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Calorimetric investigation of protein/amino acid interactions in the solid state

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Abstract

Possible protein/amino acid interactions and the physical states of amino acids after freeze-drying have been studied using isoperibol calorimetry and differential scanning calorimetry (DSC). Good linear correlations ($R^2 = 0.99$) between the enthalpies of solution and the percentage of antibody in all physical mixtures, as well as unchanging melting temperatures of amino acids for physical mixtures demonstrated that there is no interaction between the antibodies and amino acids studied upon physical mixing. On the other hand, positive deviations for antibody/histidine and antibody/arginine freeze-dried samples obtained from the isoperibol calorimetry results demonstrated that molecular level interactions, such as ion–dipole or electrostatic interactions or hydrogen bonding, occur between antibodies and histidine or arginine. The values of $\Delta H_{\text{interaction}}$ for antibody/histidine (1:1, w/w) and antibody/arginine (1:1, w/w) lyophilized samples were approximately 8 kJ/mol. These interactions were also confirmed by decreased and/or the disappearance of melting temperatures of the amino acids with DSC measurements. A negative deviation from linearity was detected for antibody/aspartic acid lyophilized samples which indicated partial amorphization of aspartic acid. No deviation from linearity as well as similar melting temperatures of antibody/glycine lyophilized samples indicated the absence of interactions between the antibodies and glycine upon freeze-drying.
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Keywords: Antibody/amino acid interactions; Calorimetry; Freeze-dried state

1. Introduction

Protein stability is a major concern for protein pharmaceuticals and this problem remains a challenge for protein chemists and the pharmaceutical industry. To overcome the protein instability problems in aqueous solutions, solid forms of proteins often have to be made to achieve an acceptable shelf life (Manning et al., 1989). Because the observed rates of chemical degradation reactions in the solid state are dramatically slower than in liquid formulations, solid formulations often possess greater long-term stability (Arakawa et al., 2001). Moreover, solid dosage forms can avoid some stability problems that occur during shipping and handling for liquid formulations due to mechanical stress. Freeze-drying is a widely accepted approach for the manufacture of protein pharmaceutical and diagnostic agents that are physically or chemically unstable in aqueous solution (Pikal, 1990; Cleland et al., 1993; Fox, 1995; Carpenter et al., 1997). Despite the attractiveness of freeze-drying, this process generates a variety of freezing and drying stresses, such as solute concentration, formation of ice crystals, pH changes, dehydration stresses, etc. (Franks, 1990; Anchordoquy and Carpenter, 1996; Chang et al., 1996; Nagendra et al., 1998). All of these stresses can cause proteins to change their native conformations or lose their biological activities to various degrees. Minimization of damage to proteins due to stresses arising from the freeze-drying process still remains a major challenge in the development and manufacture of such products. In order to prevent these freeze-drying induced protein structural alterations and the loss of protein biological activity, stabilizers are often required in a protein formulation to preserve protein structure and activity during both freezing and drying processes. Various stabilizers (or excipi-

Abbreviations: rh-DNase, recombinant human deoxyribonuclease I protein; rh-GH, recombinant human growth hormone; rh-MetGH, recombinant human methionyl growth hormone; rh-IGF-1, recombinant human insulin-like growth factor 1

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ents), such as sugars/polyols, polymers, surfactants, and amino acids, have been found to successfully prevent protein inactivation during freeze-drying. Two (or more) stabilizers may have to be used to protect proteins from conformational changes during freeze-drying if a single stabilizer does not serve as both a cryo- and lyo-protectant. Carbohydrates, such as sucrose, trehalose, and mannitol, are commonly used as both cryoprotectants and lyoprotectants in freeze-dried protein formulations. Numerous FTIR studies have shown that carbohydrates stabilize many proteins during freeze-drying (Carpenter et al., 1989, 1999; Allison et al., 1999; Souillac et al., 2002b). Sucrose and trehalose are usually the primary stabilizers for lyophilized protein formulations. In order to better understand the stabilization mechanisms of protein by additives upon lyophilization, a calorimetric method was developed previously in our laboratory to evaluate protein/carbohydrate interactions in the solid state (Souillac et al., 2002a). The direct molecular level interactions between proteins (rh-DNase, rh-GH, rh-MetGH, and rh-IGF-1) and some carbohydrates (e.g., sucrose, trehalose) were detected by isoperibol calorimetry and differential scanning calorimetry.

A recent study performed by Genentech showed that histidine protected an antibody against lyophilization induced structural perturbation and increased the stability of the antibody during subsequent storage (Sane et al., 2004). It is of interest to examine the protective effects of this and other amino acids on the molecular level. The same calorimetric techniques utilized by Souillac et al. (2002a) can be used to investigate the interactions between proteins and amino acids. For comparison with the two basic amino acids (histidine and arginine), glycine (which has a neutral side chain), and aspartic acid (which has a negative side chain) were chosen to study the possibility of side chain effects on protein/amino acid interactions.

In this paper, we explore the freeze-drying behavior of several L-amino acids (e.g., L-histidine, L-arginine, glycine, and L-aspartic acid) and investigate the existence of protein/amino acid interactions. Isoperibol (or solution) calorimetry and differential scanning calorimetry were employed to characterize the nature of such interactions which should help in understanding the mechanisms of the stabilizing effects.

The reasons to examine antibodies in this study are: (i) to understand the effects of histidine, arginine, glycine, and aspartic acid on the physical stability of antibodies during the freeze-drying process; (ii) to help develop a stable lyophilized antibody formulation.

2. Materials and methods

Therapeutic recombinant humanized anti-CD11a monoclonal antibody and anti-IgE monoclonal antibody (aka rhuMAb-E25), were produced and provided for this study by Genentech, Inc. (South San Francisco, CA). The protein bulk solution contained ~30 mg/mL antibody in 5 mM histidine at pH 6.0. Recombinant human deoxyribonuclease 1 protein (rh-DNase) was used as a model compound in this study. rh-DNase produced by Genentech, Inc., was provided as an excipient-free powder after dialysis against pure water followed by lyophilization (Costantino et al., 1997; Overcashier et al., 1997).

In the previous studies performed in our laboratory by Souillac et al. (2002a), rh-DNase, was used as a model compound to study protein/carbohydrate interactions upon lyophilization. The same model compound was used in this study to allow comparison of protein/histidine interactions with previous protein/carbohydrate studies. Crystalline L-histidine, L-arginine, glycine, and L-aspartic acid and their complementary salts were of tissue culture grade and obtained from either Fisher or Sigma. 1.0N hydrochloric acid and 1.0N sodium hydroxide were obtained from Fisher. All of these compounds were tissue culture grade or higher grade and used without further purification.

3. Methods

3.1. Sample preparation

All buffers were prepared with Milli-Q 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 strength of the protein solutions was not controlled.

L-Histidine solution was obtained by combining L-histidine with L-histidine monohydrochloride monohydrate. To prepare 300 mM histidine buffer at pH 6.0, 34.2 mg/mL L-histidine monohydrochloride monohydrate and 21.3 mg/mL L-histidine were used. Three hundred millimolar of L-arginine solution was titrated using 1.0N HCl to pH 6.0 at room temperature. 300 mM glycine and 300 mM L-aspartic acid solutions were titrated to pH 6.0 using 1.0N NaOH. All solutions of the amino acids investigated were filtered through 0.22-μm syringe filters (Osmonics Inc., Cameo 17F, Teflon).

The antibody solutions obtained from Genentech were dialyzed against distilled water using dialysis cassettes (Slide-A-Lyzer, 10,000 Da cut-off, Pierce Chemical Company, Rockford, IL) for at least 12 h at 4 °C with gentle stirring. The elimination of former histidine excipients was confirmed by analyzing UV absorbance of a placebo. The antibody concentrations were determined at 280 nm using absorptivity of 1.46 mg^{-1} mL cm^{-1} for anti-CD11a antibody (obtained from Genentech, Inc.) and 1.6 mg^{-1} mL cm^{-1} for anti-IgE antibody (Gray et al., 1994). Formulations were prepared by adding an appropriate amount of a concentrated excipient solution to the excipient-free protein solution, and a final protein concentration of 10 mg/mL (except for a 5 mg/mL protein concentration at the highest excipient concentration) was obtained by dilution with Milli-Q water. The ionic strength of the solutions was not controlled. Excipient-free protein solution (10 mg/mL) and pure amino acid solutions (10 mg/mL) were also prepared. Less than 5 mL aliquots of the formulated protein solutions in 10-cm^3 glass lyophilization vials (Wheaton Company) were lyophilized in a freeze drier (Virtis, Genesis). Table 1 provides a summary of the excipients and their concentrations in the seven lyophilized formulations.

Physical mixtures were prepared by triturating appropriate amounts of antibodies and amino acids under a dry argon envi-

Table 1
Final antibody and excipient concentrations in the lyophilized formulations

Antibody concentration (mg/mL)	Histidine concentration (mM)	Antibody in the formulation (%)	Histidine in the formulation (%)
10	0	100	0
10	10	84	16
10	20	73	27
10	60	47	53
10	150	26	74
5	200	12	88
0	60	0	100

	Arginine concentration (mM)		Arginine in the formulation (%)
10	0	100	0
10	10	85	15
10	20	74	26
10	60	49	51
10	150	28	72
5	200	13	87
0	60	0	100

	Glycine concentration (mM)		Glycine in the formulation (%)
10	0	100	0
10	10	93	7
10	20	87	13
10	60	69	31
10	150	47	53
5	200	25	75
0	150	0	100

	Aspartic acid concentration (mM)		Aspartic acid in the formulation (%)
10	0	100	0
10	10	85	15
10	20	74	26
10	60	49	51
10	144	29	71
5	188	13	87
0	60	0	100

ronment in a glove-box. Sampled antibodies and amino acids were freeze-dried, respectively, prior to the trituration. The trituration step was performed with a spatula in a glass vial for 2 min. Pure amino acids and pure antibodies, which were used in the physical mixture studies, were also triturated for 2 min to avoid possible variations introduced from trituration. The results obtained for the physical mixtures were used as controls throughout the study.

3.2. Freeze-drying procedure

Freeze-drying was performed using a Virtis (Genesis) model unit. Vials were loaded onto the lyophilizer shelf at 5 °C and the shelf temperature was reduced by 20 °C/h to −35 °C. The shelf temperature was maintained at −35 °C for 9 h. Next, the shelf temperature was increased to 0 °C at 12 °C h^{-1}, and the primary drying was achieved at a shelf temperature of 0 °C for 40 h. Finally, the shelf temperature was raised to 20 °C at a rate of 10 °C h^{-1}, and held at this temperature for 8 h (secondary drying). A vacuum of less than 150 mTorr was maintained throughout the freeze-drying cycle. All dried samples were stoppered under vacuum. The lyophilized vials were sealed using aluminum crimper caps and stored at 2–6 °C in a vacuum desiccator over drierite until testing. The lyophilized samples were tested within two months.

3.3. Calorimetry

A Hart Scientific Isoperibol Calorimetry (Model 4285) was used to determine the enthalpies of solution for the protein/amino acid mixtures in 25 mL Milli-Q water at 25 °C. About 10 mg of samples were prepared for each run. The detailed methodology was explained in previous work that was done in our laboratory (Souillac et al., 2002a). At least triplicate measurements were carried out until a good reproducibility was obtained to determine the enthalpy of solution for each protein/amino acid mixture.

3.4. Differential scanning calorimetry

Differential scanning calorimetric analysis of the lyophilized formulations was conducted with a Perkin-Elmer Pyris-1 calorimeter. Pure indium was used as a standard for temperature and heat flow calibrations. The physical mixtures and lyophilized samples for DSC measurements were prepared in a glove box filled with dry argon. Approximately 1–2 mg of each sample was loaded into a disposable aluminum sample pan and sealed, then placed in the calorimeter. An empty pan was used as reference. A temperature range from 25 to 280 °C was employed with a heating rate of 10 °C/min. Thermograms of all protein/amino acid mixtures (freeze-dried and physically mixed) were obtained in duplicate.

3.5. Karl–Fisher titration

The residual moisture content was assayed by the Karl–Fisher (Brinkmann, model 652) titration method. Measurements were made by an electrochemical technique that utilizes the conductive state of a hydranal Coulomat AG solution. Sample weights ranging from 1 to 3 mg were used. To avoid interference from moisture in the air, the sample was prepared by transferring the lyophilized powder to a 1.5-mL plastic centrifuge tube in a glove-box filled with dry argon. The tube was capped and then taken out under a flush of dry argon. Two moisture measurements were conducted per lyophilized vial.

3.6. Circular dichroism

CD experiments were performed using an AVIV CD-60DS instrument. Samples were prepared after reconstitution in Milli-Q water at a protein concentration of 0.2 mg/mL. Data were collected in the range of 240–200 nm in steps of 1 nm and smoothed. The wavelength of 222 nm was used to monitor the CD spectrum changes in the temperature range of 25–80 °C.

4. Results

4.1. Residual water contents for the freeze-dried mixtures

The residual water contents of various freeze-dried amino acids and lyophilized protein formulations were obtained. Despite some batch-to-batch differences, the residual water contents achieved when antibodies were freeze-dried in the absence of excipients (amino acids) were $3.7 \pm 0.4\%$ (w/w) for anti-CD11a antibody and $5.7 \pm 0.2\%$ (w/w) for anti-IgE antibody, respectively. These data indicate that the residual moisture of the pure anti-IgE antibody was close to the values of the water monolayer, which has been determined to consist of 5.7 g of water per 100 g of dry protein (Costantino et al., 1997).

Moisture contents ranging from 2 to 5% (w/w) (g water/100 g solid) were detected among the freeze-dried samples containing various amino acid/antibody ratios. This range of moisture content is close to other lyophilized proteins in carbohydrate systems (Sarciaux et al., 1999; Souillac et al., 2002a).

4.2. Heats of solution for the pure materials

Enthalpies of solution in water for pure amino acids and pure antibodies were measured (Fig. 1), in which crystalline amino acids showed large endothermic values, indicating their crystalline natures. In addition, the crystallinity of these materials was detected by DSC with distinct melting peaks and significant heats of fusion. Freeze-dried glycine was characterized by the same endothermic value as found for the crystalline glycine sample as received. The same one sharp melting peak of glycine was found in DSC scans before and after lyophilization. These results suggest that glycine remains fully crystallized upon freeze-drying. The other freeze-dried amino acids showed relatively small endothermic enthalpies of solution compared to their crystal forms, indicating the amorphous character of these compounds following lyophilization. These findings were consistent with DSC results, where the presence of exotherms of re-crystallization for lyophilized histidine and lyophilized arginine were observed.

All freeze-dried pure antibodies were characterized by large exothermic enthalpy values, showing a more complete amorphous character.

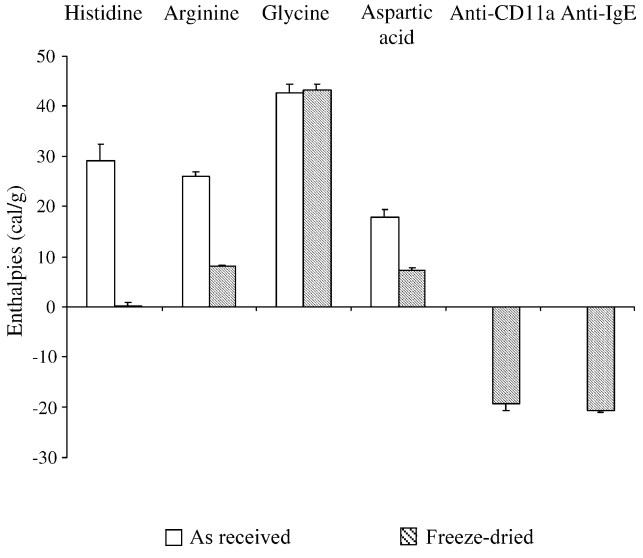

Fig. 1. Enthalpies of solution in water for pure amino acids and pure antibodies.

4.3. Antibody/amino acid mixtures

The interactions between antibodies (anti-CD11a and anti-IgE) and different amino acids (L-histidine, L-arginine, glycine, and L-aspartic acid) were investigated by isoperibol calorimetry and differential scanning calorimetry using physical and freeze-dried mixtures.

4.3.1. Histidine- and arginine-containing mixtures

4.3.1.1. Solution calorimetry. A variety of different molar ratios of L-histidine to antibodies and L-arginine to antibodies were used, respectively, to produce lyophilized antibody formulations. The enthalpies of solution were examined for both physically mixed samples and lyophilized mixtures.

As illustrated in Fig. 2a, a linear relationship between the enthalpies of solution and the weight percent of antibody in anti-CD11 a/histidine physical mixtures was observed with correlation coefficient values of 0.99. Similar correlations were observed for physical mixtures of anti-IgE/histidine, anti-CD11a/arginine, and anti-IgE/arginine, respectively (data not shown). These close to 1.00 linear correlations indicate that there are no interactions between L-histidine (or L-arginine) and any of the antibodies upon physical trituration. As shown in Eq. (1) below, theoretically the total enthalpy of solution is the sum of

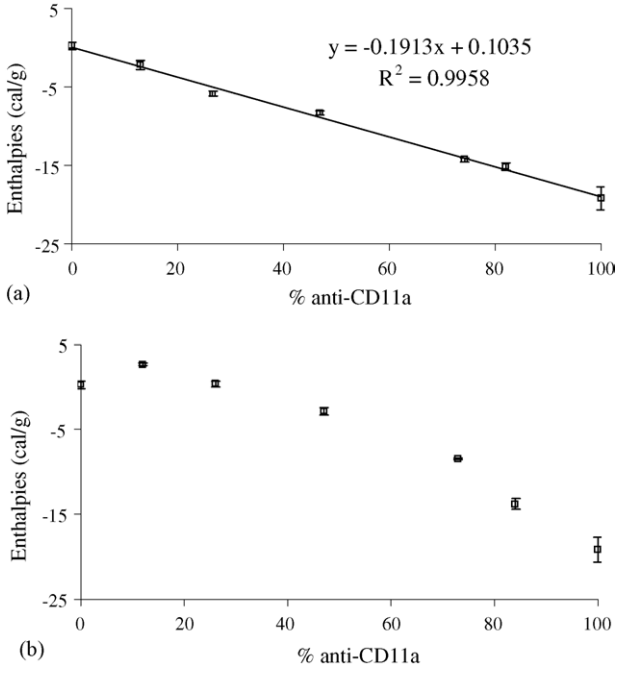

Fig. 2. (a) Enthalpies of solution for physical mixtures of anti-CD11a/histidine in water and (b) enthalpies of solution for freeze-dried mixtures of anti-CD11a/histidine in water.

the enthalpies of the individual components (Guillory and Erb, 1985; Craig and Newton, 1991; Gao and Rytting, 1997; Souillac et al., 2002a), for non-interacting binary systems:

$$\Delta H_{\text{Total}} = X_{\text{A}}\,\Delta H_{\text{A}} + X_{\text{B}}\,\Delta H_{\text{B}} \quad (1)$$

where X is the weight fractions and ΔH is the enthalpies of solution of two non-interacting substances, respectively. Hence, in the absence of interactions, a linear relationship between the enthalpies of solution and the percentage of one of the components is expected.

Unlike the linear relationship obtained from physical mixtures, the relationships between the enthalpies of solution and the percentages of anti-CD11a antibody showed positive deviations from linearity for the freeze-dried mixtures (Fig. 2b). Similar positive deviations from linearity were also obtained for lyophilized mixtures of anti-IgE/histidine, anti-CD11a/arginine and anti-IgE/arginine, respectively (data not shown). These positive deviations may be the result of interactions between the antibodies and histidine or the antibodies and arginine. In such circumstances, a higher energy would be required to dissolve the co-freeze-dried formulations than predicted for non-interacting molecules. Similar observations were found previously in freeze-dried protein/sucrose and protein/trehalose formulations (Souillac et al., 2002a). It is evident that the measured enthalpies of solution for the lyophilized mixtures were less exothermic (more endothermic) than expected for a system containing non-interacting components.

4.3.1.2. Differential scanning calorimetry. DSC thermographs were also obtained for both physically mixed and co-freeze-dried antibody/histidine and antibody/arginine samples, respectively. Fig. 3a showed distinct crystallization exotherms and melting endotherms of histidine throughout the entire antibody concentration range for the physical mixtures. The melting temperature of histidine showed no significant changes with increasing amounts of anti-CD11a antibody in the mixtures. Similar results were obtained for physically mixed anti-IgE antibody/histidine and antibody/arginine samples (data not shown). These observations suggest the absence of antibody/histidine and the absence of antibody/arginine interactions upon physical trituration. Additionally, the apparent enthalpy of crystallization versus the percentage of histidine (or arginine) present in the sample was plotted for the physical mixtures (data not shown). An observed linear relationship also confirms that there are no interactions between histidine (or arginine) and the antibody molecules upon physical mixing.

On the other hand, endothermic melting and exothermic crystallization of L-histidine were detected in co-freeze-dried samples containing up to 47% antibody (Fig. 3b). However, the melting and crystallization of L-histidine were not observed for the mixtures containing more than 47% antibody. Moreover, significant decreases in the melting temperature of histidine were observed with increases in the concentration of the antibodies. For instance, the melting peak of histidine for 12% anti-CD11a-containing sample went down to approximately 241 °C from 248 °C for the pure histidine sample, and finally decreased

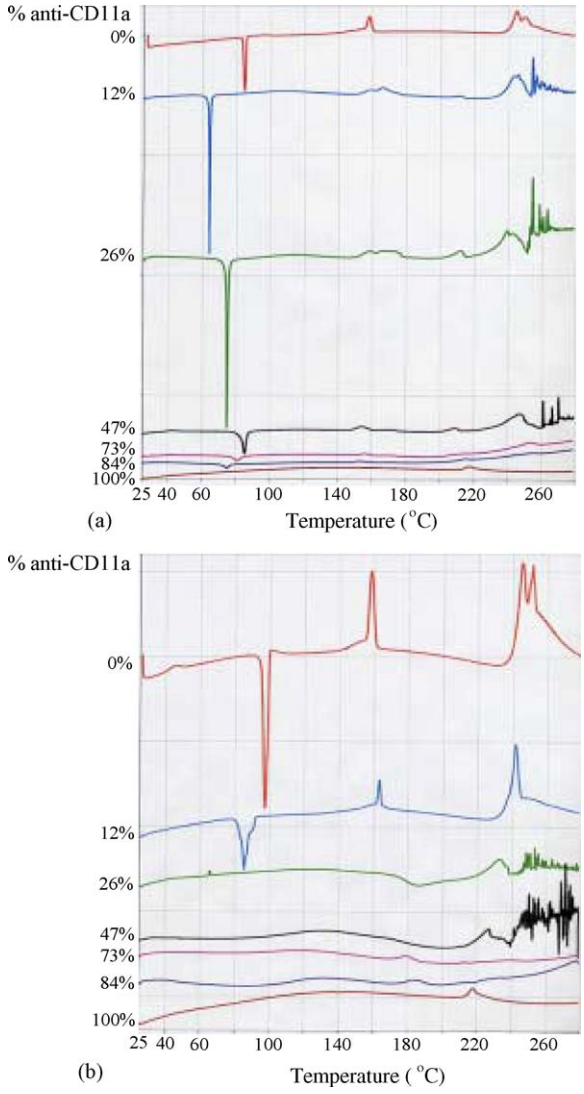

Fig. 3. DSC thermograms of anti-CD11a/histidine mixtures: (a) physical mixtures and (b) freeze-dried mixtures (endotherm up).

to 226 °C for 47% anti-CD11a/histidine lyophilized mixtures. Similar observations were displayed in the different ratios of freeze-dried anti-IgE/histidine samples (data not shown).

The freeze-dried L-arginine mixtures showed melting endotherms as well as crystallization exotherms of L-arginine at 12% antibody concentration (data not shown). The melting peak could not be detected in freeze-dried L-arginine mixtures containing 26% or more antibody concentrations. Similar observations as shown in antibody/histidine freeze-dried samples demonstrate that there are interactions between antibody and L-arginine upon lyophilization (data not shown).

The disappearance of a crystallization peak and an endothermic melting peak at high antibody concentration, as well as the decrease in the melting temperature of histidine (or arginine) with increasing antibody/histidine or antibody/arginine ratios, confirmed the presence of interactions between histidine (or arginine) and the antibody upon freeze-drying, as suggested by the results of the solution calorimetry studies.

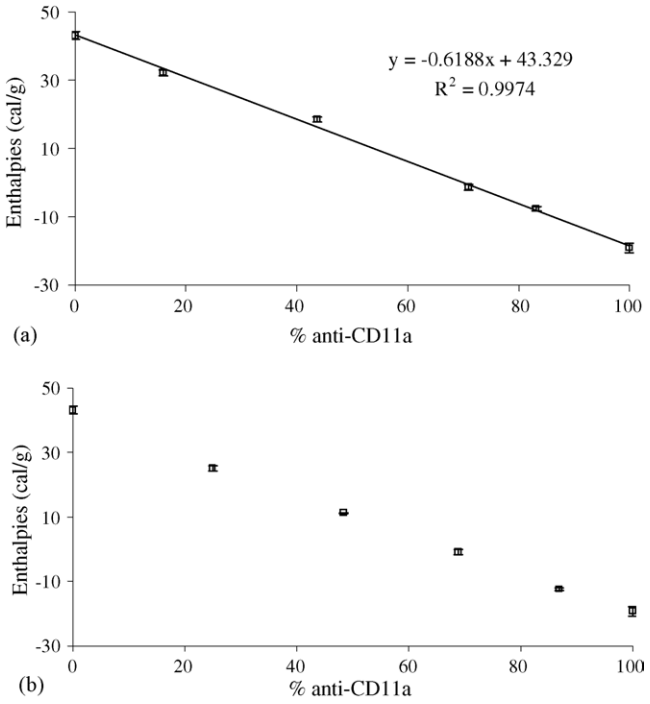

Fig. 4. (a) Enthalpies of solution for physical mixtures of anti-CD11a/glycine in water and (b) enthalpies of solution for freeze-dried mixtures of anti-CD11a/glycine in water.

4.3.2. Glycine-containing mixtures

4.3.2.1. Solution calorimetry. A linear relationship between the enthalpies of solution and the percentage of antibody in the glycine systems again suggests that there is no interaction between antibody and glycine for the physical mixtures (Fig. 4a). For the freeze-dried antibody/glycine mixtures, a slightly negative deviation from linearity seemed to indicate very little if any interaction between glycine and protein in the lyophilized mixtures (Fig. 4b). As pointed out before, no significant differences were found between the enthalpy of solution values between freeze-dried and crystalline glycine. Glycine remains in a crystalline form during the freeze-drying process, making it a good bulking agent for lyophilized formulations.

4.3.2.2. Differential scanning calorimetry. During the DSC scans, the melting peaks of glycine were detected at all protein concentrations for both the physical mixtures and the lyophilized samples (Fig. 5a and b). Neither physical nor lyophilized mixtures showed significant differences in the melting temperature of glycine (ca. 242 °C) for all examined antibody concentrations. Linear relationships between the apparent enthalpies of fusion of glycine and the amounts of glycine in the systems were observed. Furthermore, these two lines almost overlap (have very similar slopes and intercepts) (data not shown). The DSC scan indicates that there is little if any interaction between antibodies and glycine upon freeze-drying.

4.3.3. Aspartic acid-containing mixtures

4.3.3.1. Solution calorimetry. Similar to antibody/histidine physical mixtures, a linear relationship between the enthalpies

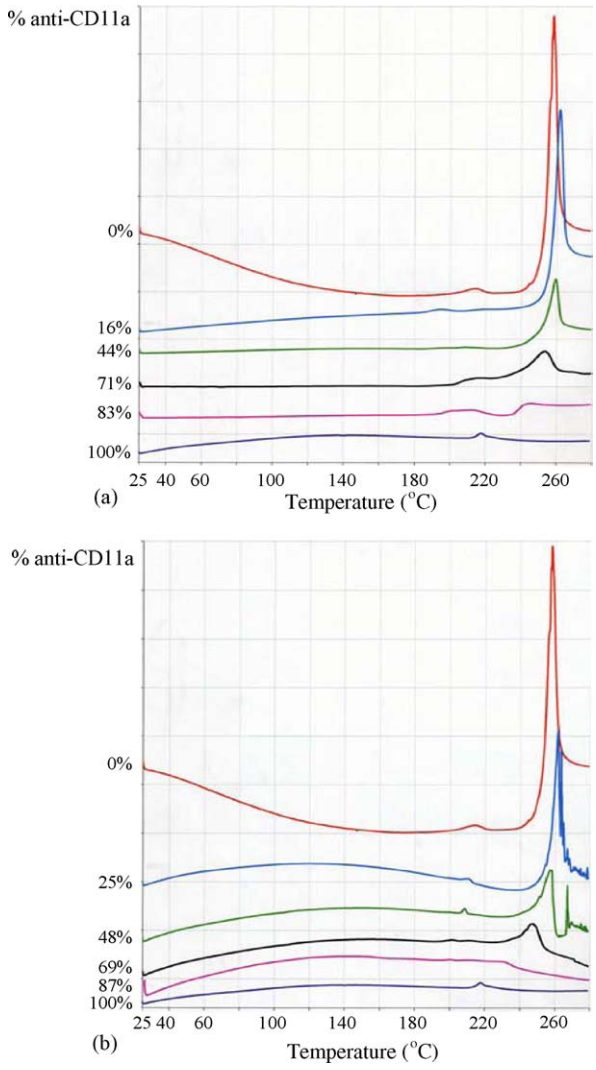

Fig. 5. DSC thermograms of anti-CD11a/glycine mixtures: (a) physical mixtures and (b) freeze-dried mixtures (endotherm up).

of solution and the percentage of antibody was obtained for physical mixtures of antibody/aspartic acid using isoperibol calorimetry, indicating the absence of interactions between the antibody and L-aspartic acid upon physical trituration (Fig. 6a). For the freeze-dried mixtures, a large negative deviation from linearity was observed in Fig. 6b. This negative deviation suggests that there is a larger portion of amorphous character in the mixtures than expected in the case of no interactions. Part of the L-aspartic acid could remain amorphous when co-freeze-dried with antibody during the freeze-drying process.

4.3.3.2. Differential scanning calorimetry. DSC scans did not show significant differences among any of the melting endotherms of L-aspartic acid for all physical mixtures (Fig. 7a).

For the anti-CD11a antibody/L-aspartic acid co-lyophilized mixtures, the melting peak at 95 °C disappeared as shown in Fig. 7b. Two endotherms were detected for the melting of L-aspartic acid for the 32% protein-containing mixture, indicating the possible presence of interactions between aspartic acid and the antibody. The melting of L-aspartic acid was not detected at

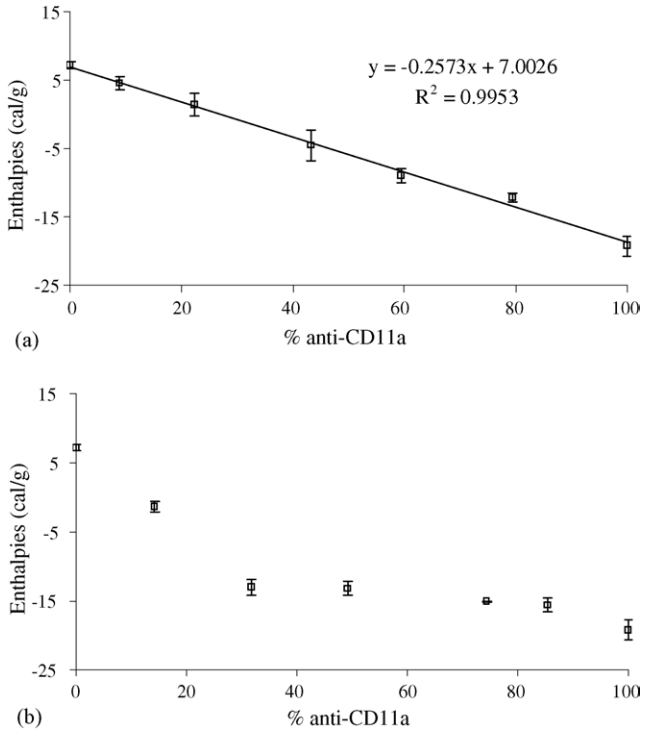

Fig. 6. (a) Enthalpies of solution for physical mixtures of anti-CD11a/aspartic acid in water and (b) enthalpies of solution for freeze-dried mixtures of anti-CD11a/aspartic acid in water.

higher protein concentrations (above 32%), thereby, confirming the formation of amorphous material during lyophilization.

4.4. rh-DNase/histidine mixtures

4.4.1. Differential scanning calorimetry

rh-DNase has been shown to have direct interactions with several carbohydrates (i.e., sucrose, trehalose, mannitol) upon lyophilization (Souillac et al., 2002a), and these interactions contribute to the preservation of secondary structure of rh-DNase during freeze-drying (Souillac et al., 2002b). In order to compare one of the potential lyo-protectants, L-histidine, a model compound, rh-DNase, which was used in the previous study (Souillac et al., 2002a) was used to conduct some preliminary studies with L-histidine. Differential scanning calorimetry was used to explore the interactions between rh-DNase and L-histidine upon lyophilization.

For physical mixtures, the detectable melting peaks of histidine showed very close temperatures for all different concentrations of protein samples (Fig. 8a). In addition, a linear correlation between apparent enthalpies of histidine crystallization versus the percentage of histidine was observed (data not shown). These observations indicate the absence of interaction between rh-DNase and L-histidine upon physical trituration.

For the freeze-dried mixtures, melting and crystallization peaks of L-histidine could not be detected except for two low protein concentrations (10% and 25% rh-DNase) (Fig. 8b). Furthermore, significant decreases in the melting temperature were observed in the histidine buffer, which dropped from

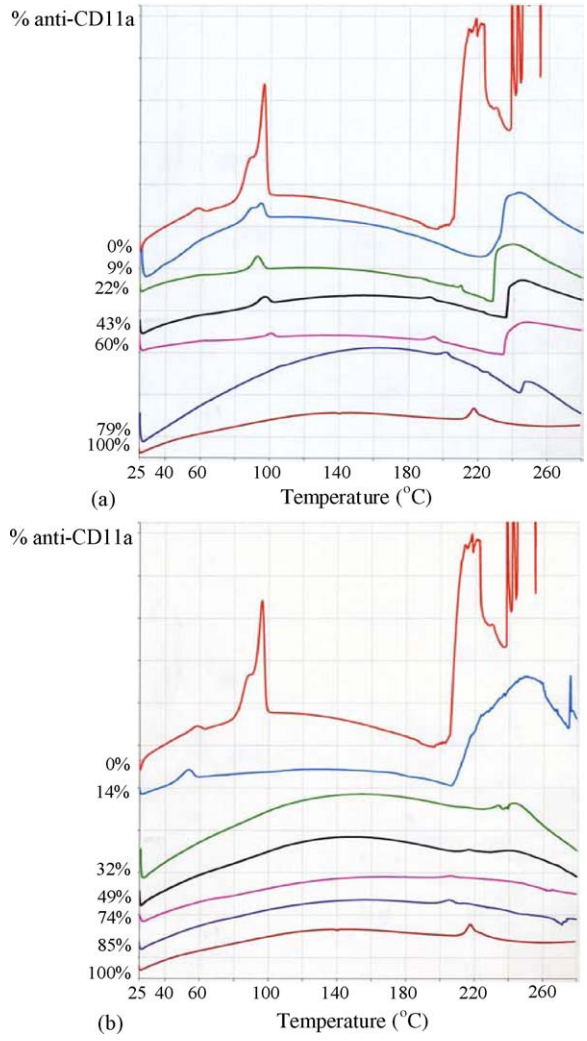

Fig. 7. DSC thermograms of anti-CD11a/aspartic acid mixtures: (a) physical mixtures and (b) freeze-dried mixtures (endotherm up).

242 °C for the pure L-histidine to approximately 240 °C for the 10% rh-DNase-containing sample and 230 °C for the 25% rh-DNase-containing sample. This decreased melting temperature of histidine at low protein concentration as well as the loss of endothermic melting for histidine at high protein concentration suggest the presence of interactions between L-histidine and rh-DNase upon freeze-drying.

The results obtained here are similar to the observations performed before using rh-DNase with sucrose (Souillac et al., 2002a), confirming the formation of interactions between rh-DNase and histidine upon freeze-drying.

4.4.2. Circular dichroism

Reconstituted samples of rh-DNase/histidine and rh-DNase/sucrose were used for monitoring the protein conformation changes upon temperature increases. The CD spectrum of rh-DNase revealed a negative broad peak at about 213 nm. Since rh-DNase is composed of α-helix and β-sheet, the wavelength of 222 nm was selected to monitor the CD spectrum changes. A temperature range from 25 to 80 °C was used to detect the

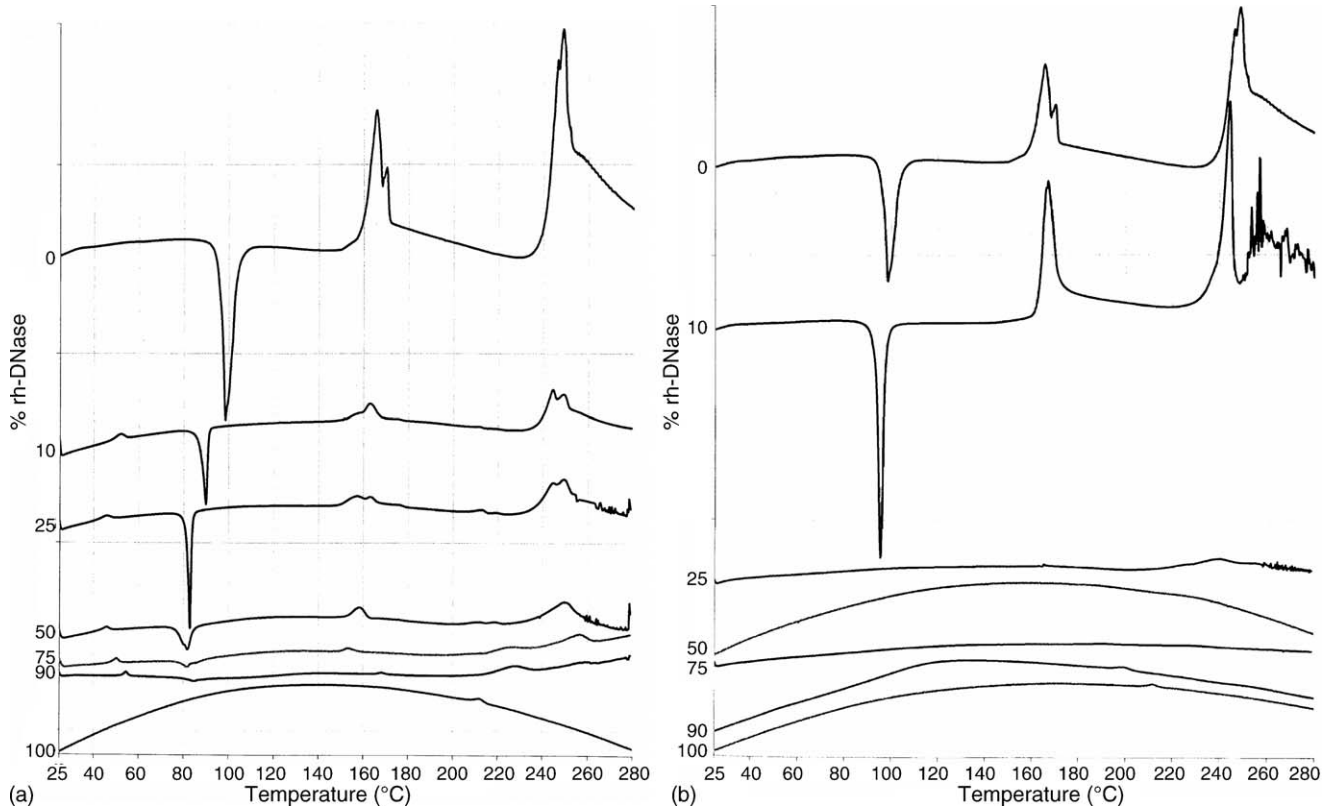

Fig. 8. (a) DSC thermographs of rh-DNase/histidine physical mixtures and (b) DSC thermographs of rh-DNase/histidine freeze-dried mixtures (endotherm up).

transition temperature of rh-DNase from its native structure to a completely unfolded state. The CD spectrum of the buffer solution was subtracted.

A transition temperature of pure rh-DNase was detected around 48 °C (Fig. 9a). Using the reconstituted sample of rh-DNase in L-histidine (1:1, w/w ratio), the transition temperature increased to about 55 °C (Fig. 9b). The increased transition temperature of protein (∼55 °C) was also obtained using the reconstituted sample of rh-DNase in sucrose (1:1, w/w ratio) (Fig. 9c). Previous studies showed that there were interactions between rh-DNase and sucrose in the solid state (Souillac et al., 2002a,b). The CD results presented here show that the native structure of rh-DNase was more stable in the presence of sucrose in solution. The similar result for rh-DNase in L-histidine system indicates that histidine improves the thermal stability of rh-DNase in solution, as does sucrose.

Furthermore, 75% rh-DNase (weight ratio) in L-histidine and sucrose were examined, respectively. Both excipients increased the transition temperature of rh-DNase, showing that the presence of histidine (or sucrose) improved the protein thermal stability.

5. Discussion

5.1. Pure excipients (amino acids)

The enthalpy of solution for the pure crystalline sample usually corresponds to the highest value, whereas the enthalpy of solution for the pure amorphous sample will be the lowest value. Partially amorphous samples, therefore, will produce an enthalpy of solution between these two extreme values based on the combined contributions from both crystalline and amorphous characteristics.

The experimental values of enthalpies of solution for the pure amino acids illustrated that L-histidine, L-arginine, and L-aspartic acid became partially amorphous during the freeze-drying process, whereas glycine remains fully crystallized.

Glycine is commonly used as a bulking agent in pharmaceutical protein formulations because of its crystallization during freezing (Akers et al., 1995; Pyne and Suryanarayanan, 2001; Pikal-Cleland et al., 2002). In analyzing the freeze-drying behavior of L-amino acids, Mattern et al. (1999) found that most amino acids (e.g., glycine) crystallized during freeze-drying, however, some basic amino acids (e.g., L-histidine, L-arginine) and their chloride salts exhibited an amorphous form. These phenomena support what was observed in this calorimetric study. The different solid forms (crystalline, amorphous) may impact the formation of interactions, which could occur between protein and amino acids during the freeze-drying process.

5.2. Protein/amino acid mixtures

All physical mixtures of the antibodies with different amino acids gave good linear correlations ($R^2 = 0.99$) between the enthalpy of solution and the ratio of protein/excipient in the sample. These results demonstrate that there is no interaction between antibodies and amino acids upon physical mixing (based on Eq. (1)). This conclusion was also confirmed by DSC

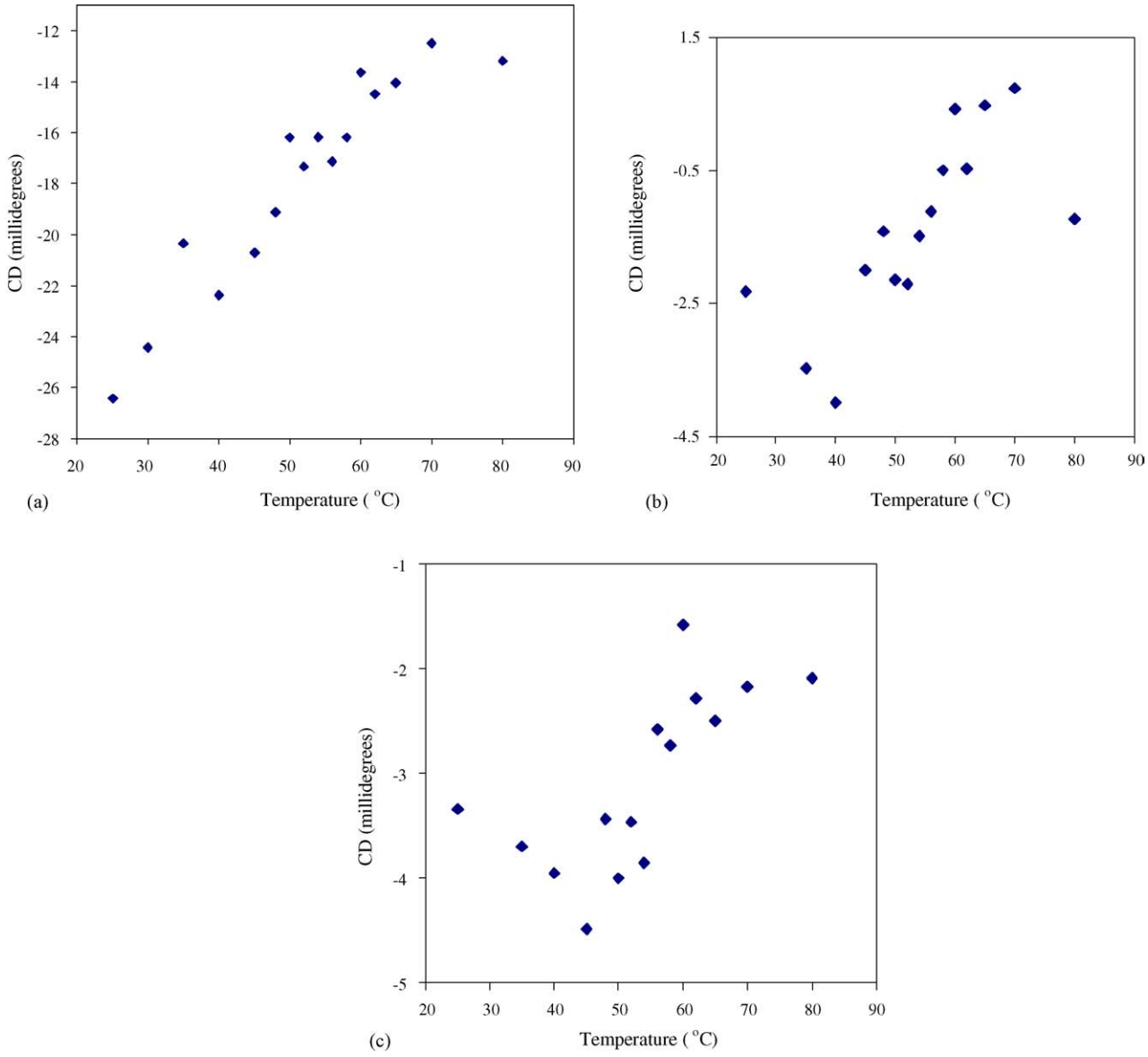

Fig. 9. (a) Transition temperature of pure rh-DNase in aqueous solution. Monitored at 222 nm by circular dichroism. Transition temperature of 50% (w/w) rh-DNase in the presence of (b) 50% (w/w) histidine and (c) 50% (w/w) sucrose aqueous solutions. Monitored at 222 nm by circular dichroism.

with the lack of noticeable change in the melting temperature of amino acids.

Theoretically, for an interacting binary system, the enthalpy due to interactions between two interacting materials should be additive, as shown in Eq. (2):

$$\Delta H_{\text{Total}} = X_{\text{A}}\,\Delta H_{\text{A}} + X_{\text{B}}\,\Delta H_{\text{B}} + \Delta H_{\text{int}} \quad (2)$$

where ΔH_{int} represents the enthalpy change due to interactions between two interacting substances. The enthalpy change due to interactions is considered to be composed of multiple terms and depends on the different types of interactions in the system. The theoretical enthalpy values of non-interacting systems can be calculated from the extrapolation of a straight line between the enthalpy of the pure protein and that of the pure amino acid. Thus, the enthalpy of solution values can be theoretically obtained for each corresponding protein/amino acid sample (with different weight ratios) in the absence of any interactions. In this case, only dissolution processes will influence the enthalpy values because of the independence of sample weight on ΔH_{sol} of pure proteins and pure amino acids. The values of ΔH_{int} can be assessed by subtracting the theoretical values from the experimental data. The determined number represents the magnitude of ΔH_{int} and the positive or negative sign indicates endothermic or exothermic of ΔH_{int} for each protein/amino acid mixture.

5.2.1. Physical mixtures

The enthalpies, due to interactions between the protein and the amino acid, obtained for physical mixtures were relatively small and randomly distributed as expected. This pattern indicates the absence of interactions between proteins and amino acids upon physical trituration.

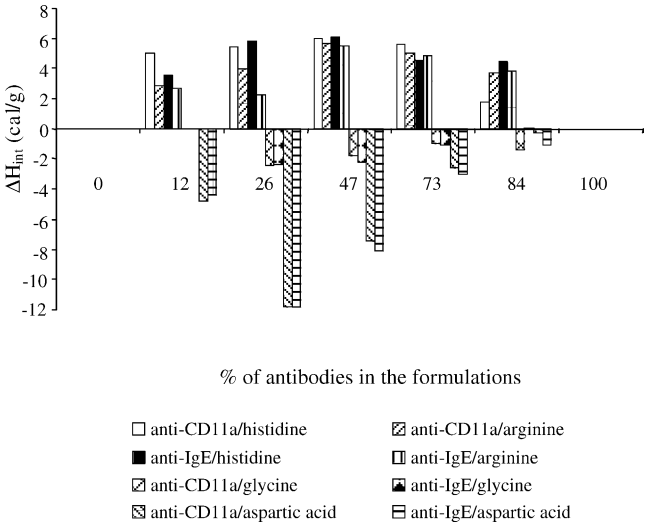

Fig. 10. The enthalpy changes of interactions between protein and amino acid for lyophilized mixtures.

The melting endotherms of amino acids in different concentrations of protein/amino acid mixtures displayed similar temperatures for all physical mixtures. These observations suggested that there was no interaction between the amino acids and proteins upon physical mixing. In addition, a straight line was obtained from plotting the apparent enthalpy of crystallization for histidine and arginine (or heat of fusion for glycine) versus the percentage of proteins in the mixtures, confirming the absence of interactions for physically mixed samples. This conclusion is supported by similar observations found in the work of Souillac et al. (2002a) for physical mixtures of proteins and carbohydrates using isoperibol calorimetry and DSC.

5.2.2. *Freeze-dried mixtures*

Compared with the physical mixtures, the changes in enthalpy of interactions between the proteins and amino acids obtained from freeze-dried samples showed larger values in either positive (endothermic) or negative (exothermic) directions (Fig. 10).

For example, the changes of enthalpy of interaction for the histidine- and arginine-containing mixtures exhibit positive values. For those two mixtures, the experimental values are more endothermic than those predicted for the non-interacting systems, suggesting more energy is required during the dissolution process. These positive deviations from linearity provided evidence for the formation of interactions between the antibody and amino acid molecules. These interactions could be ion–dipole interactions and/or hydrogen bonding based on their small enthalpic values. Other types of interactions should be also considered.

When the values of ΔH_{int} for 1:1 weight ratio lyophilized samples were considered, a ΔH_{int} of approximately 8 kJ/mol was obtained for both protein/histidine and protein/arginine samples. These values for the change in enthalpy are very close to those of weak interactions, such as ion–dipole interactions or hydrogen-bonding, with energy changes around 9 kJ/mol (Takano et al., 1999). Takano et al. (1999) examined the contribution of hydrogen bonds to the conformational stability of human lysozyme. Their results showed that hydrogen bonds between protein atoms and between a protein atom and a bound water molecule favorably contribute to the protein stability. The net contribution of an intramolecular hydrogen bond to protein stability was about 9 ± 2.6 kJ/mol. On the other hand, the contribution of hydrogen bonds between a protein atom and a bound water molecule was smaller than that between protein atoms. This is consistent with other studies, showing an enthalpy change of hydrogen bond formation in water between −8 and −13 kJ/mol (Schellman, 1955; Susi et al., 1964; Gill and Noll, 1972). In addition, some weak ion–dipole interactions also give similar energy ranges. Several studies indicate that disaccharides (i.e., sucrose, trehalose) form hydrogen bonds with proteins to inhibit the unfolding of proteins during drying (Carpenter et al., 1989, 1991; Tanaka et al., 1991; Lippert and Galinski, 1992; Allison et al., 1999; Souillac et al., 2002a,b). The ΔH_{int} values, which were obtained from previous studies conducted in our laboratory (Souillac et al., 2002a), were from 7 to 14 kJ/mol for all four different proteins (rh-DNase, rh-GH, rh-MetGH, and rh-IGF-1) with sucrose at a 1:1 weight ratio of freeze-dried samples. These energy changes were also within the scope of the hydrogen bond energy range, suggesting the formation of H-bonds between proteins and sucrose. These phenomena are consistent with hydrogen bonding (or ion–dipole) contributing primarily to the excipient–protein interactions.

In the current study, the protonated guanidine motif on the side chain of arginine and protonated imidazole motif on histidine side chain are present in the protein formulations (formulated at pH 6). These positively charged side-chains might form ion–dipole interactions or hydrogen bonding (good H-bond donors) with antibodies. Therefore, the protein/amino acid interactions may help to stabilize the native conformation of the protein molecule in the absence of water. In fact, the enthalpic energy required to destroy the pseudo-lattice structure that formed by weak interactions between protein and basic amino acid molecules gives more endothermic (less exothermic) values, leading to an unfavorable enthalpic effect upon dissolution. This was supported by Habermann's work (Habermann and Murphy, 1996), which showed that the overall enthalpy of dissolution produced positive values for cyclic dipeptides that contain amide–amide and amide–hydroxyl hydrogen bonds. Some calorimetric studies also found a negative contribution to ΔH during protein–ligand binding, indicating a favorable enthalpy change upon formation of such interactions (McGraw and Lindenbaum, 1990; St. Hilaire et al., 1994; Kornblatt et al., 2001; Hicks et al., 2003).

Compared with previous work (Souillac et al., 2002a), the lyophilized antibodies in the presence of histidine (or arginine) showed positive values of ΔH_{int} in the same range as those observed in protein/sucrose and protein/trehalose mixtures, suggesting the existence of direct interactions between antibodies and these basic amino acids. The rh-DNase/histidine interactions were also detected upon DSC scans, and an increased T_m of rh-DNase was observed by CD experiments. These data suggest that the antibody/histidine interaction is non-specific and may affect protein stability.

The value of ΔH_{int} for the aspartic acid-containing mixtures showed negative magnitudes, which are similar to protein/mannitol lyophilized samples (Souillac et al., 2002a). A possible reason for these negative values (more exothermic) is the partial amorphization of L-aspartic acid. As discussed for the lyophilized protein/mannitol systems (Souillac et al., 2002a), protein/mannitol interactions may have in fact occurred, but the endothermic enthalpy generated by the formation of interactions may have been overcompensated by the exothermic enthalpy generated by the amorphization process. A similar explanation can be applied to the antibody/aspartic acid formulations.

The ΔH_{int} for the glycine-containing lyophilized mixtures has the lowest value compared to other amino acids. As illustrated previously, glycine tends to fully crystallize with itself and be excluded from the protein surface during the fireeze-drying process. The formation of interactions between glycine and protein is very limited. The amorphous character shown for histidine, arginine, and aspartic acid, however, suggests that such interactions occur more easily with amorphous protein.

6. Conclusions

Differential scanning calorimetry and isoperibol calorimetry provide methods to detect whether there are interactions between proteins and amino acids at the molecular level. The isoperibol calorimetry results showed that a linear correlation ($R^2 = 0.99$) was obtained between the enthalpies of solution and the percentage of protein in the formulations for all physical mixtures, indicating the absence of interactions between amino acids and proteins upon physical mixing. This finding was also confirmed by DSC measurements that displayed similar melting temperatures for all physical mixtures.

Upon analyzing the data obtained from the freeze-dried mixtures using solution calorimetry, positive deviations for the histidine- and arginine-containing samples indicate that molecular level interactions, such as ion–dipole interactions or hydrogen bonding, occurred between histidine (or arginine) and the proteins. This was also confirmed by decreased and/or the disappearance of melting temperatures of the amino acids with DSC measurements. A negative deviation from linearity was found for aspartic acid-containing lyophilized mixtures using isoperibol calorimetry. The presence of partial amorphization of aspartic acid is a plausible explanation for the negative value of ΔH_{int}. Interactions between aspartic acid and protein probably exist, but the endothermic enthalpy generated by the formation of the molecular level interaction may have been overcompensated by the exothermic enthalpy generated by the amorphization of aspartic acid. This hypothesis, however, could not be totally proved by the calorimetric data which show negative deviations from linearity and undetectable transition peaks. Glycine-containing samples exhibited almost no deviation from linearity as well as similar melting temperatures, indicating the absence of interactions between protein and glycine upon freeze-drying. Further studies have been done to evaluate the effects of these interactions on protein secondary structural changes in the solid state using FTIR spectroscopy, and to detect the specific interaction site in the solid state using solid state NMR spectroscopy.

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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.

Anchordoquy, T.J., Carpenter, J.F., 1996. Polymers protect lactate dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state. Arch. Biochem. Biophys. 332, 231–238.

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.

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., Izutsu, K., Randolph, T.W., 1999. Freezing and drying-induced perturbations of protein structure and mechanisms of protein protection by stabilizing additives. In: Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products, pp. 123–160.

Carpenter, J.F., Pikal, M.J., Chang, B.S., Randolph, T.W., 1997. Rational design of stable lyophilized protein formulations: some practical advice. Pharm. Res. 14, 969–975.

Chang, B.S., Kendrick, B.S., Carpenter, J.F., 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci. 85, 1325–1330.

Cleland, J.L., Powell, M.F., Shire, S.J., 1993. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Crit. Rev. Ther. Drug Carrier Syst. 10, 307–377.

Costantino, H.R., Curley, J.G., Hsu, C.C., 1997. Determining the water sorption monolayer of lyophilized pharmaceutical proteins. J. Pharm. Sci. 86, 1390–1393.

Craig, D.Q.M., Newton, J.M., 1991. Characterisation of polyethylene glycols using solution calorimetry. Int. J. Pharm. 74, 43–48.

Fox, K.C., 1995. Putting proteins under glass. Science 267, 1922–1923.

Franks, F., 1990. Freeze-drying: from empiricism to predictability. Cryo-Letters 11, 93–110.

Gao, D., Rytting, J.H., 1997. Use of solution calorimetry to determine the extent of crystallinity of drugs and excipients. Int. J. Pharm. 151, 183–192.

Gill, S.J., Noll, L., 1972. Calorimetric study of association of diketopiperazine in water. J. Phys. Chem. 76, 3065–3068.

Gray, R.A., Stern, A., Bewley, T.A., McRorie, D., Voelker, P., Liu, J., Shire, S.J., 1994. Determination of protein concentration by improved optical methods. Pharm. Res. 11, 76.

Guillory, J.K., Erb, D.M., 1985. Using solution calorimetry to quantitate binary mixtures of three crystalline forms of sulfamethoxazole. Pharm. Manuf. 9, 29–33.

Habermann, S.M., Murphy, K.P., 1996. Energetics of hydrogen bonding in proteins: a model compound study. Protein Sci. 5, 1229–1239.

Hicks, S.N., Smiley, R.D., Hamilton, J.B., Howell, E.E., 2003. Role of ionic interactions in ligand binding and catalysis of R67 dihydrofolate reductase. Biochemistry 42, 10569–10578.

Kornblatt, J.A., Rajotte, I., Heitz, F., 2001. Reaction of canine plasminogen with 6-aminohexanoate: a thermodynamic study combining fluorescence, circular dichroism, and isothermal titration calorimetry. Biochemistry 40, 3639–3647.

Lippert, K., Galinski, E.A., 1992. Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl. Microbiol. Biotechnol. 37, 61–65.

Manning, M.C., Patel, K., Borchardt, R.T., 1989. Stability of protein pharmaceuticals. Pharm. Res. 6, 903–917.

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.

McGraw, S.E., Lindenbaum, S., 1990. The use of microcalorimetry to measure thermodynamic parameters of the binding of ligands to insulin. Pharm. Res. 7, 606–611.

Nagendra, H.G., Sukumar, N., Vijayan, M., 1998. Role of water in plasticity, stability, and action of proteins: the crystal structures of lysozyme at very low levels of hydration. Proteins: Struct. Funct. Gen. 32, 229–240.

Overcashier, D.E., Brooks, D.A., Costantino, H.R., Hsu, C.C., 1997. Preparation of excipient-free recombinant human tissue-type plasminogen activator by lyophilization from ammonium bicarbonate solution: an investigation of the two-stage sublimation phenomenon. J. Pharm. Sci. 86, 455–459.

Pikal, M.J., 1990. Freeze-drying of proteins. Part I: process design. Biopharmaceutics 3, 18–27.

Pikal-Cleland, K.A., Cleland, J.L., Anchordoquy, T.J., Carpenter, J.F., 2002. Effect of glycine on pH changes and protein stability during freeze-thawing in phosphate buffer systems. J. Pharm. Sci. 91, 1969–1979.

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

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.

Sarciaux, J.M., Mansour, S., Hageman, M.J., Nail, S.L., 1999. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. J. Pharm. Sci. 88, 1354–1361.

Schellman, J.A., 1955. The thermodynamics of urea solutions and the heat of formation of the peptide hydrogen bond. C. R. Trav. Lab. Carlsberg. (Chim.) 29, 223–229.

Souillac, P.O., Costantino, H.R., Middaugh, C.R., Rytting, J.H., 2002a. Investigation of protein/carbohydrate interactions in the dried state. 1. Calorimetric studies. J. Pharm. Sci. 91, 206–216.

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

St. Hilaire, P.M., Boyd, M.K., Toone, E.J., 1994. Interaction of the Shiga-like toxin type 1 B-subunit with its carbohydrate receptor. Biochemistry 33, 14452–14463.

Susi, H., Timasheff, S.N., Ard, J.S., 1964. Near infrared investigation of interamide hydrogen bonding in aqueous solution. J. Biol. Chem. 239, 3051–3054.

Takano, K., Yamagata, Y., Kubota, M., Funahashi, J., Fujii, S., Yutani, K., 1999. Contribution of hydrogen bonds to the conformational stability of human lysozyme: calorimetry and X-ray analysis of six Ser → Ala mutants. Biochemistry 38, 6623–6629.

Tanaka, R., Takeda, T., Miyajima, R., 1991. Cryoprotective effect of saccharides on denaturation of catalase during freeze-drying. Chem. Pharm. Biol. 39, 1091–1094.