Influence of Histidine on the Stability and Physical Properties of a Fully Human Antibody in Aqueous and Solid Forms

Bei Chen,[1,2] Raquel Bautista,[1] Kwok Yu,[1] Gerardo A. Zapata,[1] Michael G. Mulkerrin,[1] and Steven M. Chamow[1]

Received August 22, 2003; accepted September 3, 2003

Purpose. The aim of the study was to investigate the effect of histidine on the stability and physical properties of a fully human anti-IL8 monoclonal antibody (ABX-IL8) in aqueous and solid forms.

Methods. Using a fractional factorial design, we tested many excipients, including histidine, sucrose, and other commonly used excipients, on the stability and physical properties of the antibody in both liquid and lyophilized forms. Antibody stability and physical properties were evaluated using size-exclusion high-performance liquid chromatography (SEC-HPLC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and a viscometer. Residual moisture content was determined by coulometric Karl Fischer titrator. Differential scanning calorimetry (DSC) was used to detect the glass transition temperatures (T_g) of the solid cakes and melting temperatures (T_m) of the antibody in liquid formulations. Fourier-transform infrared (FTIR) spectroscopy was used to examine the overall secondary structure.

Results. Increasing the histidine concentration in the bulk solution inhibited the increases of high-molecular-weight (HMW) species and aggregates upon lyophilization and storage. In addition, histidine bulk enhanced solution stability of the antibody under freezing and thermal stress conditions, as evidenced by the lower levels of aggregates. Furthermore, histidine reduced viscosity of the antibody solution, which is desirable for the manufacture of the dosage form. However, high concentrations of histidine in liquid formulations led to coloration of the solution and high levels of aggregates on storage at elevated temperature (40°C) after the formulations were exposed to stainless steel containers during bulk freezing-thawing.

Conclusions. Histidine enhanced the stability of ABX-IL8 in both aqueous and lyophilized forms. Histidine also improved the physical properties such as reducing the solution viscosity. Liquid formulations containing high concentrations of histidine should not be stored in stainless steel tanks at elevated temperatures.

KEY WORDS: histidine; stability; liquid formulation; lyophilized formulation; viscosity; fully human antibody.

INTRODUCTION

Histidine is a unique amino acid with three ionization sites on the molecule, with pK_1' of 1.78, pK_2' of 5.97, and pK_3' of 8.97. Histidine has been used as a buffer at pH from 5.5 to 6.5 in parenteral formulations. However, as a potential stabilizing additive, it has been studied and described sparingly in the literature.

Osterberg *et al.* described the development of a stable freeze-dried formulation for recombinant factor VIII-SQ (r-VIII SQ) without the addition of albumin. The authors found that a combination of noncrystallizing excipients (L-histidine and sucrose), a nonionic surfactant (polysorbate 80) and a crystalline bulking agent (sodium chloride) preserve factor VIII activity during formulation, freeze-drying and storage. It was also stated that L-histidine, besides functioning as a buffer, also protected r-VIII SQ during freeze-drying and storage. However, there were no data shown supporting the effect of histidine (1).

Cleland *et al.* (2) studied the lyophilization of a monoclonal antibody, rhuMAb HER2. The authors compared the stability profile of rhuMAb HER2 formulated at 25 mg/ml in either 5 mM succinate, pH 5, or 5 mM histidine, pH 6, before lyophilization, immediately after lyophilization, and on storage of the lyophilized protein at 40°C. They found that in the absence of sugar, a greater extent of aggregation was observed in the histidine formulation than in the succinate formulation after 3 months at 40°C. They concluded that the potential stabilizing effects of histidine during lyophilization and storage were not observed with rhuMAb HER2. It is noteworthy that the succinate and histidine formulations used had different pHs.

Sugars, on the other hand, have been studied extensively as cryoprotectants and lyoprotectants to stabilize proteins against denaturation and aggregation during freezing and lyophilization, respectively. For example, it has been reported that hydrogen bonding between a sugar and a protein is responsible for inhibition of dehydration-induced protein unfolding (2). Cleland *et al.* demonstrated that a specific molar ratio of sugars (sucrose or trehalose) to an antibody is required for storage stability of a lyophilized antibody. Specifically, a 360:1 molar ratio of sugars to the antibody was required for storage stability (2).

Furthermore, sugars have been extensively studied as thermodynamic stabilizers. Specifically, Timasheff *et al.* have established a preferential exclusion mechanism explaining the stabilizing effects of sugars in protein solutions (3). Kendrick *et al.* have proposed that the degree of preferential exclusion and increase in chemical potential are directly proportional to the protein surface area and that the system will favor the protein state with the smallest surface area (4). A recent study showed that sucrose could inhibit oxidation of subtilisin, a serine protease (5). The study demonstrated the limited conformational mobility of the enzyme in the presence of sucrose by hydrogen–deuterium exchange measurements.

The antibody in the current study, ABX-IL8, is a fully human IgG_2 antibody generated using Abgenix' proprietary XenoMouse® technology (6), which involves transgenic mouse strains. The mouse antibody-producing genes have been inactivated and functionally replaced by most of the human antibody-producing genes. The antibody is specific for human interluekin-8 (IL8), a potent chemotactic cytokine with a K_d of 2.1×10^{-10} M (7).

During our investigation, we used Design of Experiment (DOE), specifically, a fractional factorial design, to study the effects of histidine on the stability of ABX-IL8 in lyophilized form. We also examine the stabilizing effect of histidine on the antibody in liquid formulations under freezing and ther-

[1] Department of Process Sciences, Abgenix, Inc., Fremont, CA 94555.
[2] To whom correspondence should be addressed. (email: Bei.Chen@abgenix.com)

0724-8741/03/1200-1952/0 © 2003 Plenum Publishing Corporation

mal stress conditions, in comparison to sucrose, succinate, and citrate. The data revealed that histidine enhanced the stability profile of the antibody in both aqueous and lyophilized forms. In addition, it significantly reduced the solution viscosity. The possible mechanisms of the effects of histidine were also explored.

MATERIALS AND METHODS

Materials

ABX-IL8 is a fully human monoclonal IgG_2 antibody generated using Abgenix' proprietary XenoMouse® technology and manufactured by Lonza Biologics, plc (Slough, UK). The antibody has κ light chains and a molecular weight of approximately 150 kDa with pI range of 7.3–8.5. All chemicals including glycine, glutamic acid, histidine, arginine, sucrose, mannitol, succinate, citrate, and polysorbate 20 are of USP/NF or ACS analytical reagent grade and from commercial sources. All references in the text to solute concentrations are on a weight-to-volume (w/v) percentage basis unless specifically stated otherwise.

Experimental Design

A modified fractional factorial (2^{7-4}) design, as described by Youden (8), was used. The design is a two-level design with seven variables. It is a resolution 3 design, in which main effects are not aliased with other main effects. The experimental design matrix is shown in Table I.

Table I. 2^{7-4} Fractional Factorial Design Matrix and Study Results of Lyophilized Formulations

	Formulation variables							Results[a]	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Percentage aggregates[b]	Percentage HMW bands[c]
1	+	+	−	−	−	−	−	4.3	4.4
2	+	+	+	−	+	+	+	3.0	3.3
3	+	−	−	**+**	−	+	+	**1.8**	**0**
4	+	−	+	**+**	+	−	−	**1.7**	**0**
5	−	+	−	**+**	+	−	+	**1.5**	**0**
6	−	+	+	**+**	−	+	−	**1.5**	**0**
7	−	−	−	−	+	+	−	3.8	2.1
8	−	−	+	−	−	−	+	4.8	2.9

The Variables and Their Settings Were as Follows[d]

	Variables	−	+
X_1	Freeze-drying cycle[e]	Cycle I	Cycle II
X_2	pH	5.7	6.3
X_3	Glycine (mM)	15	20
X_4	**Histidine (mM)**	**4**	**6**
X_5	Mannitol (%)	0.2	0.3
X_6	Glutamic acid (mM)	16	21
X_7	Polysorbate 20 (%)	0.02	0.03

[a] The samples were stored at 37°C for one month.
[b] Percentage aggregates were determined by SEC-HPLC.
[c] Percentage HMW bands were determined by non-reducing SDS-PAGE gel.
[d] The concentration of ABX-IL8 is 50 mg/ml. The concentration of each excipient represents that in the bulk solution before lyophilization.
[e] Cycle I: Precool the shelf to –45°C; primary drying at –20°C for 75 h; secondary drying at 20°C for 50 h. Cycle II: Freeze at 1°C/min to –45°C; then the same drying processes as cycle I.

Lyophilized Formulation Preparation

Seven formulation variables were evaluated in the study, including pH, freeze-dry cycle, histidine, glycine, mannitol, glutamic acid, and polysorbate 20. The purified antibody was buffer-exchanged into eight different formulations using PD10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden) based on the design matrix in Table I. For instance, formulation 1 contains 4 mM histidine, pH 6.3, 15 mM glycine, 0.2% mannitol, 16 mM glutamic acid, and 0.02% polysorbate 20 and is lyophilized with cycle II. Each formulation of 0.8 ml was dispensed into 3-ml type 1 glass vials with 13-mm lyophilization stoppers. Lyophilization was carried out in a DuraDry MP freeze-dryer (FTS Systems, Stone Ridge, NY). The lyophilized vials were stored at different temperatures for different periods of time. The samples were reconstituted with 0.2 ml water for injection (WFI) before characterization using the assays listed below.

An additional study was performed by spiking histidine into the formulation matrix (17.5 mM glycine, 0.25% mannitol, 18.8 mM glutamic acid, and 0.025% polysorbate 20) to final histidine concentrations of 5 mM, 15 mM, and 40 mM, respectively, all at pH 6.0 (Table II). The samples were freeze-dried, and the dried cakes were stored at 40°C for 2 weeks.

Liquid Formulation Preparation

Solution Stability Studies

The purified antibody was formulated at 100 mg/ml into either formulation A, consisting of 40 mM histidine, 40 mM arginine, and 150 mM sucrose, or formulation B, containing 15 mM histidine, 15 mM arginine, and 200 mM sucrose, using a Tangential Flow Filtration (TFF) system with Biomax 30 membrane (Millipore, Bedford, MA). Each formulation of 1 ml was dispensed into 3-ml type 1 glass vials with 13-mm serum stoppers. Samples were stored at 2–8°, 25°, and 40°C.

In addition, the effect of histidine on the stability of ABX-IL8 was studied by freezing-thawing of samples containing different concentrations of histidine three times. Then the samples were analyzed by SEC-HPLC. Furthermore, the effect of histidine on the stability of the antibody was evaluated by formulating the antibody into histidine or succinate or citrate buffer, pH 6, and incubating at 40°C and 50°C.

Solution Viscosity Study

The purified antibody was concentrated using a TFF system with Biomax 30 membrane to 150 mg/ml in 5 mM histidine, pH 6. Stock solutions of histidine (0.5 M) were aliquoted and concentrated (~4×) in a Speed-Vac (Savant Instruments, Farmingdale, NY). Concentrated salt was spiked into the antibody solution to yield appropriate final concentrations for viscosity measurement. The volume change at the highest spiking concentration is less than 5%. Therefore, the antibody concentration was maintained upon spiking of the concentrated salts, which was confirmed by the measurement of the

Table II. Titration Study and Results

	Components[a]	Percentage aggregates[b]	Percentage aggregates[c]
1	17.5 mM glycine, 0.25% mannitol, 18.8 mM glutamic acid, 0.025% polysorbate 20, **5 mM histidine,** pH 6	3.24 ± 0.18	3.23 ± 0.16
2	17.5 mM glycine, 0.25% mannitol, 18.8 mM glutamic acid, 0.025% polysorbate 20, **15 mM histidine,** pH 6	2.21 ± 0.04	1.63
3	1.75 mM glycine, 0.25% mannitol, 18.8 mM glutamic acid, 0.025% polysorbate 20, **40 mM histidine,** pH 6	1.89	1.58 ± 0.01

[a] The concentrations of excipients represent those in bulk solution before lyophilization.
[b] The samples were freeze-dried using cycle II: Precool the shelf to −45°C; primary drying at −20°C with a ramping rate of 0.5°C/min from −45°C to −20°C and hold for 75 h at a chamber pressure of 70 mTorr for 75 h; secondary drying at 20°C with a ramping rate of 0.5°C/min from −20°C to 20°C and hold for 44 h at a chamber pressure of 50 mTorr; total cycle time is ~120 h. The dried cakes were stored at 40°C for 2 weeks.
[c] The samples were freeze-dried using cycle III: Freeze at a rate of 0.35°C/min to −45°C; primary drying at 20°C with a ramping rate of 0.16°C/min from −45°C to 20°C and hold for 25 h at a chamber pressure of 200 mTorr; secondary drying at 30°C with a ramping rate of 0.5°C/min and hold for 50 h at a chamber pressure of 50 mTorr; total cycle time is ~50 hours. The dried cakes were stored at 40°C for 2 weeks.
Mean ± SD, N = 6.

concentrations using A_{280}. Thus, any effect of the antibody concentration on the viscosity could be excluded.

Characterization Assays

UV-Vis Spectrophotometer

Protein concentration was determined spectrophotometrically using $A_{280,\ 1\ mg/ml,\ 1\ cm} = 1.58$. Sufficient WFI was added to the sample to adjust the antibody concentration to a range between 0.5 to 1 mg/ml.

SEC-HPLC

Size exclusion chromatography was performed using a Water LC system with a diode array detector. A TSK-Gel 3000 SW_{XL} column (0.78 × 30 cm; TosoHaas) was used with an elution buffer consisting of 500 mM sodium chloride, 50 mM borate, pH 8.0, with a flow rate of 0.5 ml/min. Mass load of the antibody was 50 μg, and detection was at 215 nm.

SDS-PAGE

The purity of the antibody was also examined using SDS-PAGE, which was carried out on 10% Bis-Tris Novex ready gels (Invitrogen, Carlsbad, CA) using a Bio-Rad mini Protean II eletrophoresis system. Samples were diluted with 2× glycine/SDS solution, either with or without DTT, to a protein concentration of 0.5 mg/ml. Protein samples (10 μg per lane) were applied and subjected to electrophoresis at 100 mA for approximately 60 min. Protein bands were visualized using Coomassie blue followed by destaining until the backgrounds were clear. The intensity of the protein bands was determined by densitometry [AGFA Arcus II gel scanner (Scanalytics, Fairfax, VA) with ONE-Dscan software] and calculated as a percentage of the total intensity of the sample.

Coulometric Karl Fischer Assay

The residual moisture content was determined by Karl-Fischer titration (756 KF Coulometer; Metrohm) according to the procedure described previously (9).

Inductively Coupled Plasma-Atomic Emission Spectrometry

The determination of concentrations of iron was carried out by Industrial Analytical Services Inc. (a subsidiary of AAA Environmental Labs) using the inductively coupled plasma (ICP) method, which is an EPA method (10).

Differential Scanning Calorimetry

The glass transition temperatures (T_g) of the solids were determined using Perkin Elmer DSC-7 as previously described (11). Measurements of melting temperature (T_m) were carried out using MicroCal VP-DSC as previously described (12).

Fourier-Transform Infrared Spectroscopy

The IR spectra of lyophilized cakes were acquired as previously described using a Bomem Prota Spectrometer and software (13).

Viscometry

Solution viscosity was measured using a Cannon-Fenske capillary viscometer (Brinkmann, Westbury, NY). A sample solution was dispensed into an appropriate size capillary using a 10-ml syringe. The capillary loaded with the sample solution was placed vertically and secured. The solution was allowed to flow freely down past two marks. The time taken to flow through the upper mark to the lower mark was recorded as efflux time in seconds. The kinematic viscosity of the solution was calculated by multiplying the efflux time by the constants.

RESULTS AND DISCUSSION

Effect of Histidine on the Stability of ABX-IL8 in Lyophilized Form

There have been numerous reports about the stabilizing effects of sugars, such as sucrose and trehalose, on the proteins during lyophilization and storage in the dried cakes. The current investigation aims to screen excipients (other than sugars) for the ability to stabilize the antibody during lyophilization and on storage. Selection of the excipients and levels is based on the knowledge from literature, experience, and preliminary formulation development. Gly/mannitol/Glu/His was selected as a leading formulation matrix. In addition, it has been taken into consideration that the total excipient concentration in the bulk solution was four-fold below isotonic

concentration in order to make the final reconstituted solution (four times higher than the bulk solution) isotonic (~300 mOsm).

The first "eyeball approximation" of data in Table I revealed two distinct data sets. Formulations 1, 2, 7, and 8 (4 mM histidine in bulk solution) had high levels of aggregates as well as HMW bands, whereas formulations 3, 4, 5, and 6 (6 mM histidine in bulk solution) had lower levels. The results show that the stability of ABX-IL8 is highly dependent on the concentration of histidine in the prelyophilization bulk. There were no high-molecular-weight (HMW) bands observed in the formulations containing high histidine concentrations (6 mM) after storage of the dried cakes at 37°C for 1 month, whereas there were an average of 3.2% HMW [(4.4 + 3.3 + 2.1 + 2.9)/4] bands in the formulations containing lower histidine concentrations (4 mM). Figure 1A depicts the profile of nonreducing SDS-PAGE gel.

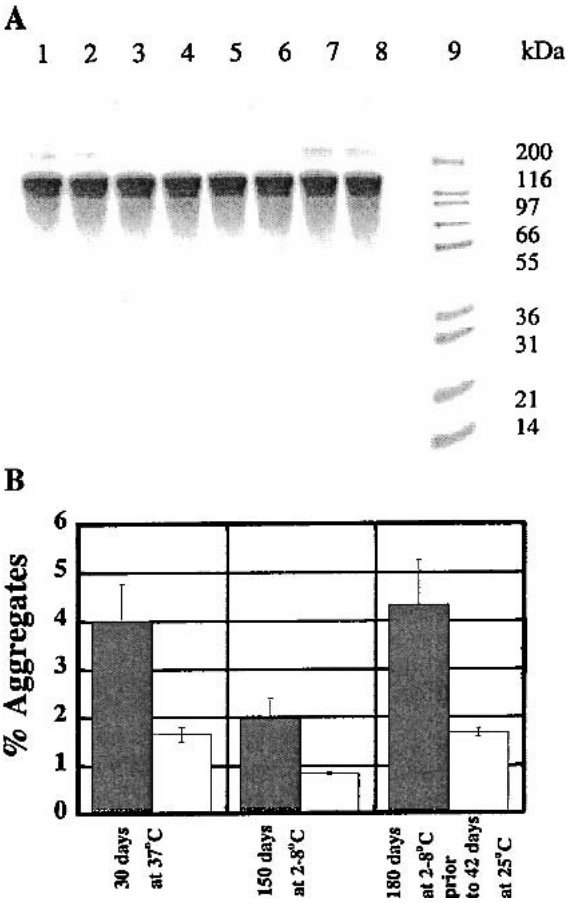

Fig. 1. The effect of histidine on (A) the formation of high-molecular-weight bands determined by nonreducing SDS-PAGE and (B) the formation of soluble aggregates determined by SEC-HPLC. (A) ABX-IL8 was lyophilized from formulations 1, 2, 7, 8 containing 4 mM histidine (lane 1, 2, 7, 8) and formulations 3, 4, 5, 6 containing 6 mM histidine (lane 3, 4, 5, 6). Lyophilized ABX-IL8 cakes were incubated at 2–8°C for 6 months and followed by 25°C for 42 days. Samples were analyzed after reconstitution with WFI. Lane 9: molecular weight standard. (B) Average percentage aggregates for formulations containing 4 mM histidine (*solid symbols*) and formulations containing 6 mM histidine (*open symbols*), respectively. The lyophilized ABX-IL8 cakes were incubated for various periods of time. Samples were analyzed after reconstitution with WFI.

Concomitant with the inhibition of HMW formation observed by SDS-PAGE, there was inhibition of soluble aggregate formation determined by SEC-HPLC for samples containing higher levels of histidine. Figure 1B shows the effect of histidine on the inhibition of aggregation determined by SEC-HPLC assay. Again, the same trend was observed; i.e., the samples containing higher concentrations of histidine in the bulk solutions had lower levels of soluble aggregates than those containing lower concentrations of histidine, regardless of the storage temperatures or incubation length. It should be noted that the aggregate levels determined by SDS-PAGE and SEC-HPLC are different (Table I) and represent different aspects of the aggregates observed. A fraction of aggregates can be dissociated by SDS treatment, suggesting the noncovalent force holding the aggregates together.

We further analyzed the data in Table I using the Youden technique (8), which states that the effect of a variable on a response can be determined by taking the average of the responses at the higher level (+) minus the average of the response at the low (–) level.

$$\text{Effect} = \Delta(\text{high} - \text{low}) = \Sigma \text{ responses on high setting}/4 - \Sigma \text{ responses on low setting}/4.$$

For instance, for the variable *histidine concentration*, the effect on the response *percentage aggregates* can be determined as follows:

$$\text{Effect} = \Sigma \text{ formulation 3, 4, 5, 6}/4 - \Sigma \text{ formulation 1, 2, 7, 8}/4$$

$$= (1.8 + 1.7 + 1.5 + 1.5)/4 - (4.3 + 3.0 + 3.8 + 4.8)/4 = -2.35$$

If the obtained value is relatively high, it has more significant effects on the response than a small one does. Furthermore, the negative sign indicates that the variable is helpful to the response (less aggregation), whereas variables with positive values have adverse effects on the response (more aggregation). By using the technique, we analyzed the effect of each formulation variable on aggregate formation. We found that among five excipients tested (histidine, glycine, mannitol, glutamic acid, and polysorbate 20), histidine is the most critical excipient for the solid-state stability of the antibody, minimizing formation of aggregates.

Furthermore, we titrated histidine from 5 mM, 15 mM, to 40 mM in the formulation matrix consisting of 17.5 mM glycine, 0.25% mannitol, 18.8 mM glutamic acid, and 0.025% polysorbate 20 (Table II), all at pH 6. Again, the antibody is more stable in the formulation matrix containing higher histidine concentration on lyophilization and storage regardless of freeze-drying cycle. The pH did not account for the stabilization of the antibody, because it was fixed at 6 in the study.

The possible mechanisms of the effect of histidine on the antibody were explored. First, we examined the residual moisture contents in different formulations, which contained two different levels of histidine (4 mM vs. 6 mM in prelyophilized bulk) and had different stability profiles. It has been demonstrated in published studies that the stability profile of proteins correlates with residual moisture levels in dried cakes. For example, a study with a monoclonal recombinant antibody of lyophilized formulation demonstrated that high moisture levels reduced chemical stability of the antibody in a

glassy or rubbery state but not physical stability when stored below the T_g value (14).

In our study, we discovered that the residual moisture levels of all samples were ~1%. No significant differences were observed among the samples. Therefore, the potential cause of the different stability profiles related to the difference in residual moisture content in the lyophilized cakes was excluded.

Secondly, we examined T_g using DSC. It has been proposed that the mechanism of protein lyoprotection by various excipients is related to glass formation in the dried state (15). That is, immobilization of the protein and additives in a solid, amorphous (i.e., glassy) state results in protection of the protein from chemical and conformational degradation because diffusion rates, and therefore reaction rates, are slow. It has been suggested that it is necessary for a formulation to have a T_g well above the highest anticipated storage temperature for storage stability (16). One might speculate that a glass is more "solid" with a higher T_g and therefore more stable, which was not found to be the case for histidine lyoprotection on the antibody. The values of T_g determined by DSC were 57.5 ± 0.16°C and 57.6 ± 0.14°C for dried samples lyophilized from bulk formulations containing 4 mM histidine and formulations containing 6 mM histidine, respectively. It has also been reported that T_g may not be a good indicator for stability. For example, human growth hormone freeze-dried in stachyose had a higher T_g (134°C) than in trehalose (115°C) but did not show superior stability. Conversely, trehalose offers slightly better stability than does stachyose (16). Hatley (17) has reported that T_0, zero mobility temperature, is a better measure of stability than T_g and represents the safe storage temperature for an active material in an amorphous state. It may be an interesting investigation in the future.

Furthermore, we used FTIR spectroscopy to probe the secondary structure of the antibody in different formulation matrices. FTIR has been used extensively to explore the secondary structure of dried proteins and the interactions between proteins and additives (18). The results of the FTIR studies (data not shown) revealed that the secondary structure profiles appear to be identical for the dried antibody lyophilized from formulations with 4 mM or 6 mM histidine in bulk solution. Estimation of the secondary structure content suggests that ABX-IL8 in both formulations has approximately 69% β-sheet, which is typical of antibody structures analyzed by IR spectroscopy (2). It has been documented that lyoprotection of proteins by stabilizers correlates directly with retention of the native structure in the dried solid. For example, the spectrum of interferon-γ dried in the presence of 1 M sucrose is similar to that of the native aqueous protein, whereas that of the protein dried alone is greatly altered (19). Thus, the mechanism by which stabilizing additives such as sugars minimize aggregation during lyophilization is to prevent unfolding during freezing and drying, which is not apparent here in the case of histidine. Therefore, the potential mechanism of the stabilizing effect of histidine remains unknown.

We further studied the effect of histidine as a potential cryoprotectant. ABX-IL8 was formulated at 55 mg/ml at pH 6 (5 mM histidine) first. Then histidine and sucrose stock solutions were spiked into the above formulation to the final concentrations of 15 mM (41 molar ratio of excipient to antibody) to 138 mM (376 molar ratio excipient to antibody), respectively, on top of the starting concentration of 5 mM histidine. Samples were frozen at –70°C and thawed at room temperature, which constituted one freezing-thawing cycle. Three cycles were carried out. The data (Fig. 2) show that addition of histidine inhibited aggregation under freezing stress in a concentration-dependent manner, suggesting a cyroprotection property of histidine. Minimum aggregate formation was observed at approximately 60 mM (109 molar ratio of histidine:antibody), indicating maximal protection at the concentration of histidine. Quite interestingly, the protective effect afforded by histidine is comparable to that conferred by sucrose.

It is noteworthy that the histidine concentration was much higher here than in the study of lyoprotection of histidine (15 to 138 mM vs. 4 to 40 mM). For the lyoprotection study, histidine was one of the five excipients (histidine, glycine, glutamic acid, mannitol, and polysorbate 20) in the formulations and exhibited the most effective stabilizing effect among them. On the other hand, the presence of these excipients provides spatial separation between the antibody molecules, i.e., "dilution" of the molecules within the glassy matrix to prevent aggregation. The current cryoprotection study was intended to single out histidine and evaluate its effect. In addition, freezing-thawing poses only freezing stress, which is a less stressful condition than lyophilization (dehydration is another stress). Therefore, direct correlation between the cryoprotection and lyoprotection studies is hard to make.

Numerous reports have been published about sucrose's properties as a cryoprotectant as well as a lyoprotectant for proteins. Lyophilization often presents stability problems because of the conformational instability of many proteins when

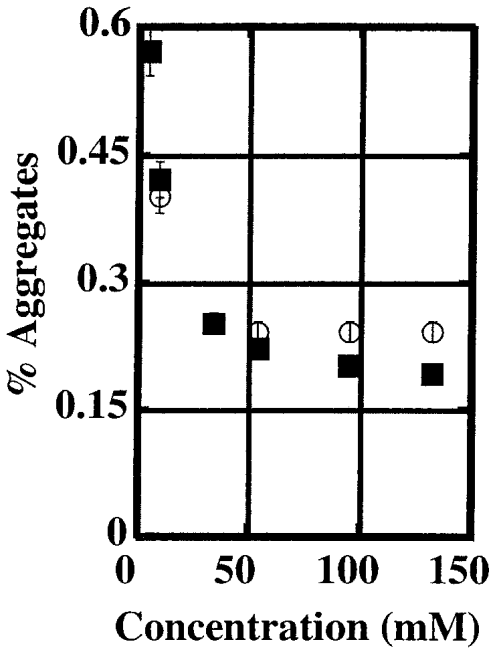

Fig. 2. Effect of histidine and sucrose on the stability of ABX-IL8 under freezing stress. ABX-IL8 was formulated at 55 mg/ml at pH 6 with different levels of histidine (*solid symbols*) or sucrose (*open symbols*). The concentrations of the excipient ranged from 15 mM to 138 mM. Samples were frozen at –70°C and thawed at room temperature for three cycles and assayed with SEC-HPLC.

subjected to the stress of freezing and subsequent dehydration (20). The protein must be stabilized against both of the two fundamentally different stresses (21). Sucrose has been shown to confer protection against both (22,23), but by two different mechanisms. Specifically, sucrose affords protection for proteins through the mechanism of preferential exclusion of sucrose from the protein surface in the frozen state. Timasheff and Lee have established that thermodynamic stabilization of proteins by sugars occurs through preferential exclusion of the sugar from the protein's surface, which increases the protein chemical potential (24). All presently known cryoprotectants have been shown to be preferentially excluded from the surface of proteins (25). The preferential exclusion mechanism for the stabilization of proteins in nonfrozen aqueous systems applies equally well to protein cryopreservation (26).

On the other hand, at least two nonexclusive mechanisms have been proposed for the stabilization of dried proteins by sucrose. First, one mechanism states that proteins are mechanically immobilized in the glassy solid matrix (vitrification) provided by sugars, which restricts translational and relaxation processes, leading to prevention of protein unfolding (15). The second mechanism is often referred to as the water replacement mechanism, which states that sugars protect labile proteins during drying by hydrogen-bonding to polar and charged groups as water is removed and, thus, preventing drying-induced denaturation of proteins (27).

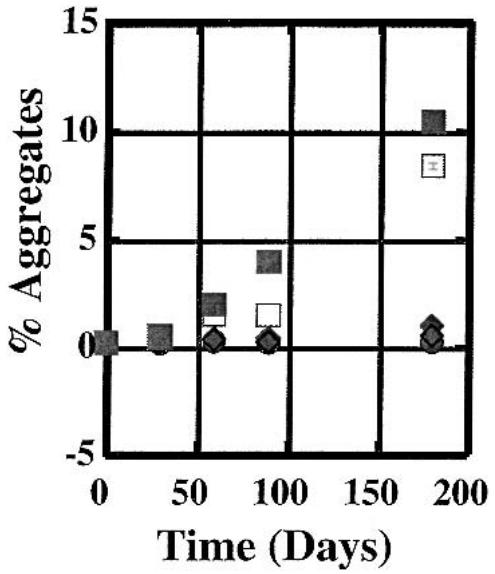

Fig. 3. Comparison of the effect of histidine/arginine and sucrose on the solution stability of ABX-IL8 under thermal stress. *Open symbols* represent ABX-IL8 in formulation A (40 mM histidine, 40 mM arginine, 150 mM sucrose, pH 6). *Solid symbols* represent ABX-IL8 in formulation B (15 mM histidine, 15 mM arginine, 200 mM sucrose, pH 6). Samples were stored at various temperatures: 2–8°C (*circle*), 25°C (*diamond*), and 40°C (*square*).

Effect of Histidine on the Solution Stability of ABX-IL8

A common approach for stabilizing proteins in the aqueous state is the addition of a sugar or salt (28,29). To this end, we have attempted to examine the effect of histidine on the solution stability. ABX-IL8 was formulated at 100 mg/ml into either formulation A consisting of 40 mM histidine, 40 mM arginine, 150 mM sucrose, or formulation B containing 15 mM histidine, 15 mM arginine, 200 mM sucrose. The difference is that 50 mM sucrose was substituted with 25 mM histidine/25 mM arginine in formulation A. The formulations were stored at 2–8°, 25°, and 40°C for 6 months. The data showed that ABX-IL8 in formulation B had higher levels of soluble aggregates than in formulation A when samples were stored at 25°C or 40°C, with samples at 40°C having more pronounced increases of aggregates over time (Fig. 3). The results suggest that histidine combined with arginine conferred better protection than sucrose at the concentrations tested.

Furthermore, we compared the protective effect of histidine to succinate and citrate under thermal stress. Samples of ABX-IL8 formulated in 15 mM histidine or 15 mM succinate or 15 mM citrate, all at pH 6.0, were incubated at 40°C and 50°C. The results (Fig. 4) show that histidine provided a better protection for the antibody against thermal stress than citrate or succinate at the same pH. It is possible that the effect of histidine on enhancing solution stability may be caused by the increase of transition temperature (T_m) of the antibody, which was under investigation using Microcal VP-DSC. VP-DSC was performed from 10° to 100°C at a scan rate of 60°C/h. It was found that the antibody in citrate and succinate buffers was aggregating and precipitating on the thermal unfolding, which caused the posttransition baseline to drop down sharply in the scans (data not shown). On the other hand, the antibody in histidine buffer did not exhibit such shifting of baseline, indicating that the antibody was more thermally stable in histidine buffer than in citrate or succinate buffer. The data also showed that the antibody in histidine buffer exhibited multiple transitions with apparent T_ms at 71°, 76.3°, and 82.9°C, whereas the antibody in succinate or citrate had at least two apparent T_ms centered at *ca.* 72° and 75°C followed by a sharp drop of baseline afterward.

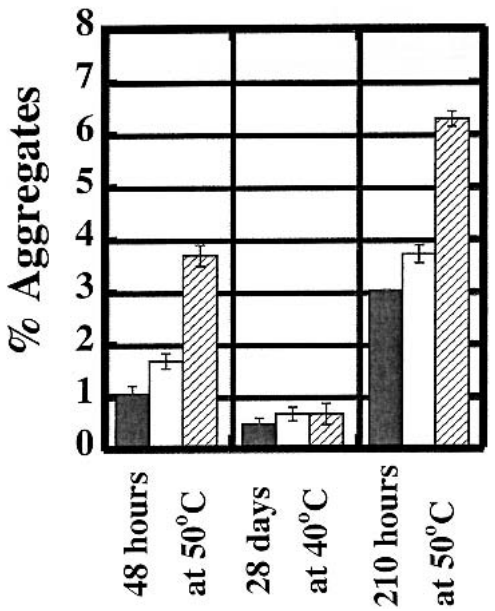

Fig. 4. Comparison of the effect of histidine to succinate and citrate on solution stability of ABX-IL8 under thermal stress. ABX-IL8 was formulated at 55 mg/ml at pH 6 in 15 mM histidine (*solid bars*), 15 mM succinate (*open bars*), or 15 mM citrate buffer (*striped bars*). Samples were stored at 40°C or 50°C and assayed by SEC-HPLC.

It has been documented that disaccharides and sugar alcohols improve the thermal stability of proteins by increasing the transition temperature for heat denaturation of the proteins (30).

Effect of Histidine on the Solution Viscosity of ABX-IL8

In addition to the beneficial effects of histidine on the stability of the antibody in both aqueous and solid states, we further investigated the effect of histidine on solution viscosity, an important property for high-concentration protein formulations. Because proteins or antibodies tend to reversibly associate, formulations containing high concentrations of proteins become viscous (31), which makes it difficult to scale up and manufacture the dosage form. Therefore, any means by which the solution viscosity of a formulation containing a high protein concentration can be effectively reduced would be desirable.

To this end, we have tested the solution viscosity of ABX-IL8 liquid formulations containing different levels of histidine (5 mM to 60 mM). The data (Fig. 5) showed that increasing the concentration of histidine in the formulation led to decreases in viscosity in a concentration-dependent manner.

It has been reported (32) that because of weak self-associations of an antibody, formulations containing the highly concentrated antibody become viscous. The viscosity of the formulation can be reduced significantly by adding salts such as sodium chloride. The study further demonstrated that the self-association of the antibody was not observed by HPLC and sedimentation equilibrium analysis at diluted and high-salt conditions, suggesting that the self-association is reversible and mainly mediated by charge–charge interaction. The observation here on the reduction of viscosity by histidine may also be caused by the disruption of charge–charge interactions between the antibody molecules.

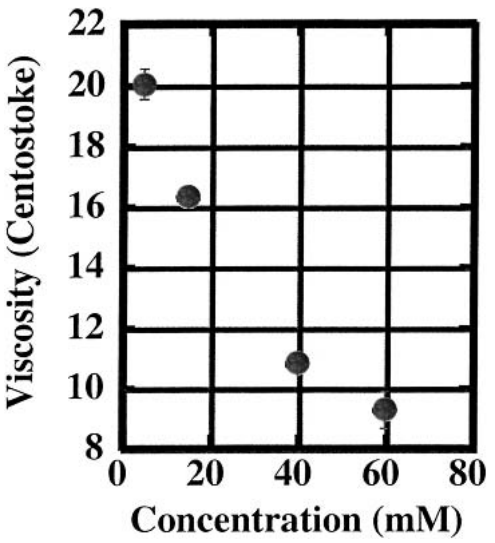

Fig. 5. The effect of histidine on the solution viscosity of ABX-IL8. ABX-IL8 was formulated at 150 mg/ml in 5 mM histidine, pH 6. An aliquot of stock solution (0.5 M histidine, pH 6) was concentrated in a Speed-Vac and added into ABX-IL8 formulations to yield appropriate final concentrations. Viscosity measurement was carried out at room temperature using a Cannon-Fenske capillary viscometer as described in the text.

Researchers have used B_{22}, the osmotic second virial coefficient, to correlate molecular interactions with protein precipitation and crystallization. It was found that the repulsive protein interactions for BSA and myoglobin at high concentration of sodium chloride agreed with the high solubility of the proteins (33).

It should be noted that the potential complexity of the protein–protein interactions in different solution conditions can be caused by the magnitude and distribution of the charges of the proteins. Nevertheless, a study of the correlation of B_{22} with the observed solution viscosity may present an interesting story.

Something to Be Aware of About Histidine Formulations

We have discussed the effects of histidine on the stability of ABX-IL8 in both aqueous and solid states, and its desirable influence on the properties such as solution viscosity. We also observed undesirable effects of histidine, to which one should pay attention. We have noticed that liquid formulations containing a high concentration of histidine (60 mM) had a higher level of aggregates (data not shown) when they went through bulk freezing-thawing in a stainless steel vessel and subsequently were stored at 40°C over time. On the other hand, the same formulation without going through freezing-thawing in the stainless steel vessel had a lower level of aggregates after storage at the same condition. Further investigation showed that the formulation after freezing-thawing in the stainless steel vessel had a higher level of iron (0.01 ppm) than the one without exposure to the stainless steel vessel (not detected; minimum detection limit = 0.003 ppm), suggesting the leaching of iron from the stainless steel vessel.

To confirm that high levels of aggregates observed in samples that went through freezing-thawing in stainless steel vessels were not caused by the stress induced by freezing-thawing. We tested samples in the same formulation that went through freezing-thawing in glass vials and subsequently were stored at 40°C. There were no differences in aggregate levels in the samples compared to the control (samples without freezing-thawing prior to storage at 40°C). To further confirm that the formation of trace metal (i.e., iron ions) as a result of corrosion of stainless steel was the major cause for the aggregation of the antibody at high temperatures, a metal chelating agent (EDTA) was added to the samples that went through freezing-thawing in a stainless steel vessel before incubation. After incubation at 40°C for 4 weeks, aggregate levels in samples containing either 0.02 or 0.05 mM EDTA were comparable to that in samples without freezing-thawing and lower than in the control sample (without the chelating agent). The results suggest that the formation of metal ions, most likely iron ions in the antibody formulation caused by the contact with stainless steel was the main cause for the observed increases in aggregation.

It has been known (34,35) that stainless steel is prone to corrosion in the presence of chloride. A study of recombinant humanized monoclonal antibody, rhuMAb HER2, in liquid formulations demonstrated that the presence of NaCl in the rhuMAb HER2 formulation caused an increase in oxidation of the antibody on storage at higher temperatures after contact with stainless steel containers or stainless steel components in the filling process. Iron ions generated from the corrosion of stainless steel by chloride ions in the low-pH-

formulation buffer catalyzed methionine oxidation in rhuMAb HER2 (35). Therefore, formulations containing higher concentrations of histidine HCl should avoid being stored at elevated temperatures in a stainless steel vessel. Alternatively, sulfate ion can be used as an alternative counterion in order to avoid corrosion issues associated with chloride.

CONCLUSIONS

We have demonstrated that histidine enhances both solid and solution stability of the antibody. In comparison with sucrose, histidine affords comparable protection for the antibody as a cryoprotectant. Furthermore, histidine improves the physical properties of the liquid dosage form of the antibody. Specifically, it reduces solution viscosity. Such an effect on the improvement of physical properties is very desirable for liquid formulations containing a high concentration of proteins or antibodies.

ACKNOWLEDGMENTS

The authors wish to thank Kent Yao, Pannaga Parthasarathy, Celeste Espasandin, and Mike Raffo for excellent technical support, Dr. Rajiv Nayar for carrying out FTIR analysis, Dr. Ruoheng Zhang for his valuable input, and Melanie Schneider and Juan Li for their excellent editorial support.

REFERENCES

1. T. Osterberg, A. Fatouros, and M. Mikaelsson. Development of freeze-dried albumin-free formulation of recombinant factor VIII SQ. *Pharm. Res.* **14**:892–898 (1997).
2. J. L. Cleland, X. Lam, B. Kendrick, J. Yang, T. H. Yang, D. Overcashier, D. Brooks, C. Hsu, and J. F. Carpenter. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. *J. Pharm. Sci.* **90**:310–321 (2001).
3. J. C. Lee and S. N. Timasheff. The stabilization of proteins by sucrose. *J. Biol. Chem.* **256**:7193–7201 (1981).
4. B. S. Kendrick, B. S. Chang, T. Arakawa, B. Peterson, T. W. Randolph, M. C. Manning, and J. F. Carpenter. Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: role in restricted conformational mobility and compaction of native state. *Proc. Natl. Acad. Sci. USA* **94**:11917–11922 (1997).
5. R. A. DePaz, C. C. Barnett, D. A. Dale, J. F. Carpenter, A. L. Gaertner, and T. W. Randolph. The excluding effects of sucrose on a protein chemical degradation pathway: methionine oxidation in subtilisin. *Arch. Biochem. Biophys.* **384**:123–132 (2000).
6. L. L. Green. Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies. *J. Immunol. Methods* **231**:11–23 (1999).
7. X. D. Yang, J. R. Corvalan, P. Wang, C. M. Roy, and C. G. Davis. Fully human anti-interleukin-8 monoclonal antibodies: potential therapeutics for the treatment of inflammatory disease states. *J. Leukoc. Biol.* **66**:401–410 (1999).
8. W. J. Youden and E. H. Steiner. *Statistical Manual of the Association of Official Analytical Chemists,* AOAC International, Arlington, VA, pp. 51–58 (1987).
9. J. C. May, E. Grim, R. M. Wheeler, and J. West. Determination of residual moisture in freeze-dried viral vaccines: Karl Fischer gravimetric and thermogravimetric methodologies. *J. Biol. Stand.* **10**:249–259 (1982).
10. *E. P. A. Manual. 200.7. Determination of Metals and Trace Elements in Water and Wastes by Inductively Coupled Plasma-Atomic Emission Spectrometry,* U.S. Environmental Protection Agency, Cincinnati (1994).
11. M. J. Pikal, K. M. Dellerman, M. L. Roy, and R. M. Riggin. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm. Res.* **8**:427–436 (1991).
12. R. L. Remmele Jr., N. S. Nightlinger, S. Srinivasan, and W. R. Gombotz. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. *Pharm. Res.* **15**:200–208 (1998).
13. L. Kreilgaard, S. Frokjaer, J. M. Flink, T. W. Randolph, and J. F. Carpenter. Effects of additives on the stability of recombinant human factor XIII during freeze-drying and storage in the dried solid. *Arch. Biochem. Biophys.* **360**:121–134 (1998).
14. E. D. Breen, J. G. Curley, D. E. Overcashier, C. C. Hsu, and S. J. Shire. Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. *Pharm. Res.* **18**:1345–1353 (2001).
15. F. Franks, R. H. M. Hatley, and S. F. Mathias. Material science and the production of shelf stable biologicals. *BioPharm* **4**:38–55 (1991).
16. M. J. Pikal. *Mechanisms of Protein Stabilization During Freeze-Drying and Storage: the Relative Importance of Thermodynamic Stabilization and Glassy State Relaxation Dynamics*, Freeze-drying/lyophilization of pharmaceutical and biological products. Drugs and the pharmaceutical sciences. Volume 96. Marcel Dekker, New York (1999).
17. R. H. Hatley. Glass fragility and the stability of pharmaceutical preparations—excipient selection. *Pharm. Dev. Technol.* **2**:257–264 (1997).
18. B. Chen, H. R. Costantino, J. Liu, C. C. Hsu, and S. J. Shire. Influence of calcium ions on the structure and stability of recombinant human deoxyribonuclease I in the aqueous and lyophilized states. *J. Pharm. Sci.* **88**:477–482 (1999).
19. A. Dong, S. J. Prestrelski, S. D. Allison, and J. F. Carpenter. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. *J. Pharm. Sci.* **84**:415–424 (1995).
20. S. D. Allison, T. W. Randolph, M. C. Manning, K. Middleton, A. Davis, and J. F. Carpenter. Effects of drying methods and additives on structure and function of actin: mechanisms of dehydration-induced damage and its inhibition. *Arch. Biochem. Biophys.* **358**:171–181 (1998).
21. J. F. Carpenter, S. J. Prestrelski, and T. Arakawa. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies. *Arch. Biochem. Biophys.* **303**:456–464 (1993).
22. R. L. Remmele Jr., C. Stushnoff, and J. F. Carpenter. Real-time *in situ* monitoring of lysozyme during lyophilization using infrared spectroscopy: dehydration stress in the presence of sucrose. *Pharm. Res.* **14**:1548–1555 (1997).
23. S. D. Allison, A. Dong, and J. F. Carpenter. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying, and rehydration. *Biophys. J.* **71**:2022–2032 (1996).
24. G. Xie and S. N. Timasheff. The thermodynamic mechanism of protein stabilization by trehalose. *Biophys. Chem.* **64**:25–43 (1997).
25. T. Arakawa, S. J. Prestrelski, W. C. Kenney, and J. F. Carpenter. Factors affecting short-term and long-term stabilities of proteins. *Adv. Drug Deliv. Rev.* **46**:307–326 (2001).
26. J. F. Carpenter and J. H. Crowe. The mechanism of cryoprotection of proteins by solutes. *Cryobiology* **25**:244–255 (1988).
27. S. D. Allison, B. Chang, T. W. Randolph, and J. F. Carpenter. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Arch. Biochem. Biophys.* **365**:289–298 (1999).
28. T. Arakawa, Y. Kita, and J. F. Carpenter. Protein–solvent interactions in pharmaceutical formulations. *Pharm. Res.* **8**:285–291 (1991).
29. B. S. Chang, R. M. Beauvais, T. Arakawa, L. O. Narhi, A. Dong, D. I. Aparisio, and J. F. Carpenter. Formation of an active dimer during storage of interleukin-1 receptor antagonist in aqueous solution. *Biophys. J.* **71**:3399–3406 (1996).
30. A. Fatouros, T. Osterberg, and M. Mikaelsson. Recombinant factor VIII SQ—inactivation kinetics in aqueous solution and the influence of disaccharides and sugar alcohols. *Pharm. Res.* **14**: 1679–1684 (1997).

31. R. I. Senderoff, K. M. Kontor, L. Kreilgaard, J. J. Chang, S. Patel, J. Krakover, J. K. Heffernan, L. B. Snell, and G. B. Rosenberg. Consideration of conformational transitions and racemization during process development of recombinant glucagon-like peptide-1. *J. Pharm. Sci.* **87**:183–189 (1998).
32. J. Liu, E. D. Breen, B. Chen, and S. J. Shire. Investigation of thermodynamic properties and stability of a highly concentrated antibody in aqueous solution. *American Association of Pharmaceutical Scientists (AAPS) Annual Meeting*, Indianapolis, Indiana, 2000.
33. P. M. Tessier, H. R. Johnson, R. Pazhianur, B. W. Berger, J. L. Prentice, B. J. Bahnson, S. I. Sandler, and A. M. Lenhoff. Predictive crystallization of ribonuclease A via rapid screening of osmotic second virial coefficients. *Proteins* **50**:303–311 (2003).
34. J. C. Wataha, S. K. Nelson, and P. E. Lockwood. Elemental release from dental casting alloys into biological media with and without protein. *Dent. Mater.* **17**:409–414 (2001).
35. X. M. Lam, J. Y. Yang, and J. L. Cleland. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. *J. Pharm. Sci.* **86**:1250–1255 (1997).