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Spectroscopic evaluation of the stabilization of humanized monoclonal antibodies in amino acid formulations

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Abstract

The protective effects of amino acids on stabilizing protein secondary structure were evaluated using diffuse reflectance FTIR spectroscopy, and interactions between proteins and arginine were detected using solid-state NMR spectroscopy. Upon freeze-drying, excipient-free anti-CD11a and anti-IgE antibodies underwent significant changes in their secondary structures. For both antibodies, the amount of intermolecular β-sheet substantially increased and the native conformation of intramolecular β-sheet content decreased considerably. The addition of amino acids to the formulations reduced protein secondary structure alterations in a concentration-dependent manner. Histidine and arginine appeared to be the most protective excipients (of the amino acids studied) in inhibiting protein secondary structural changes. Solid-state NMR illustrated that non-covalent interactions (e.g., hydrogen bonding, ion–dipole interactions) were formed between the arginine side chain and the protein. Glycine is the least effective additive of those studied in preventing secondary structure changes upon freeze-drying.

Despite secondary structural changes, freeze-dried protein in the presence and absence of amino acids refolded back into its native conformation upon reconstitution in water.

Keywords: Antibodies; Protein; FTIR; NMR; Stabilization; Amino acids; Histidine; Arginine; Glycine

1. Introduction

Shelf-life is a major concern in the development of protein-based therapeutic agents. Ideally, a protein formulation will not only protect the protein against acute damage arising during processing and shipping, but will also permit storage for 24 months or longer. Lyophilization (freeze-drying) is the method most commonly used to prepare dehydrated proteins to allow the product to be stored longer and handled conveniently.

Protein-based therapeutic agents may exist in different solid forms (crystalline, amorphous or mixtures of crystalline and amorphous) before and after lyophilization (Pikal and Rigsbee, 1997). These forms sometimes display very significant differences in physical/chemical stability, solubility and biological activity. Sometimes, the extreme conditions of processing (i.e., freeze-drying, spray-drying) the formulation into a dosage form can alter the native conformations of protein drugs, and significantly impact the stability and bioactivity of the protein (Cleland et al., 2001; Pikal-Cleland and Carpenter, 2001; Taschner et al., 2001; Patro et al., 2002). Hence, sensitive and specific solid-state analytical techniques are necessary for the characterization of new formulations during drug development.

Recent infrared spectroscopic studies have documented that the acute freezing and dehydration stresses of lyophilization can induce protein conformational alterations (Prestrelski et al., 1993a,b, 1994; Dong et al., 1995). This can lead to irreversible protein structural alterations for the rehydrated sample, and also reduce storage stability in the dried solid (Prestrelski et al., 1995; Chang et al., 1996). Therefore, vibrational spectroscopy (FTIR and Raman) provides a convenient means for determining perturbed secondary structures in the dried state.

Intensive studies have been conducted to investigate the protective effect of carbohydrates on freeze-drying induced

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alterations in protein conformation (Ressing et al., 1992; Li et al., 1996; Carrasquillo et al., 2000; Johnson et al., 2002; Souillac et al., 2002; Andya et al., 2003; Gloger et al., 2003; Imamura et al., 2003; Sane et al., 2004). Several mechanisms of protection and stabilization of proteins by carbohydrates have been proposed. Preferential exclusion/hydration is the most common mechanism proposed for most known cryoprotectants. Vitrification (glass formation) and water substitution are the other two prevailing theories of protein stabilization by carbohydrates in the solid state. The first involves the immobilization of proteins in a glassy matrix, which mechanically hinders degradative protein–protein interactions. Moreover, increased viscosity in the glassy state restricts intramolecular movements of a protein, thereby reducing protein unfolding and chemical reactions (Hagen et al., 1995, 1996). The other stabilization mechanism is the water replacement hypothesis (Carpenter et al., 1989, 1991; Arakawa et al., 1991; Crowe et al., 1998). This mechanism involves the formation of hydrogen bonds between a protein and an excipient(s) at the end of the drying process to satisfy hydrogen bonding requirements of polar groups on a protein's surface (Carpenter et al., 1989, 1990). Thus, such excipients are proposed to preserve the native structures of proteins by serving as water substitutes (Carpenter et al., 1990, 1993; Prestrelski et al., 1995). The plausibility of such protein/excipient interactions is supported by many studies that include IR spectroscopy of carbohydrate containing freeze-dried proteins (Chapman et al., 1980; Fell, 1983; Byler and Susi, 1986; Dong and Caughey, 1994; Prestrelski et al., 1995; Souillac et al., 2002).

A few studies have shown that certain amino acids can also be used as cryoprotectants in protein formulations (Carpenter et al., 1986). In addition, amino acids can also be used as lyoprotectants to prevent proteins from dehydration-induced unfolding/aggregation. For example, phenylalanine:arginine:H_3PO_4 in a 1:1:0.5 molar ratio has been found to increase lactate dehydrogenase (LDH) activity by 20% during vacuum-drying (Mattern et al., 1997). The mechanism of stabilizing effects by amino acids, however, is still unclear.

The objective of the current study is to investigate the effects of amino acids as excipients on the structure of humanized monoclonal antibodies in the dried state. Two spectroscopic technologies, diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS) and solid-state NMR spectroscopy were used to this end. DRIFTS was used as a non-invasive spectroscopic method to assess changes in secondary structure of antibodies upon freeze-drying in both the presence and absence of different amino acids (Souillac et al., 2002). Solid-state NMR spectroscopy was used to evaluate potential specific sites of amino acid–protein interactions.

2. Materials and methods

2.1. Materials

A therapeutic recombinant humanized anti-CD11a monoclonal antibody and anti-IgE monoclonal antibody, were produced and provided for this study by Genentech Inc. (San Francisco, CA). The bulk protein solution contained ~30 mg/mL antibody in 5 mM histidine at pH 6.0. L-Histidine, L-arginine, glycine and L-aspartic acid and their complimentary salts were of tissue grade and obtained from either Fisher or Sigma. Uniformly ^{13}C, ^{15}N-labeled arginine chloride, ^{13}C-labeled glycine and ^{15}N-labeled glycine were purchased from Cambridge Isotope Laboratory, Inc. One normal hydrochloric acid and 1.0N sodium hydroxide were obtained from Fisher.

All buffers were prepared with distilled water and filtered through 0.22 μm filters. The buffer pH was adjusted using 1.0N sodium hydroxide or 1.0N hydrochloric acid solutions. The ionic strengths of the protein solutions were not controlled.

2.2. Experimental methods

2.2.1. Freeze-drying conditions

Ten milligram per millilitre anti-CD11a and anti-IgE antibodies containing different excipient concentrations (10, 20, 60, 150 and 200 mM) were transferred into 10-mL flat-bottomed lyophilization vials (Wheaton Co.) and placed on the lyophilizer shelf. Samples were freeze-dried in a Virtis (Genesis) freeze-drier according to the following cycle: samples were frozen at a shelf temperature of −35 °C for 9 h. The chamber vacuum was decreased to 120 mTorr, and the shelf temperature was increased to 0 °C at 12 °C/h. Primary drying was maintained for 40 h at 0 °C. Secondary drying was allowed to proceed for 8 h after the shelf temperature was increased to 20 °C at 10 °C/h. The vacuum remained at 120 mTorr during the entire process. Vials were capped under vacuum, sealed with an aluminum crimp, and stored with Drierite at −20 °C until assayed. Samples were assayed by FTIR and solid-state NMR within 1 week of lyophilization.

2.2.2. FTIR

Infrared spectra were recorded at room temperature using a Nicolet Magna-FTIR 560 spectrometer equipped with a mercury–cadmium–telluride (MCT) detector (Madison, WI). For each spectrum, an accumulation of 256 interferograms was collected in single beam mode with a 2 cm^{-1} resolution from 4000 to 400 cm^{-1}.

For the measurement of aqueous solutions, a 10 mg/ml antibody solution was used to reduce the influence of adsorbed proteins on the spectra (Swedberg et al., 1990; Jackson and Mantsch, 1992; Oberg and Fink, 1998). The solution sample was placed directly on a ZnSe ATR plate (Thermal A.R.K., SpectraTech, Shelton, CT). The effective pathlength of the ZnSe plate employed is 12 μm. For ATR experiments, water subtraction was performed until a straight baseline was obtained between 2000 and 1750 cm^{-1} using Nicolet Omnic 4.0 software. Baseline correction was also applied from 1800 to 900 cm^{-1}.

The FTIR spectra of antibodies in the solid samples were obtained by diffuse reflectance spectroscopy. The diffuse reflectance accessory used was a Graseby Specac Minidiff™ PN 4500 (Graseby Specac Inc., Fairfield, USA). Due to the small quantity of sample available, the lyophilized mixture was simply placed on top of a sample cup that was filled with ground KBr and the top surface was gently leveled so that it was even with the top of the sample cup. Alignment of the

system was performed using powdered KBr. Baseline correction (1800–900 cm^{-1}) was applied to all spectra as well as a Kubelka-Munk correction after referemce subtraction. Curve fitting was performed using a mixed Gaussian/Lorentzian function (Grams/32 software, Galactic Industries). Second-derivative and Fourier self-deconvoluted (FSD) spectra were used as peak position guides for the curve fitting procedure. A bandwidth at half height of 20 cm^{-1} and an enhancement factor of 2.0 were used in the FSD function of the Omnic™ software. The overlapping bands in the deconvoluted Amide I band region were resolved using the PeakSolve software program (Galactic Ind., Salem, NH). The secondary structure content of each sample was measured three times, and the standard deviations were within ±3% of the mean value.

After each sample was placed in the instrument, the sample compartment was purged for at least 20 min before collecting spectra. The spectrum of freeze-dried pure amino acid buffer was subtracted from each sample spectrum. Peaks at ∼1495 cm^{-1} for histidine, ∼1705 cm^{-1} for arginine, ∼1509 cm^{-1} for glycine and ∼1685 cm^{-1} for aspartic acid were subtracted separately.

2.2.3. Solid-state NMR spectroscopy

All ^{13}C spectra were acquired on a Chemagnetics CMX-300 spectrometer using cross polarization and magic-angle spinning. Samples were packed into 7.5 mm zirconia rotors and spun at 4 kHz in a Chemagnetics probe outfitted with a Pencil™ spinning module. Kel-F endcaps were used to hold the sample in the rotor. A variable-amplitude cross-polarization experiment (Peersen et al., 1993) was used. Pulse delays of 5 and 10 s were used for glycine and arginine, respectively. A ^{1}H 90° pulse width of 4.8 μs was used. The ^{13}C NMR spectra were externally referenced to tetramethylsilane using the methyl peak of hexamethyl benzene at 17.35 ppm. The ^{15}N spectra were externally referenced to nitromethane ($\delta = 0.0$ ppm) by using the amino peak of ^{15}N labeled glycine at −346.4 ppm.

3. Results

3.1. FTIR evaluation of antibodies

A mixed Gaussian/Lorentzian curve fitting analysis was used in both the Amide I and II regions because of band overlap. Only the Amide I region was employed, however, to evaluate secondary structural changes upon freeze-drying.

3.1.1. Anti-CD11a antibody

3.1.1.1. Pure antibody. The secondary structure of the anti-CD11a antibody in solution was analyzed using ATR spectroscopy. Fig. 1a shows the deconvoluted spectrum (with curve fitting in the Amide I and II frequency regions) of anti-CD11a antibody in aqueous solution. In the Amide I region, peaks were present at 1690, 1674, 1662, 1652, 1637 and 1617 cm^{-1}. The position of these peaks was consistent with previous measurements of immunoglobulins (Wasacz et al., 1987). The intense major peak at 1637 cm^{-1} and the signal at 1674 cm^{-1} indicate the expected predominantly β-sheet structure (Susi et al., 1967; Susi, 1969; Parker, 1971).

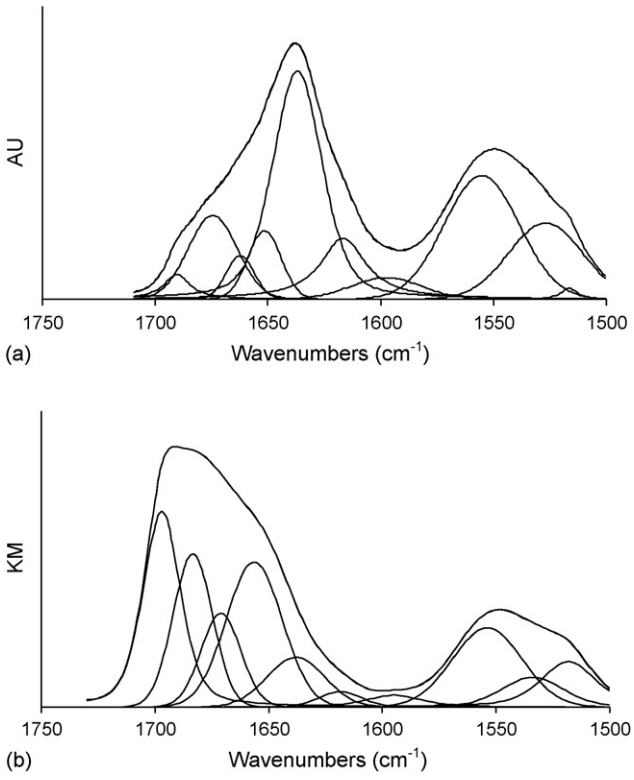

Fig. 1. FTIR spectra of anti-CD11a antibody in the Amide I and II regions. (a) In aqueous solution and (b) freeze-dried.

The peaks located at 1690 and 1662 cm^{-1} can be attributed to β-turns with the feature at 1652 cm^{-1} probably due to a small amount of disordered structure and helix. The peak at 1617 cm^{-1} is probably due to side-chain vibrations (Chan et al., 1996; Griebenow and Klibanov, 1997), but may also arise from intermolecular β-sheets (Dong et al., 1995; Prestrelski et al., 1995).

Overall, the secondary structure of anti-CD11a antibody was estimated to be composed of 67% β-sheet and 33% other structures such as turns and disordered regions (Table 1). This result is in agreement with the X-ray crystallographically estimated secondary structure of IgG which shows 65% β-sheet and 32% other structures (Przybycien and Bailey, 1991).

The structure of freeze-dried anti-CD11a antibody in the absence of excipients was assessed by diffuse reflectance spectroscopy (Fig. 1b). When compared with the spectrum of the native anti-CD11a antibody in aqueous solution, the freeze-dried FTIR spectrum of the freeze dried protein shows substantial structural differences including broadening and significant shifts toward higher wavenumbers of the Amide I spectral components. These changes indicate a significant loss of order in the individual elements of the protein's secondary structure as a result of the drying procedure (Prestrelski et al., 1993b; Dong et al., 1995; Griebenow and Klibanov, 1995, 1997; Wellner et al., 1996; Souillac et al., 2002). Similar observations were shown in Souillac's (2002) work with two different proteins (rh-DNase and rh-IGF-1). Changes in the secondary structure of lyophilized proteins seem to be somewhat independent of the protein's predominant conformation since generally

Table 1
Secondary structure contents of pure anti-CD11a and anti-CD11a/excipient mixtures based on the Amide I region

Protein samples	β-sheet		Unordered (%)	Others (%)
	Intermolecular (%)	Intramolecular (%)		
Pure anti-CD11a				
Solution	0	67	9	24
Freeze-dried	28	23	27	22
15% Excipient				
Histidine	21	24	22	33
Arginine	24	26	30	20
Glycine	32	14	38	16
Aspartic acid	29	18	18	34
51% Excipient				
Histidine	14	32	15	39
Arginine	11	39	33	17
Glycine	35	14	39	12
Aspartic acid	24	26	18	32
71% Excipient				
Histidine	5	58	20	17
Arginine	7	53	23	16
Aspartic acid	20	29	21	30
Physically mixed (1:1, w/w)				
Histidine	29	24	19	28
Arginine	28	24	21	27
Pure anti-CD11a				
Reconstituted	0	65	15	20

the structure that forms in the absence of lyoprotectants is intermolecular β-sheet. Several significant peaks in the Amide I region are observed at 1697, 1683, 1671, 1656, 1638 and 1618 cm^{-1}. The most obvious difference is the appearance of a peak at about 1697 cm^{-1} for the lyophilized anti-CD11a antibody. It has been claimed that the presence of a peak at this wavenumber is indicative of intermolecular β-sheet formation as a result of increased protein–protein contact in the solid state (van de Weert et al., 2001). The formation of intermolecular β-sheet arising from aggregation has been observed for many other proteins upon drying (Prestrelski et al., 1993a; Dong et al., 1995; van de Weert et al., 2001; Souillac et al., 2002). The peak at 1656 cm^{-1} is attributed to unordered structures. Quantification of the secondary structure by mixed Gaussian/Lorentzian curve fitting of the Amide I FTIR spectrum revealed a large decrease in the intramolecular β-sheet content from 67 to 23%, and an increase in the intermolecular β-sheet content from zero to 28% upon freeze-drying. The content of disordered structure also increased from 9 to 27% after freeze-drying (Table 1). Thus, the secondary structure of the anti-CD11a antibody is significantly changed upon freeze-drying. Similar observations were previously reported for other proteins containing β-sheet (Dong et al., 1998; Sane et al., 2004), as well as for α-helical containing proteins (Souillac et al., 2002).

3.1.1.2. Histidine- and arginine-containing mixtures. Various ratios of protein/histidine freeze-dried sample mixtures were characterized by DRIFTS. The concentrations of 15, 51 and 71% (w/w) histidine were chosen based on previous calorimetric studies (Tian et al., 2006). The FTIR spectra obtained indicate a significant progressive shift of the Amide I region toward lower wavenumbers in the presence of increased amounts of histidine (Fig. 2). The changes observed in secondary structure that were found for the lyophilized protein in the absence of histidine are partially inhibited. After Gaussian/Lorentzian curve fitting (Table 1), the intensities of the peaks corresponding to intramolecular β-sheet (at 1638 and around 1674 cm^{-1}) increase gradually from 24% (for 15% histidine) to 58% (for 71% histidine). Simultaneously, the high frequency intramolecular β-sheet band shifts from 1696 cm^{-1} for 15% histidine to 1693 cm^{-1} for 51% histidine, then to 1691 cm^{-1} for 71% histidine with a four-fold decrease in band area. Thus, the presence of relatively large amounts of histidine significantly inhibits the secondary structure changes of the anti-CD11a antibody upon freeze-drying.

FTIR spectra were also obtained for a physical mixture of the anti-CD11a antibody with histidine at a 1:1 weight ratio. There was no significant difference in the Amide I region compared to that of the pure freeze-dried anti-CD11a antibody (Table 1). Similar results to those found with the freeze-dried anti-CD11a/histidine were observed when analyzing the anti-CD11a in the presence of arginine freeze-dried samples (Table 1).

3.1.1.3. Glycine-containing mixtures. Table 1 lists the assignments of the peaks obtained from curve fitting in the Amide I region for glycine-containing samples. Quantitative analysis shows that 15 and 51% glycine produced even greater

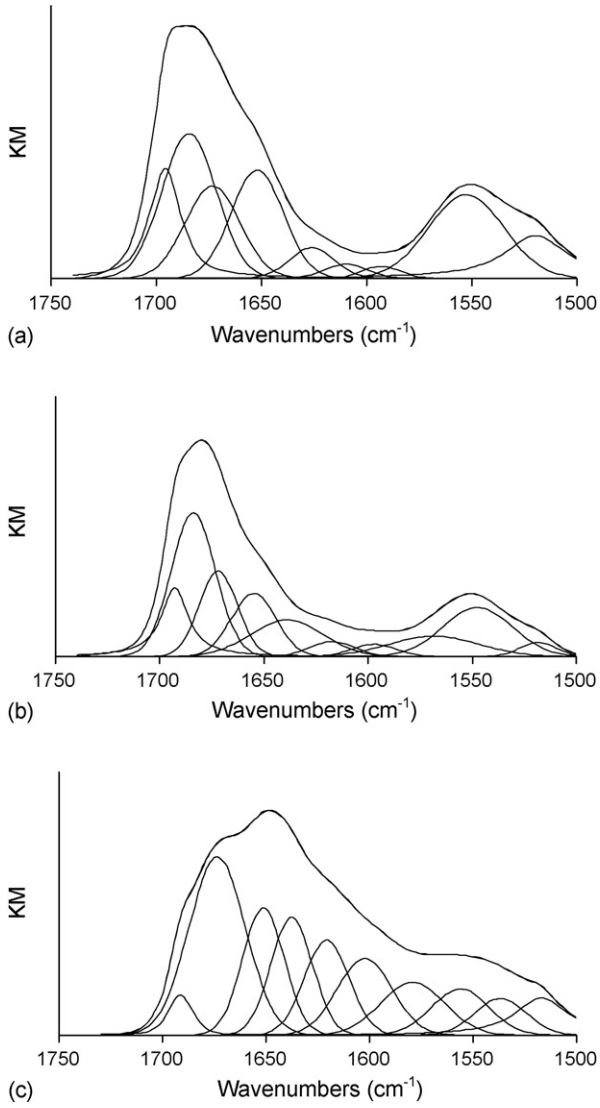

Fig. 2. FTIR spectra of anti-CD11a/histidine freeze-dried samples in the Amide I and II regions. (a) 15% Histidine, (b) 51% histidine and (c) 71% histidine.

amounts of intermolecular β-sheet (due to protein unfolding and aggregation during the drying phase) and lower amounts of intramolecular β-sheet (representing the native conformation of the protein) compared to the FTIR spectrum of the anti-CD11a antibody in the absence of amino acids. Furthermore, the content of unordered structure increased in the glycine formulations. These results may be caused by the crystallization of glycine during the freeze-drying process. When glycine molecules are excluded from contact with protein molecules and form crystals, the concentration of protein will increase, enhancing the tendency to form protein–protein interactions. These FTIR results agreed with the absence of protective effects seen with glycine in previous calorimetric studies (Tian et al., 2006). Among all of the amino acids examined, glycine appears to be the least effective in preventing protein secondary structure changes upon freeze-drying.

Due to the interference of glycine signals, FTIR spectral analysis could not be interpreted in antibody samples containing more than 51% glycine. Meyer also demonstrated that reliable

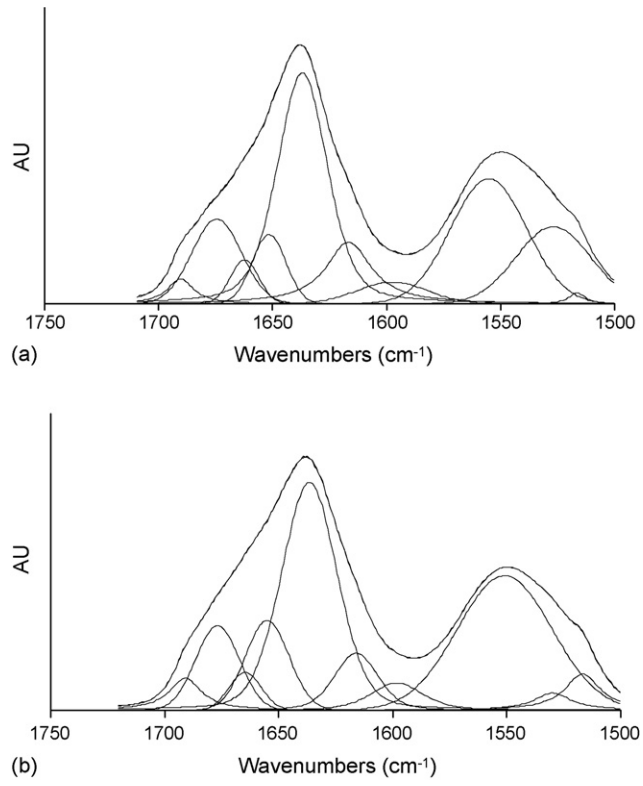

Fig. 3. FTIR spectra of anti-CD11a antibody in the Amide I and II regions. (a) In aqueous solution and (b) after reconstitution.

Amide I spectra can be obtained by accurate subtraction of glycine signals only if the protein-to-glycine ratio was ≥1:1 (Meyer et al., 2004).

3.1.1.4. Aspartic acid-containing mixtures. The results of curve fitting of the original spectra from lyophilized anti-CD11a/aspartic acid formulations reveal decreasing amounts of intermolecular β-sheet and increasing amount of native-like intramolecular β-sheet as the percentage of aspartic acid increases (Table 1). Even though there were protective effects seen in the 71% aspartic acid sample, a significant amount of aggregation (intermolecular β-sheet) is still detected in the sample. Thus, FTIR experiments indicate that aspartic acid is not as effective as histidine or arginine in preserving the native conformation of these antibodies during the freeze-drying process.

3.1.1.5. Reconstituted antibody. ATR spectroscopy was also used to obtain the FTIR spectrum of anti-CD11a antibody reconstituted in solution. To evaluate the retention of secondary structure alteration of the anti-CD11a antibody upon freeze-drying in the absence of amino acids, the sample was prepared after reconstitution of freeze-dried pure protein in distilled water. Fig. 3 indicates that there was no noticeable difference in the Amide I region of the reconstituted anti-CD11a antibody spectrum compared to that of the protein initially seen in solution.

After curve fitting, no significant differences in the secondary structure of the anti-CD11a antibody were observed in the Amide I region of the reconstituted sample (Table 1). Thus, the altered conformation appears to completely refold back into

Table 2
Secondary structure contents of pure anti-IgE and anti-IgE/excipient mixtures based on the Amide I region

Protein samples	β-sheet		Unordered (%)	Others (%)
	Intermolecular (%)	Intramolecular (%)		
Pure anti-IgE				
Solution	0	75	6	19
Freeze-dried	31	21	14	34
16% Excipient				
Histidine	31	23	12	35
Arginine	20	23	17	40
Glycine	34	17	25	24
Aspartic acid	28	18	21	34
51% Excipient				
Histidine	20	33	14	33
Arginine	7	34	20	39
Glycine	34	17	31	18
Aspartic acid	23	23	16	38
72% Excipient				
Histidine	5	64	17	13
Arginine	2	47	17	34
Aspartic acid	21	35	21	23
Physically mixed (1:1, w/w)				
Histidine	32	23	11	34
Arginine	29	21	13	37
Pure anti-IgE				
Reconstituted	0	67	7	25

native protein after re-dissolution in water. This result indicates the reversibility of the lyophilization-induced secondary structural changes upon rehydration of the freeze-dried samples. Although this altered conformation in the dried protein can fold back into a native-like form after freeze-drying, long-term storage could still result in more permanent physical or chemical changes to the antibody.

3.1.2. Anti-IgE antibody

As shown in Table 2, similar results were obtained for the anti-IgE antibody with the same amino acids. Like the anti-CD11a antibody, the presence of a large amount of histidine (72% histidine, w/w) significantly inhibits changes in the secondary structure of anti-IgE antibody upon lyophilization. The addition of 72% arginine, however, is less effective for anti-IgE than for the anti-CD11a antibody. After reconstitution, the anti-IgE antibody also refolded primarily into its native structure.

3.2. Solid-state NMR

3.2.1. Arginine-containing mixtures

3.2.1.1. Anti-CD11a antibody. Weak interactions between molecules, such as hydrogen bonding or ion–dipole interactions, can often be detected by monitoring the changes in chemical shifts of specific nuclei using solid-state NMR spectroscopy. In the current study, ^{13}C and ^{15}N solid-state NMR spectroscopy were performed to evaluate the interactions between arginine and the anti-CD11a antibody (at a 1:1 weight ratio of lyophilized sample). As shown in Fig. 4b, the chemical shifts of the carbonyl

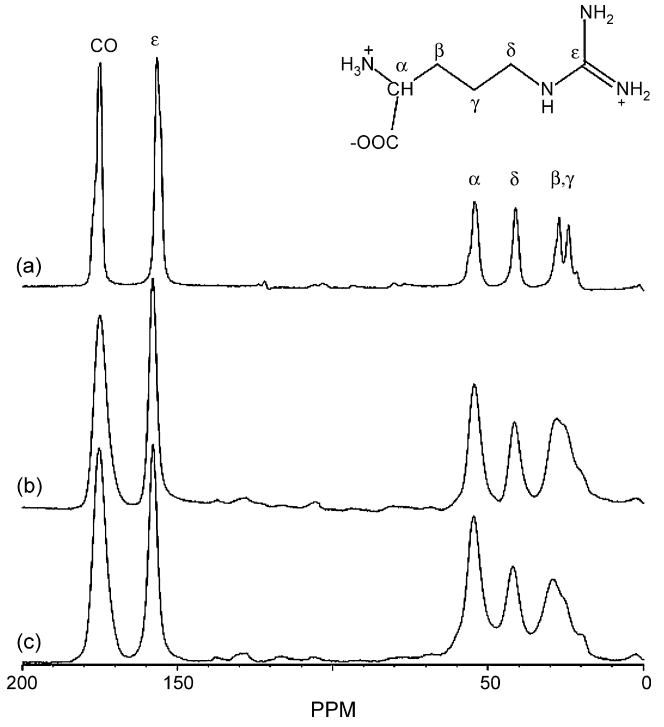

Fig. 4. (a) ^{13}C NMR spectrum of lyophilized arginine in the absence of protein, (b) ^{13}C NMR spectrum of lyophilized arginine in the presence of anti-CD11a antibody (1:1, w/w) and (c) ^{13}C NMR spectrum of lyophilized arginine in the presence of anti-IgE antibody (1:1, w/w). All spectra acquired using a 10 s pulse delay, 4 kHz spinning speed, and 2.00 ms contact time.

Table 3
^{13}C, ^{15}N NMR chemical shifts for pure lyophilized amino acids and protein/amino acid lyophilized formulations (1:1, w/w)

	Chemical shifts		
	Pure lyophilized amino acids	Anti-CD 11a/amino acid (1:1, w/w)	Anti-IgE/amino acid (1:1, w/w)
C-13 Gly			
δ_{CO}	176.8	175.6	175.8
N-15 Gly			
δ_{NH}	−346.3	−347.5	−347.4
C-13 Arg			
δ_{CO}	175.4	175.4	175.3
δ_{ε}	156.7	158.3	158
δ_{α}	54.4	54.8	54.7
δ_{δ}	41.2	41.9	42.1
δ_{β}	27.3	28.9	28.7
δ_{γ}	24.2 21.6		
N-15 Arg			
N_4	−288.7	−294.5	−294.3
N_2	−296.5	−303.2	−304.2
N_3	−311.2		
N_1	−333.8	−335	−335.3

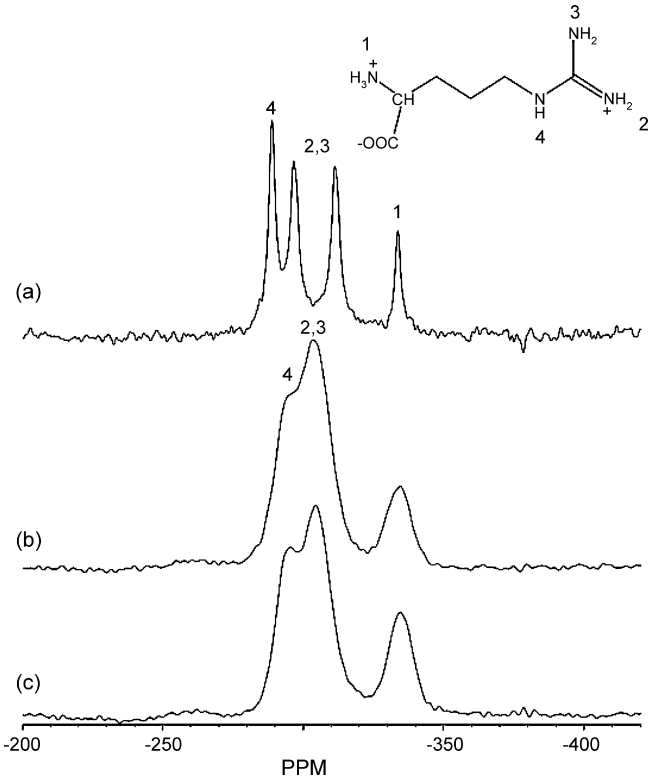

Fig. 5. (a) ^{15}N NMR spectrum of lyophilized arginine in the absence of protein, (b) ^{15}N NMR spectrum of lyophilized arginine in the presence of anti-CD11a antibody (1:1, w/w) and (c) ^{15}N NMR spectrum of lyophilized arginine in the presence of anti-IgE antibody (1:1, w/w). All spectra acquired using a 10 s pulse delay, 4 kHz spinning speed, and 1.00, 0.5 and 1.00 ms contact time for (a), (b) and (c), respectively.

carbon (C=O) and C_α of arginine did not show any significant changes except for a broadening of their line-widths, indicating more amorphous character. The absence of changes in chemical shifts suggests no interaction of the protein with the backbone of arginine. The increase in overlap and shifts of the arginine C_β and C_γ signals are probably due to the more amorphous environment formed when the amino acid is co-freeze-dried with the antibody. This is supported by the partial amorphization displayed in Fig. 4a with the behavior of the shoulder peaks along with the $C_{c=o}$, C_α and C_δ signals when pure arginine was lyophilized. The chemical shift of C_δ does not show any significant changes except for peak broadening. In contrast, the chemical shift of C_ε (the carbon at the end of guanidine side chain of arginine) moved about 2 ppm downfield (Table 3). This change is, in fact, consistent with weak intermolecular interactions between the excipient arginine side chain and the protein.

In the ^{15}N NMR spectra, the chemical shift of N_1 on the arginine backbone remained unchanged (Fig. 5), confirming the absence of interactions between the protein and the backbone of arginine suggested by ^{13}C NMR. The other three nitrogens on the guanidine side chain of arginine shifted with N_2 and N_3 combining into a single peak at 303.2 ppm and N_4 shifted downfield (Table 3). These changes can be explained by the conjugated resonance formed among these three nitrogens in the more amorphous form. The interaction of one of the nitrogens with the antibody will perturb the other two, causing changes in chemical shifts. Thus, the ^{15}N solid-state NMR results provide further evidence that interactions occur between the side chain of arginine and the protein surface. All of the protein/arginine (1:1, w/w) lyophilized samples produced broader ^{15}N resonances than pure arginine, again suggesting the presence of more amorphous material being formed when co-freeze-dried with the protein.

3.2.1.2. Anti-IgE antibody. ^{13}C and ^{15}N solid-state NMR measurements were also obtained for anti-IgE antibody in the presence and absence of arginine (Figs. 4c and 5c). Similar results were obtained, suggesting the presence of direct interactions between the guanidine side chain and the anti-IgE antibody. Furthermore, the larger shifts of the C_β and C_γ of arginine in freeze-dried anti-IgE suggest greater changes in the surrounding environment compared to those of anti-CD11a (Fig. 4b and c).

3.2.1.3. Glycine-containing mixtures. For glycine-containing lyophilized samples of both anti-CD11a and anti-IgE antibodies, ^{13}C NMR resonances of the glycine carbonyl carbon ($\delta_{c=o}$) shifted upfield approximately 1 ppm (Fig. 6), while the ^{15}N NMR resonances of the nitrogen (δ_{NH}) shifted downfield by ~1 ppm (Fig. 7). The chemical shifts of a 1:1 weight ratio of lyophilized antibody/glycine are summarized in Table 3. These small changes in NMR resonances between pure glycine and antibody/glycine could be due to either the presence of different polymorphs of glycine or an interaction with the protein. Akers et al. (1995) showed that a neutral glycine solution with no additives formed a β-polymorph during freezing. Increased ionic strength significantly slows the crystallization of neutral glycine and promotes formation of the γ-polymorph. Other studies (Shalaev et al., 1992; Pyne and Suryanarayanan, 2001) have shown that crystallization of β-glycine occurs upon cooling of

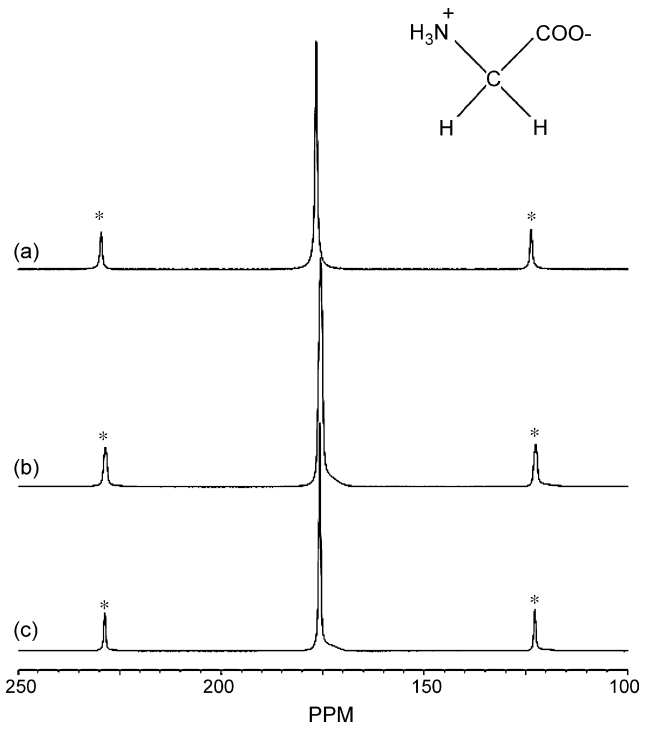

Fig. 6. (a) ^{13}C NMR spectrum of lyophilized glycine in the absence of protein, (b) ^{13}C NMR spectrum of lyophilized glycine in the presence of anti-CD11a antibody (1:1, w/w) and (c) ^{13}C NMR spectrum of lyophilized glycine in the presence of anti-IgE antibody (1:1, w/w). All spectra acquired using a 5 s pulse delay, 4 kHz spinning speed, and 0.5 ms contact time. *Denotes spinning sidebands.

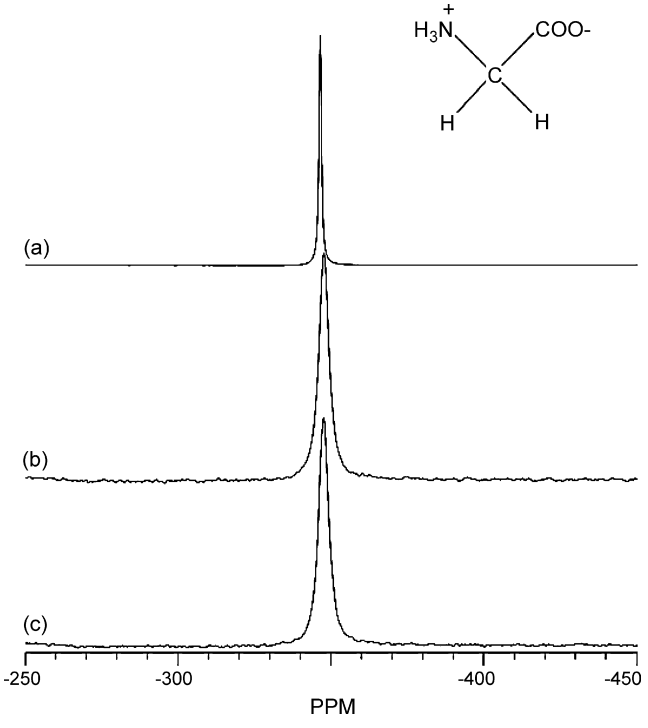

Fig. 7. (a) ^{15}N NMR spectrum of lyophilized glycine in the absence of protein, (b) ^{15}N NMR spectrum of lyophilized glycine in the presence of anti-CD11a antibody (1:1, w/w) and (c) ^{15}N NMR spectrum of lyophilized glycine in the presence of anti-IgE antibody (1:1, w/w). All spectra acquired using a 5 s pulse delay, 4 kHz spinning speed, and 2.25 ms contact time.

aqueous solutions. Although β-glycine was usually found in the freeze-dried systems, it converts readily to mixtures of γ- and α-glycine at room temperature in the presence of moisture (Chongpresart, 1998). Since previous calorimetric studies (Tian et al., 2006) suggested that there was no interaction between glycine and these same proteins upon freeze-drying, these chemical shift differences are most likely due to the formation of a polymorph when glycine was co-freeze-dried with protein. Further solid-state NMR studies of glycine polymorphs would be of interest in this regard.

4. Discussion

A variety of previous studies have monitored the secondary structural changes of proteins induced by freeze-drying (Prestrelski et al., 1993b; Griebenow and Klibanov, 1995; Souillac et al., 2002; Andya et al., 2003). FTIR spectroscopy has proven to be a valuable technique for determining differences in protein conformation in the solid state. It has been argued that perturbing the conformation of predominantly β-sheet proteins would decrease the intensity of the intramolecular β-sheet (native) peaks and increase those of intermolecular β-sheet (unfolding/aggregation) and turn bands (Allison et al., 1999). This was also observed here. Lyophilization of the antibodies examined here in the absence of amino acids (used as excipients) induced dramatic structural changes in which β-sheets are altered from an intramolecular to an intermolecular form (aggregation). These results demonstrate that the secondary structures of antibodies are substantially distorted during the freeze-drying process. These changes may impact the stability of such pharmaceutically important proteins during both short- and long-term storage (Arakawa et al., 2001).

To detect any stabilizing effect of amino acids on lyophilization-induced protein secondary structure changes, the structural consequences of the presence of different weight ratios of antibodies to various amino acids were probed in the solid state. Substantial amounts of intramolecular β-sheet and relatively small amounts of protein aggregation (intermolecular β-sheet) were observed in the histidine- and arginine-containing samples at high excipient concentration, indicating some inhibition of freezing and drying stresses. The shapes and positions of the FTIR bands for the highest amount of histidine- and arginine-containing dried samples, however, were different than that of the protein in solution. The estimated secondary structures of dried antibodies in the presence of various amounts of aspartic acid showed less protective effects upon lyophilization, while in the presence of glycine, the secondary structures of dried antibodies were significantly different than their native structures in solution, indicating the least protective effect among the four amino acids tested. Some previous studies have also indicated a protective effect of histidine and arginine on protein stability and biological activity during lyophilization and storage. Osterberg et al. (1997) found that L-histidine produced good recovery of recombinant factor VIII-SQ after freeze-drying with good storage stability. Izutsu et al. (1991) discovered that L-arginine·HCl prevented inactivation of β-galactosidase upon freeze-drying and during storage. Similar results were also

obtained using L-histidine·HCL. However, glycine had no effect on the inhibition of β-galactosidase inactivation. A recent study (Mattern et al., 1999) suggested that lactate dehydrogenase activity was protected in a Phe/Arg/H_3PO_4 (1:1:1) formulation during vacuum-drying and storage.

Excipient induced preservation of native structure has often been explained in terms of a preferential exclusion mechanism when protein solutions are frozen (Carpenter et al., 1999; Arakawa et al., 2001). This proposes that the native conformations of proteins are stabilized by the preferential exclusion of many additives, since the free energy of the system increases as the chemical potential of the solvent increases at the protein surface (Arakawa and Timasheff, 1982). For example, glycine increased the recovery of protein activity when inactivation occurred by freezing rabbit skeletal muscle phosphofructokinase (PFK) in liquid nitrogen for 30 s (Carpenter et al., 1986). In our studies, stabilizing effects (arginine ≥ histidine > aspartic acid) on the native structure of the proteins were found in the presence of charged amino acids. Glycine, used as a neutral amino acid, however, did not show any significant inhibition of protein secondary structural changes. These results suggest that weak interactions, such as ion–dipole interactions, may be present between protein and charged amino acids to preserve the native conformation of the protein. This is consistent with a preferential binding (rather than exclusion) mechanism in the freezing stage. Furthermore, even the addition of relatively large amounts of glycine produced little protective effect on protein secondary structural changes, consistent with a preferential binding mechanism.

During the dehydration phase, a preferential exclusion mechanism is no longer applicable because the hydration shell of the protein is removed (Carpenter et al., 1993; Crowe et al., 1993; Allison et al., 1996). Vitrification (glass formation) and water substitution have been proposed as two non-exclusive mechanisms for stabilizing protein during the drying stage. The vitrification (glass formation) hypothesis is purely kinetic in origin and related to molecular mobility. Proteins are immobilized in the glassy matrix to restrict mobility, thereby sterically hindering protein unfolding/aggregation. It has been hypothesized that proteins act as amorphous substrates and tend to form protein-rich aqueous concentrates interspersed among ice crystals. Amorphous excipients form part of the protein-rich glassy concentrate. These behaviors can have important implications for the stability of proteins during freezing, freeze-drying, and storage of the freeze-dried product (Hora et al., 1992). Sucrose and trehalose demonstrate a protective effect while maintaining their amorphous state during freeze-drying and storage, whereas freeze-dried mannitol with some portion of crystalline structure has a less marked protective effect (Izutsu et al., 1994; Izutsu and Kojima, 2002; Souillac et al., 2002).

As illustrated by the previous calorimetric analyses (Tian et al., 2006), histidine and arginine take on amorphous forms during freeze-drying, while glycine tends to remain in a crystalline state even when co-freeze-dried with antibodies. In this study, we found that maximal retention of native structure occurred at the highest concentration of histidine or arginine in both antibody formulations. On the other hand, glycine at all concentrations in the mixtures appears to have little if any protective effect on the native conformation of the proteins. The results obtained from both studies suggest that the protective effect in freeze-dried cakes appears to be correlated with the crystallinity of these amino acids. Histidine (or arginine) probably forms a glassy matrix, which restricts protein mobility and separates protein molecules, consequently inhibiting protein unfolding and protein aggregation. Glycine is excluded from the protein surface and crystallizes to form a more ordered, restricted structure, thus leading to phase separation. Aspartic acid shows partial amorphization (Tian et al., 2006), which suggests less protective effect on protein secondary structural changes. As a result, protein stability may be enhanced by the immobilization of protein molecules accompanied by glass formation using histidine or arginine.

The water substitution hypothesis proposes a direct interaction, such as hydrogen bonding, between protein and excipient. Thus, the excipients interact directly with proteins and replace water by hydrogen bonding to the protein's polar groups. Many IR spectroscopic studies have confirmed the formation of hydrogen bonds between carbohydrates and freeze-dried proteins (Carpenter et al., 1989; Crowe et al., 1993; Prestrelski et al., 1993b; Remmele et al., 1997; Allison et al., 1999; Souillac et al., 2002). Allison et al. (1999) argued that the extent of hydrogen bonding between sucrose/trehalose and lysozyme determined the degree of protection of protein's secondary structure. Protonated histidine and arginine are good hydrogen bonding donors since they have a protonated nitrogen on the side chain (the weakly basic imidazolium functionality for histidine and the basic guanidine group for arginine at pH 6). Protonation also produces a cation. Therefore, direct interactions, such as H-bonds or ion–dipole interactions, may form between these two basic amino acids and the antibody to facilitate stabilization of the protein in the dried state. This hypothesis is supported by calorimetric studies and solid-state NMR analysis. Isoperibol calorimetry and DSC experiments (Tian et al., 2006) indicate that such interactions are present between the proteins and histidine (or arginine) upon lyophilization. The binding enthalpy value is around 8 kJ/mol, which is in the normal range of weak interactions. Moreover, solid-state NMR reported here suggests that the guanidine side chain of arginine formed a direct interaction with the protein. The chemical shift difference of ∼2 ppm seems to indicate the formation of weak interactions with the arginine's side chain. These direct interactions between protein and arginine (or histidine) may account for stabilizing the native structure of protein through simple preferential binding to this state and subsequent mass action effects. On the other hand, glycine has no side chain. This lack of ion–dipole interaction for glycine as well as its poor hydrogen bonding ability because of its crystallization confers less stabilization relative to histidine and arginine.

Since amorphous states of proteins and stabilizers appear important to permit maximal interactions between protein and stabilizer molecules, crystallization of amorphous protein stabilizers during lyophilization often causes protein destabilization due to reduced direct interactions. Izutsu and Kojima (2002) found that mannitol had weak effects on the maintenance

of lactate dehydrogenase secondary structure during freeze-drying due to its crystallization. When mannitol crystallinity was reduced by a combination of mannitol and a phosphate buffer, protein secondary structure was retained to a greater extent. Kreilgaard et al. (1999) found that 300 mM mannitol in the formulation destabilized *Humicola lanuginosa* lipase because mannitol (at 85%) crystallized during lyophilization. In this study, it appears that glycine crystallized from the amorphous protein preventing stabilizing interactions. The molecules in a crystalline material should be able to form more ordered, restricted interactions with themselves than would molecules in an amorphous state.

The several mechanisms discussed above should all be considered in attempting to account for the stabilization of proteins conferred by amino acids against freezing and drying stresses. The amorphicity of stabilizers required in preserving protein structure can be explained by the contribution of glass formation during drying. In terms of interactions, charged amino acids gave better protection suggesting that preferential binding may occur during freezing. The water substitution mechanism may also apply during the drying phase based on the interactions detected from both calorimetric and solid-state NMR analyses.

All secondary structural changes were essentially reversible upon rehydration. This observation might be taken to suggest that the presence of excipients-like amino acids is not necessary to prevent irreversible changes, even though they decrease the extent of secondary structural alterations upon lyophilization. The presence of the appropriate amino acid in the formulation, however, could play a significant role in long-term storage stability. This has been confirmed in several recent studies (Andya et al., 2003; Sane et al., 2004). An excipient-free formulation of a recombinant humanized monoclonal antibody resulted in reversible solid-state protein structural alteration, but increased aggregation during storage. The change caused by the removal of tightly bound water from the protein surface during the lyophilization correlated with increased protein aggregates during storage. When formulated with carbohydrates, native-like solid-state protein structure was obtained and aggregation during storage was reduced.

Carpenter et al. (1999) have proposed that preservation of native protein structure in a dried form plays an important role in the improvement of the storage stability of most protein formulations. Conformationally altered protein, although it can refold upon hydration, usually exhibits a greater tendency to both physical and chemical degradation. This may lead to long-term events which result in the native structure not being re-attained after rehydration and a consequent loss of biologic activity or alteration in immunogenicity (Arakawa et al., 2001). Thus, merely achieving the preservation of protein activity over the short term but not optimizing the stabilization of the native structure may result in misleading predictions of the shelf life of protein formulations.

In comparison with carbohydrates, higher concentrations of histidine (or arginine) are required to stabilize protein conformation during the freeze-drying process. The lower effective concentration and the more native-like protein structure produced by the presence of carbohydrates (i.e., sucrose, trehalose) (Souillac et al., 2002) indicate that carbohydrates are often superior stabilizers for proteins during lyophilization. Even though sucrose and trehalose appear to be preferred stabilizers based upon their lower effective concentration (Souillac et al., 2002), histidine and arginine can still be considered as potential stabilizers to expand the choice of lyo-protectants for freeze-drying protein formulations.

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References

Akers, M.J., Milton, N., Byrn, S.R., Nail, S.L., 1995. Glycine crystallization during freezing: the effects of salt form, pH, and ionic strength. Pharm. Res. 12, 1457–1461.

Allison, S.D., Chang, B., Randolph, T.W., Carpenter, J.F., 1999. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Arch. Biochem. Biophys. 365, 289–298.

Allison, S.D., Dong, A., Carpenter, J.F., 1996. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying and rehydration. Biophys. J. 71, 2022–2032.

Andya, J.D., Hsu, C.C., Shire, S.J., 2003. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. AAPS PharmSci 5, E10.

Arakawa, T., Prestrelski, S.J., Kenney, W.C., Carpenter, J.F., 2001. Factors affecting short-term and long-term stabilities of proteins. Adv. Drug Deliv. Rev. 46, 307–326.

Arakawa, T., Timasheff, S.N., 1982. Stabilization of protein structure by sugars. Biochemistry 21, 6536–6544.

Arakawa, T., Yoshiko, K., Carpenter, J.F., 1991. Protein-solvent interactions in pharmaceutical formulations. Pharm. Res. 8, 285–291.

Byler, D.M., Susi, H., 1986. Examination of the secondary structure of proteins by deconvolved FTIR spectra. Biopolymers 25, 469–487.

Carpenter, J.F., Arakawa, T., Crowe, J.H., 1991. Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. Dev. Biol. Stand. 74, 225–239.

Carpenter, J.F., Crowe, J.H., Arakawa, T., 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry 28, 3916–3922.

Carpenter, J.F., Crowe, J.H., Arakawa, T., 1990. Comparison of solute-induced protein stabilization in aqueous solution and in frozen and dried state. J. Dairy Sci. 73, 3627–3636.

Carpenter, J.F., Hand, S.C., Crowe, L.M., Crowe, J.H., 1986. Cryoprotection of phosphofructokinase with organic solutes: characterization of enhanced protection in the presence of divalent cations. Arch. Biochem. Biophys. 250, 505–512.

Carpenter, J.F., Izutsu, K., Randolph, T.W., 1999. Freezing and drying-induced perturbations of protein structure and mechanisms of protein protection by stabilizing additives. Drugs Pharm. Sci. (Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products), Marcel Dekker, Inc., vol. 96, pp. 123–160.

Carpenter, J.F., Prestrelski, S.J., Arakawa, T., 1993. 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.

Carrasquillo, K.G., Sanchez, C., Griebenow, K., 2000. Relationship between conformational stability and lyophilization-induced structural changes in chymotrypsin. Biotechnol. Appl. Biochem. 31, 41–53.

Chan, H.K., Ongpipattanakul, B., Au-Yeung, J., 1996. Aggregation of rhDNase occurred during the compression of KBr pellets used for FTIR spectroscopy. Pharm. Res. 13, 238–242.

Chang, B.S., Beauvais, R.M., Dong, A., Carpenter, J.F., 1996. Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: glass transition and protein conformation. Arch. Biochem. Biophys. 331, 249–258.

Chapman, D., Gomez-Fernandez, J.C., Goni, F.M., Barnard, M., 1980. Difference infrared spectroscopy of aqueous model and biological membranes using an infrared data station. J. Biochem. Biophys. Methods 2, 315–323.

Chongpresart, S., 1998. Influence of drug polymorphism on the physical chemistry of freeze-drying. Ph.D. Thesis.

Cleland, J.L., Lam, X., Kendrick, B., Yang, J., Yang, T.H., Overcashier, D., Brooks, D., Hsu, C., Carpenter, J.F., 2001. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J. Pharm. Sci. 90, 310–321.

Crowe, J.H., Carpenter, J.F., Crowe, L.M., 1998. The role of vitrification in anhydrobiosis. Annu. Rev. Physiol. 60, 73–103.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., 1993. Preserving dry biomaterials: the water replacement hypothesis. Biopharmaceutical 6, 40–43.

Dong, A., Caughey, W.S., 1994. Infrared methods for study of hemoglobin reactions and structures. Methods Enzymol. 232, 139–175.

Dong, A., Matsuura, J., Manning, M.C., Carpenter, J.F., 1998. Intermolecular beta-sheet results from trifluoroethanol-induced nonnative alpha-helical structure in beta-sheet predominant proteins: infrared and circular dichroism spectroscopic study. Arch. Biochem. Biophys. 355, 275–281.

Dong, A., Prestrelski, S.J., Allison, S.D., Carpenter, J.F., 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J. Pharm. Sci. 84, 415–424.

Fell, A.F., 1983. Biomedical applications of derivative spectroscopy. Trends Anal. Chem. 2, 63–66.

Gloger, O., Witthohn, K., Muller, B.W., 2003. Lyoprotection of aviscumine with low molecular weight dextrans. Int. J. Pharm. 260, 59–68.

Griebenow, K., Klibanov, A.M., 1995. Lyophilization-induced reversible changes in the secondary structure of proteins. Proc. Natl. Acad. Sci. U.S.A. 92, 10969–10976.

Griebenow, K., Klibanov, A.M., 1997. Can conformational changes be responsible for solvent and excipient effects on the catalytic behavior of subtilisin Carlsberg in organic solvents. Biotechnol. Bioeng. 53, 351–362.

Hagen, S.J., Hofrichter, J., Eaton, W.A., 1995. Protein reaction kinetics in a room-temperature glass. Science 269, 959–962.

Hagen, S.J., Hofrichter, J., Eaton, W.A., 1996. Germinate rebinding and conformational dynamics of myoglobin embedded in a glass at room temperature. J. Phys. Chem. 100, 12008–12021.

Hora, M.S., Rana, R.K., Smith, F.W., 1992. Lyophilized formulations of recombinant tumor necrosis factor. Pharm. Res. 9, 33–36.

Imamura, K., Ogawa, T., Sakiyama, T., Nakanishi, K., 2003. Effects of types of sugar on the stabilization of protein in the dried state. J. Pharm. Sci. 92, 266–274.

Izutsu, K., Kojima, S., 2002. Excipient crystallinity and its protein-structure stabilizing effect during freeze-drying. J. Pharm. Pharmacol. 54, 1033–1039.

Izutsu, K., Yoshioka, S., Takeda, Y., 1991. The effects of additives on the stability of freeze-dried β-galactosidase stored at elevated temperature. Int. J. Pharm. 71, 137–146.

Izutsu, K., Yoshioka, S., Terao, T., 1994. Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying. Chem. Pharm. Bull. (Tokyo) 42, 5–8.

Jackson, M., Mantsch, H.H., 1992. Artifacts associated with the determination of protein secondary structure by ATR-IR spectroscopy. Appl. Spectrosc. 46, 699–701.

Johnson, R.E., Kirchhoff, C.F., Gaud, H.T., 2002. Mannitol-sucrose mixtures—versatile formulations for protein lyophilization. J. Pharm. Sci. 91, 914–922.

Kreilgaard, L., Frokjaer, S., Flink, J.M., Randolph, T.W., Carpenter, J.F., 1999. Effects of additives on the stability of Humicola lanuginosa lipase during freeze-drying and storage in the dried solid. J. Pharm. Sci. 88, 281–290.

Li, S., Patapoff, T.W., Overcashier, D., Hsu, C., Nguyen, T.H., Borchardt, R.T., 1996. Effects of reducing sugars on the chemical stability of human relaxin in the lyophilized state. J. Pharm. Sci. 85, 873–877.

Mattern, M., Winter, G., Kohnert, U., Lee, G., 1999. Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. Pharm. Dev. Technol. 4, 199–208.

Mattern, M., Winter, G., Rudolph, R., Kohnert, U., Lee, G., 1997. Formulation of proteins in vacuum-dried glasses. I. Improved vacuum drying using crystallizing amino acids. Eur. J. Pharm. Biopharm. 44, 177–185.

Meyer, J.D., Bai, S.J., Rani, M., Suryanarayanan, R., Nayar, R., Carpenter, J.F., Manning, M.C., 2004. Infrared spectroscopic studies of protein formulations containing glycine. J. Pharm. Sci. 93, 1359–1366.

Oberg, K.A., Fink, A.L., 1998. A new attenuated total reflectance Fourier transform infrared spectroscopy method for the study of proteins in solution. Anal. Biochem. 256, 92–106.

Osterberg, T., Fatouros, A., Mikaelsson, M., 1997. Development of freeze-dried albumin-free formulation of recombinant factor VIII SQ. Pharm. Res. 14, 892–898.

Parker, F.S., 1971. Applications of infrared spectroscopy in biochemistry, biology, and medicine. Plenum, New York, NY, pp. 188–231.

Patro, S.Y., Freund, E., Chang, B.S., 2002. Protein formulation and fill-finish operations. Biotechnol. Annu. Rev. 8, 55–84.

Peersen, O.B., et al., 1993. Variable-amplitude cross-polarization MAS NMR. J. Magn. Reson., Series A 104, 334–339.

Pikal, M.J., Rigsbee, D.R., 1997. The stability of insulin in crystalline and amorphous solids: observation of greater stability for the amorphous form. Pharm. Res. 14, 1379–1387.

Pikal-Cleland, K.A., Carpenter, J.F., 2001. Lyophilization-induced protein denaturation in phosphate buffer systems: monomeric and tetrameric beta-galactosidase. J. Pharm. Sci. 90, 1255–1268.

Prestrelski, S.J., Arakawa, T., Carpenter, J.F., 1993a. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress specific stabilization. II. Structural studies using infrared spectroscopy. Arch. Biochem. Biophys. 303, 465–473.

Prestrelski, S.J., Arakawa, T., Carpenter, J.F., 1994. The structure of proteins in lyophilized formulations using Fourier-transform infrared spectroscopy. In: Formulation and Delivery of Proteins and Peptides Symposium Series No. 567.

Prestrelski, S.J., Pikal, K.A., Arakawa, T., 1995. Optimization of lyophilization conditions for recombinant human interleukin-2 by dried-state conformational analysis using Fourier-transform infrared spectroscopy. Pharm. Res. 12, 1250–1259.

Prestrelski, S.J., Tedeschi, N., Arakawa, T., Carpenter, J.F., 1993b. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys. J. 65, 661–671.

Przybycien, T.M., Bailey, J.E., 1991. Secondary structure perturbations in salt-induced protein precipitates. Biochim. Biophys. Acta 1076, 103–111.

Pyne, A., Suryanarayanan, R., 2001. Phase transitions of glycine in frozen aqueous solutions and during freeze-drying. Pharm. Res. 18, 1448–1454.

Remmele, R.L.J., Stushnoff, C., Carpenter, J.F., 1997. Realtime in situ monitoring of lysozyme during lyophilization using infrared spectroscopy: dehydration stress in the presence of sucrose. Pharm. Res. 14, 1548–1555.

Ressing, M.E., Jiskoot, W., Talsma, H., van Ingen, C.W., Beuvery, E.C., Crommelin, D.J., 1992. The influence of sucrose, dextran, and hydroxypropyl-beta-cyclodextrin as lyoprotectants for a freeze-dried mouse IgG2a monoclonal antibody (MN12). Pharm. Res. 9, 266–270.

Sane, S.U., Wong, R., Hsu, C.C., 2004. Raman spectroscopic characterization of drying-induced structural changes in a therapeutic antibody: correlating structural changes with long-term stability. J. Pharm. Sci. 93, 1005–1018.

Shalaev, E.Y., Malakhov, D.V., Kanev, A.N., Kosyakov, V.I., Tuzikov, F.V., Varaksin, N.A., Vavilin, V.I., 1992. Study of the phase diagram water fraction of the system water–glycine–sucrose by DTA and X-ray diffraction methods. Thermochim. Acta 196, 213–220.

Souillac, P.O., Middaugh, C.R., Rytting, J.H., 2002. Investigation of protein/carbohydrate interactions in the dried state. 2. Diffuse reflectance FTIR studies. Int. J. Pharm. 235, 207–218.

Susi, H., 1969. Infrared spectra of biological macromolecules and related systems. Struct. Stabil. Biol. Macromol., 2. Marcel Dekker, Inc., New York, NY, pp. 575–663.

Susi, H., Timasheff, S.N., Stevens, L., 1967. Infrared spectra and protein conformations in aqueous solutions. I. The amide I band in H_2O and D_2O solutions. J. Biol. Chem. 242, 5460–5466.

Swedberg, S.A., Pesek, J.J., Fink, A.L., 1990. Attenuated total reflectance Fourier transform infrared analysis of an acyl-enzyme intermediate of alpha-chymotrypsin. Anal. Biochem. 186, 153–158.

Taschner, N., Muller, S.A., Alumella, V.R., Goldie, K.N., Drake, A.F., Aebi, U., Arvinte, T., 2001. Modulation of antigenicity related to changes in antibody flexibility upon lyophilization. J. Mol. Biol. 310, 169–179.

Tian, F., Sane, S., Rytting, J.H., 2006. Calorimetric investigation of protein/amino acid interactions in the solid state. Int. J. Pharm. 310, 175–186.

van de Weert, M., Haris, P.I., Hennink, W.E., Crommelin, D.J., 2001. Fourier transform infrared spectrometric analysis of protein conformation: effect of sampling method and stress factors. Anal. Biochem. 297, 160–169.

Wasacz, F.M., Olinger, J.M., Jakobsen, R.J., 1987. Fourier transform infrared studies of proteins using nonaqueous solvents. Effects of methanol and ethylene glycol on albumin and immunoglobulin G. Biochemistry 26, 1464–1470.

Wellner, N., Belton, P.S., Tatham, A.S., 1996. Fourier transform IR spectroscopic study of hydration-induced structures changes in the solid state of w-gliadins. Biochem. J. 319, 741–747.