is shown in (b), and samples consists of rhuMAbE25 in PBS (------) prior to spray drying, (·····) following spray drying, and (——) following spray-drying and storage of the powder at 40 °C for 1 year in chambers equilibrated with saturated $CaCl_2$.

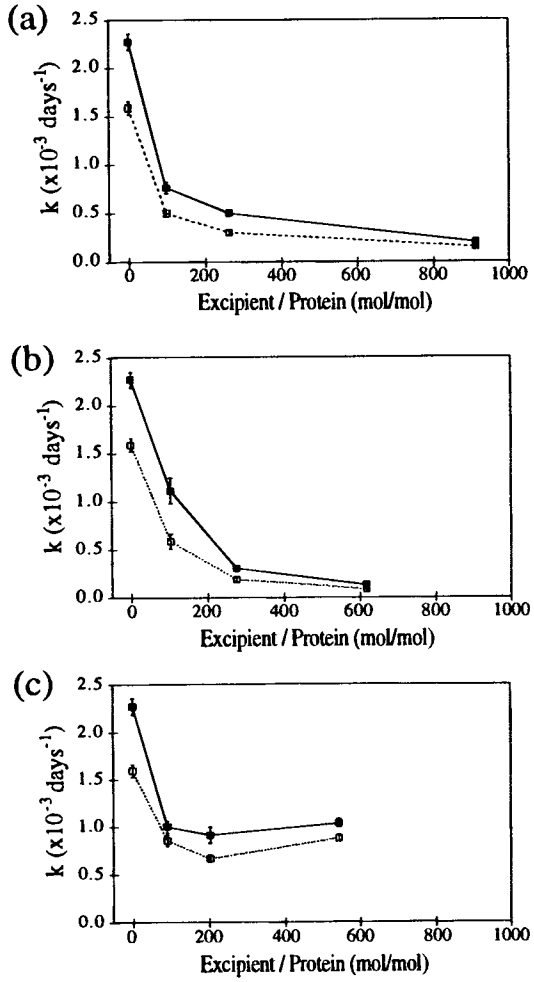

Fig. 5. The effect of carbohydrate concentration on soluble aggregation during storage of spray-dried rhuMAbE25 formulated with (a) trehalose, (b) lactose, and (c) mannitol. The first-order rate constants were obtained following storage of powders for 1 year at 30°C in chambers equilibrated with saturated (--□--) LiCl and (-■-) $CaCl_2$. Results from excipient-free spray dried rhuMAbE25 are also included in each plot.

aggregates were formed first, and were stabilized by intermolecular disulfide exchange of monomers placed in close proximity.

Aggregation Kinetics of Spray-Dried rhuMAbE25 Formulated with Carbohydrate

Aggregation kinetics of spray-dried rhuMAbE25 formulated with trehalose, lactose, and mannitol was determined. The pseudo first-order rate constants and standard error from the linear regression analysis were obtained as described above for excipient-free rhuMAbE25, and are shown *vs.* excipient concentration in Fig. 5. As was the case for the excipient-free protein, aggregation of the carbohydrate-containing formulations was more pronounced at the higher RH storage condition.

The presence of carbohydrate resulted in decreased rates of aggregation depending on the formulation. Figure 5 shows that the rate constants dropped to less than half that exhibited during storage of excipient-free protein powders when the amount of each excipient was increased to the lowest molar ratio tested (approximately 100:1 excipient:protein). In the trehalose- and lactose-containing formulations, the rate constants were further decreased when the excipient was increased beyond this level, and maximum stability was obtained at or beyond a molar ratio in the range of 300:1 to 500:1 excipient:protein. In contrast, aggregation in the mannitol-containing formulations leveled off at a molar ratio in the range of 100:1 to 200:1, and further increase in mannitol resulted in increased aggregation. Furthermore, the rate constants of the mannitol formulations were higher than those of formulations prepared with trehalose and lactose at a similar molar ratio of excipient to protein. This result correlates with the initial observation that mannitol was less efficient than the disaccharides studied at stabilizing rhuMAbE25 following spray-drying of the powders.

Although there are relatively few reports regarding the stabilizing effect of carbohydrate excipients on spray-dried proteins, there have been extensive studies conducted on freeze-dried formulations (11). Therefore, it was logical to examine mechanisms proposed for the stabilization of lyophilized proteins, and to explore their extension to spray-dried proteins as well. For instance, carbohydrate excipients may provide for a glassy matrix below the glass transition temperature (T_g) where protein mobility and flexibility are reduced (23). However, it should be noted that the excipients used in our study are low molecular weight species, and will lower the T_g of the protein-sugar formulation relative to protein alone (24). Therefore, the stabilizing effect of various carbohydrates on rhuMAbE25 cannot be explained in terms of the excipient's ability to impact mobility through changes in the T_g of the system. A similar

conclusion was made regarding the effect of various excipients on stabilizing lyophilized growth hormone against thermal unfolding (24) and aggregation (25). Even so, T_g is important because above this temperature amorphous polyols and sugars are more susceptible to crystallization due to the increased mobility that allows increased intermolecular interaction (i.e., nucleation) and crystal growth. For solid-state formulations stored in a humid environment, a high T_g is desirable because increased moisture effectively decreases the T_g, which would exacerbate crystallization. As discussed below, mannitol, which has a much lower T_g than the disaccharides studied, tends to crystallize in spray-dried powder formulations, whereas trehalose and lactose remain amorphous (26).

Another theory, the water substitution hypothesis, proposes that as drying removes the protein's hydration shell, excipients serve as a substitute for water in fulfilling the hydrogen-bonding requirements of surface amino acids (27). It has been further hypothesized that this interaction is involved in the specific ability of saccharide excipients to stabilize protein against dehydration-induced structural perturbation (28). Structural conservation during drying may result in increased protein stabilization, provided the mechanism of instability is related to structure. For excipient-free and lactose-containing formulations of spray-dried rhuMAbE25, we have previously demonstrated using fourier-transform infrared (FTIR) spectroscopy (amide I and III regions) that protein structure was not significantly altered from the native state upon spray-drying or storage of the powders (29). Furthermore, protein structure of reconstituted spray-dried rhuMAbE25 was characterized by CD in the far ultraviolet region (Fig. 4b). The results show that the overall secondary structure of rhuMAbE25 was unaltered after spray-drying. In addition, for excipient-free powder stored at 40°C for 1 year at elevated RH, a condition that resulted in substantial aggregation, the CD data indicated no significant structural change. These results correlated with FTIR analysis, and suggests that in the case of rhuMAbE25 either stabilization against solid-state aggregation cannot be explained by structural conservation, or CD and FTIR spectroscopy both lack the sensitivity to detect small changes in protein structure that may contribute to protein aggregation.

Inhibition of rhuMAbE25 aggregation may also arise from reduced protein-protein contacts as a result of protein-excipient interactions. Consistent with the water substitution hypothesis, such interactions replace those otherwise satisfied by water molecules. The number of strongly water-binding sites of a protein can be estimated from its composition, and for rhuMAbE25 the total is calculated to be about 500 (30). Interestingly, the aggregation data show the maximum stabilization effect for carbohydrate was observed when the excipient was present in this range (Fig. 5). This suggests that protein-excipient interaction at these sites may be involved in the stabilization mechanism. However, because the powders are not completely devoid of water, not all of the strongly water-binding sites may be available for interaction with formulation excipients, and competition for binding sites may occur between residual moisture and carbohydrate. Spray-dried powders of rhuMAbE25 contained residual water at a molar ratio of approximately 600:1 water:protein, and the amount of water may increase during storage in a humid environment (16). This exceeds the theoretical water monolayer of the protein (29), and the relatively high level of residual water likely plays a role in protein destabilization, as evidenced by the influence of relative humidity on aggregation rates (Fig. 2 and 5).

Physical Stability of Spray-Dried rhuMAbE25 Formulated with Carbohydrate

All of the aforementioned mechanisms of carbohydrate protection of dried protein are based on excipient being present in the amorphous protein-containing phase. Upon excipient crystallization, solid proteins may have increased instability, for example, as a result of the loss of excipient-protein interaction or by virtue of increased moisture (i.e., expelled upon formation of anhydrous crystals). We have observed that mannitol was less efficient at stabilizing rhuMAbE25 compared to the disaccharides studied (Fig. 5), and perhaps this is due to increased tendency towards crystallization. For instance, XRD of powders prepared with the highest level of trehalose (molar ratio of 900:1 excipient:protein) and lactose (molar ratio of 600:1 excipient:protein) showed that the formulations were amorphous even after storage for 1 year at the high temperature and humidity condition (Fig. 6a,b). In contrast, a similar analysis of mannitol (molar ratio of 500:1 excipient:protein, Fig. 6c) showed obvious crystallinity, and this correlated with decreased protein stability (Fig. 5c) and aerosol performance (Fig. 1c). XRD also indicated that formulations with less mannitol were amorphous (molar ratio of 100:1 and 200:1 excipient:protein, (Fig. 6d,e)). However, aggregation rate constants of these formulations (Fig. 5c) were higher than those of the disaccharide powders prepared with a similar molar ratio (Fig. 5a,b). This indicates that either mannitol, even when amorphous, was not as efficient as a protectant, or crystallization occurred at a level below the sensitivity of XRD. The latter is consistent with the observation that some crystallinity occurred in spray-dried rhuMAbE25 formulated with 30% dry weight mannitol (molar ratio of 350:1 excipient:protein, (10)). Therefore, powders prepared with mannitol at a concentration of less than 500:1 excipient:protein may be on the verge of crystallization at a level that was difficult to detect by XRD.

Protein Glycation of Spray-Dried rhuMAbE25 Formulated in Lactose

Although successful in minimizing protein aggregation during storage, lactose is a reducing sugar, and has the potential to react with the primary amine of lysine residues (20). Indeed protein structural modification was detected by IEF of spray-dried rhuMAbE25 formulated in lactose (Fig. 7a). The IEF pattern of rhuMAbE25 consists of three bands with pI's that lie between 7.4 and 8.2. Although the pattern was maintained following storage at 5°C for 9 months, an acidic shift occurred during storage at 30°C. The shift was more pronounced with increased amount of lactose in the formulation, and did not occur during storage of similar formulations prepared with the non-reducing polyol, mannitol (data not shown). This led us to suspect protein glycation, because a Mailard reaction of reducing sugar with the primary amine of lysine would result in loss of positive charge and a corresponding acidic shift in protein pI. Similar degradation has been reported during storage of spray-dried recombinant human deoxyribonuclease formulated in lactose (12).

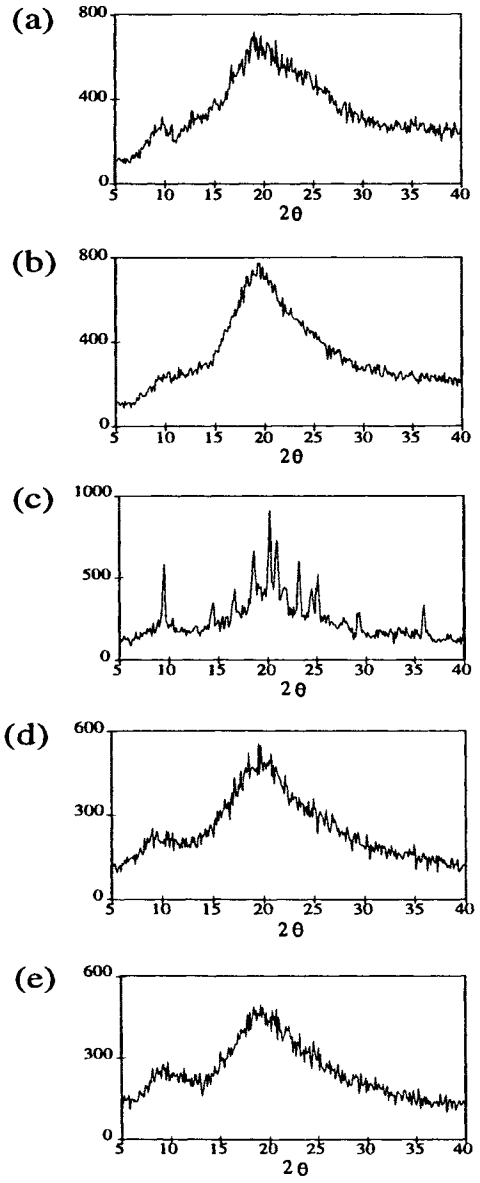

Fig. 6. XRD patterns of spray-dried powders stored at 30 °C for 1 year in chambers equilibrated with saturated $CaCl_2$. Formulations consists of rhuMAbE25 prepared with excipient at a molar ratio of approximately (a) 900:1 trehalose:protein, (b) 600:1 lactose:protein, (c) 500:1 mannitol:protein, (d) 200:1 mannitol:protein, and (e) 100:1 mannitol:protein.

Confirmation of rhuMAbE25 glycation was obtained by affinity chromatography using a boronate column. In this assay, under alkaline conditions boronate anion hydroxyl groups are replaced by the *cis*-hydroxyl groups of a reducing sugar, and carbohydrates absorbed to the column are eluted by displacement with the carbohydrate, sorbitol (31). An analysis of lactose-containing powders prepared with a molar ratio of 300:1 excipient:protein and stored for 9 months is shown in Fig. 7b. Following storage at 5°C, the majority of rhuMAbE25 flows through the column indicating that the protein was mostly unmodified (Fig. 7b, peak 1). However, following storage at 30°C the majority of rhuMAbE25 was retained by the column and eluted in the gradient, which indicated that significant protein modification had occurred (Fig. 7b, peak 2). In a separate

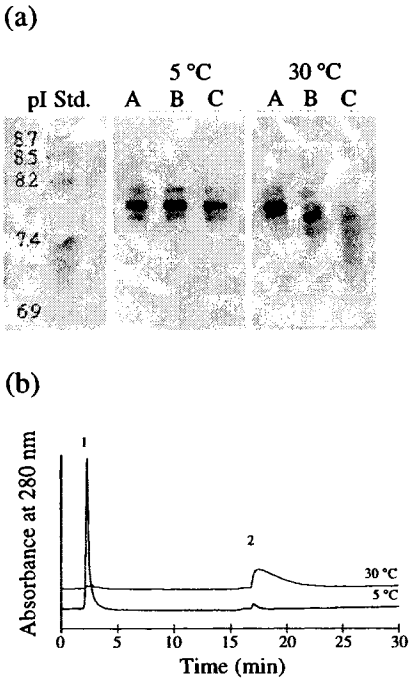

Fig. 7. Protein glycation assayed by (a) IEF and (b) boronate chromatography of powders stored for 9 months in chambers equilibrated with saturated $CaCl_2$. IEF was performed on rhuMAbE25 spray-dried in lactose at a molar ratio of (A) 100:1, (B) 275:1, and (C) 600:1 lactose:protein. Boronate chromatography was performed on powders prepared with 275:1 lactose:protein.

study, protein from the retained peak was characterized by tryptic mapping and mass spectroscopy, and it was determined that 16 out of a total of 44 lysine residues in the primary structure of rhuMAbE25 were lactosylated (32).

CONCLUSIONS

Successful formulation of a spray-dried therapeutic protein requires a balance between improving the biochemical stability, while not compromising the aerosol performance of the blended powder. In the case of rhuMAbE25, the protein was susceptible to solid-state aggregation, and improved stability was obtained by employing a strategy developed for freeze-dried proteins, namely, formulation with carbohydrate. The disaccharide trehalose was effective in stabilizing the protein. However, the cohesive nature of the spray-dried powders resulted in poor aerosol performance. In powders prepared with lactose, improved protein stability was obtained without compromising aerosol performance following spray-drying, and the balance was obtained. However, the use of lactose as an excipient for a therapeutic protein formulation was questionable due to chemical protein modification during storage (i.e. glycation). The addition of mannitol also improved protein stability, but its usefulness was limited by its susceptibility towards crystallization, which had a detrimental effect on both protein stability and aerosol performance. However, the utility of mannitol as an excipient in spray-dried protein powders may be extended by employing strategies for inhibiting crystallization (26). Finally, we note that this investigation was intended to identify candidate formulations and define appropriate storage conditions. Further evaluation of a formulation must also include the biochemical

stability and aerosol performance during storage of the actual drug product, which is the blended powder.

ACKNOWLEDGMENTS

The authors acknowledge Felicity Shen and Reed Harris for the boronate assay and the analysis of lactosylated protein, Dr. Jun Liu for assistance with on-line laser light scattering, and Bob Jones of the Geology Department at Stanford University for supporting XRD measurements. In addition, the generous support of Drs. Andy Clark, Tom Bewley, Tue Nguyen and Robert Fick is gratefully appreciated.

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