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Investigation of protein/carbohydrate interactions in the dried state. 2. Diffuse reflectance FTIR studies

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Abstract

Upon freeze-drying in the absence of lyoprotectants, Fourier transform infrared (FTIR) spectroscopy has detected changes in the secondary structures of proteins. Such FTIR studies have been typically conducted using protein/KBr pellets, where additional protein degradation could potentially occur due to pressure effects and partial dissolution of the chaotropic KBr. Diffuse reflectance FTIR spectroscopy, in which no sample preparation is necessary, was evaluated as an alternative spectroscopic method to examine protein structure upon freeze-drying. The therapeutic proteins recombinant human deoxyribonuclease I (rh-DNase) and recombinant human insulin like growth factor I (rh-IGF-I) were freeze-dried with mannitol, sucrose, trehalose, and two molecular weight dextrans (69 and 503 kDa) separately, at concentrations ranging from 0 to 100% (w/w). Upon freeze-drying, rh-DNase and rh-IGF-I underwent significant changes in their secondary structure. For both proteins, the presence of intermolecular β-sheets due to aggregation was detected and the α-helix content decreased significantly. The addition of carbohydrates to the formulations inhibited the protein secondary structure rearrangement in a concentration-dependent manner. Sucrose and trehalose appeared to be the most efficient excipients in preventing secondary structure changes. The conformational changes observed for both proteins appeared to be reversible upon rehydration.

Keywords: Freeze drying; Protein; Carbohydrate; FTIR; Solid state

1. Introduction

The need to study protein/excipient interactions in the solid state has become increasingly apparent, as freeze-drying has become the preferred process to improve the long-term stability of pharmaceutical recombinant proteins. Upon freeze-drying in the absence of lyoprotectants, changes in the secondary structures of most proteins have been observed. The addition of excipients has been found to both improve the stability of proteins upon freeze-drying and subsequent storage. It has been hypothesized that a direct interaction between the additives and the surface of proteins occur. During the secondary drying phase, the removal of water molecules is

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believed to be at least partially responsible for protein aggregation. By replacing water molecules with other hydrogen bond forming compounds such as carbohydrates, proteins can be stabilized during the secondary drying phase. This is usually referred to as the water-replacement mechanism. The presence of such protein/carbohydrate interactions has been inferred by infrared spectroscopy (Carpenter and Crowe, 1989; Prestrelski et al., 1993; Kreilgaard et al., 1998; Allison et al., 1999). Changes in the IR spectra of dried proteins compared with those of proteins in solution have been interpreted as either due to significant changes in the secondary structure (Lamba et al., 1983; Poole and Finney, 1983, 1984; Prestrelski et al., 1993; Costantino et al., 1995; Allison et al., 1998; Costantino et al., 1998) or simply due to changes in the hydration state (Careri et al., 1980; Rupley and Careri, 1991). Proteins composed of α-helices and mixtures of α-helices and β-sheets have generally shown more dramatic changes upon freeze-drying than those consisting primarily of β-structure (Prestrelski et al., 1993; Costantino et al., 1995, 1998). Most commonly, a significant decrease in α-helix content is detected, with a simultaneous increase in β-sheet formation (possibly intramolecular in nature). Most FTIR studies (Yang et al., 1987; Costantino et al., 1996; Dong et al., 1996; Carpenter et al., 1998), however, have been conducted on samples in which the protein was pressed at high pressure into a KBr pellet. Dispersing dry protein samples in material such as KBr after fine grinding and compressing this mixture to obtain a transparent disk could result in additional protein structural alterations. Chan et al. (Chan et al., 1996) demonstrated a correlation between the loss of activity (due to the formation of irreversible aggregates) of rh-DNase and the pressure used for the compression of KBr pellets. Additional protein deterioration could occur from partial dissolution of KBr in the presence of residual moisture contained in the freeze-dried samples. Bromide anions are highly chaotropic and their dissolution in residual water hydrating the protein could induce further structural alterations. Diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS) eliminates sample preparation and permits direct determinations of FTIR spectra of solid samples. Spectra obtained from the absorbed fraction of the radiation are, however, characterized by different band intensities compared with those obtained by classical transmission geometry. A correction factor can be applied to DRIFTS spectra which compensates for the artificial enhancement of weaker intensity peaks (Culler, 1993):

$$f(R_\infty) = \frac{(1-R_\infty)^2}{2R} = \frac{K}{s}$$

where R is the absolute reflectance of the absorbing layer relative to that of a non-absorbing material (KBr or KCl), s is the scattering coefficient, and K is the molar absorption coefficient. The reflectance spectrum then is defined as the ratio of the sample concentration to the scattering characteristic of the sample.

2. Materials and methods

2.1. Materials

Recombinant human deoxyribonuclease I protein (rh-DNase) and recombinant human insulin-like growth factor I (rh-IGF-I) were produced at Genentech Inc., (South San Francisco, CA). All proteins were provided as excipient free powder after dialysis against pure water or a 10 mM ammonium bicarbonate buffer followed by lyophilization (Costantino et al., 1997; Overcashier et al., 1997). Sucrose, and dextrans 69 and 503 kDa were purchased from Sigma Chemicals, St. Louis, USA, dihydrate trehalose from Acros, Pittsburgh, USA and mannitol from E.M. Industry, USA. These compounds were of the highest grade available and used without further purification.

2.2. Methods

2.2.1. Freeze-drying procedure

The freeze-drying process was performed on a Benchtop 1.5 freeze-dryer (Virtis Company, Inc.). rh-DNase and rh-IGF-I were freeze-dried with mannitol, sucrose, and trehalose separately at concentrations ranging from 0 to 100% (w/w).

Unbuffered solutions of protein/carbohydrate mixtures, containing 5 mg/ml of total solid, were prepared using nanopure water and dispensed in scintillation glass vials. Pure protein solutions (5 mg/ml) and pure carbohydrate solutions (5 mg/ml) were also prepared. Freezing of the solutions was performed by dipping the vials in liquid nitrogen for about 1 min (Carstensen and Scoik, 1990). The vials were then placed on a tray previously cooled to −45 °C. The shelf temperature was maintained at a temperature of −37 °C for a period of about 90 h during primary drying. The temperature was raised in five steps to 30 °C over a period of about 40 h during secondary drying. At the end of the freeze-drying cycle, the vacuum was broken with dry nitrogen. The vials were further refrigerated in a vacuum desiccator over P_2O_5 until testing.

2.2.2. Fourier transform infrared spectroscopy

FTIR spectra were recorded using a Nicolet Magna 560 spectrometer from 4000 to 400 cm^{-1} using 4 cm^{-1} resolution and an accumulation of 256 scans. The system was continuously purged with dry air. Original spectra were smoothed using an approximate 13 cm^{-1} span.

Spectra of aqueous samples were obtained using attenuated total reflectance (ATR) spectroscopy in H_2O. A multi bounce (45°) ZnSe crystal plate (Spectra-Tech, Shelton, USA) was used for the ATR measurements. High protein concentrations (10 mg/ml) were employed to avoid the influence of adsorbed proteins on the spectra (Swedberg et al., 1990; Jackson and Mantsch, 1992; Oberg and Fink, 1998). Smoothing and water subtraction were performed using NICOLET OMNIC 4.0 software. Water subtraction was performed until a straight baseline was obtained between 2000 and 1750 cm^{-1} (Haris et al., 1986; Olinger et al. 1986).

Diffuse reflectance spectra were measured with a Graseby Specac Minidiff™ PN 4500 (Graseby Specac Inc., Fairfield, USA). Alignment of the system was performed using powdered KBr.

The amide I region (1600–1700 cm^{-1}), corresponding primarily to CO stretching with some contribution from the stretching of CN groups as well as CCN deformations (Susi et al., 1967; Krimm and Bandekar, 1986; Bandekar, 1992; Torii and Tasumi, 1996), was used to evaluate secondary structure changes upon freeze drying. Second-derivative and Fourier self-deconvoluted spectra of the amide I region were used as peak position guides for the curve fitting procedure. Curve fitting was performed using a mixed Gaussian/Lorentzian function (Cameron and Moffatt, 1984; Markovich and Pidgeon, 1991) (GRAMS/32 software, Galactic Industries). Based on studies of proteins of known secondary structure, model polymers, and theoretical calculations, specific IR bands have been assigned to different types of secondary structures. It is generally understood that α-helices absorb between 1660 and 1650 cm^{-1}, β-sheets between 1640 and 1620 cm^{-1} as well as near 1675 cm^{-1}, and β-turns from 1695 to 1660 cm^{-1} (Susi and Byler, 1986). Peaks between 1650 and 1640 cm^{-1} have been assigned to unordered structures and α-helices (Krimm and Bandekar, 1986). Errors in estimates of secondary structure content described in Tables 1 and 2 were estimated to be of the order of ±3%.

2.2.3. Circular dichroism

CD spectra were recorded on a JASCO (J-720) spectropolarimeter from 260 to 185 nm with a resolution of 0.5 nm and an accumulation of four scans. The data were averaged and smoothed after subtraction of a water baseline. The path length of the cell was 0.1 cm and protein solution concentrations were about 0.1 mg/ml. The scan speed and the sensitivity were set at 50 nm/min and 20 mdeg, respectively.

2.2.4. Thermal gravimetry analysis

Thermogravimetric analysis (TGA) was used to determine the amount of residual moisture contained in the samples before and after freeze-drying. All measurements were performed with a Perkin-Elmer TGA-2. Sample sizes of 2–3 mg were used. To avoid interference from moisture in the air, the sample chamber was purged with dry nitrogen at a flow rate of 15–25 ml/min. A temperature range of 25–250 °C was employed with a heating rate of 10 °C/min. These determinations were performed in duplicate.

3. Results and discussion

3.1. rh-DNase-containing mixtures

The FTIR spectrum of rh-DNase in solution was obtained using ATR geometry. Curve fitting of the amide I region (Fig. 1a) revealed the presence of peaks at 1688, 1679, 1666, 1654, 1644, 1630, and 1619 cm^{-1}. The position of these peaks is consistent with previous measurements (Chan et al., 1996; Costantino et al., 1998). The peaks at 1666 and 1654 cm^{-1} were assigned to short and long α-helices, respectively (Costantino et al., 1998), while signals at 1679 and 1630 cm^{-1} arose from β-sheets. Overall, the secondary structure of rh-DNase (Table 1) was estimated to be composed of about equal amounts of α-helices, β-sheets, and other structural elements (i.e. turns and disordered regions). This result is consistent with the X-ray structure of rh-DNase (Shire, 1996).

The FTIR spectrum of freeze-dried rh-DNase was obtained using a diffuse reflectance spectroscopic technique (DRIFTS). A significant shift toward higher wavenumbers is evident in the amide I region of the lyophilized protein (Fig. 1b). This shift resulted in some absorbance above 1700 cm^{-1}. A peak in this position is rarely seen for proteins, except in the case of protonated carboxylic groups (Chirgadze and Brazhnikov, 1974; Casal et al., 1988). Such shifts are also consistent with a previously reported spectrum of freeze-dried rh-DNase (Chan et al., 1996), which was interpreted to indicate the presence of aggregated material. This latter spectrum was obtained by transmission IR of KBr pellets. Thus, the presence of this absorbance above 1700 cm^{-1} found by the two different IR sampling techniques suggests that such results are not an artifact of the diffuse reflectance geometry and validate the use of K factor corrections for possible band distortions. Thus, employing peak assignments, obtained by transmission or ATR FTIR, to assign peaks in diffuse reflectance spectrum seem justified. Since there are differences in the overall amide I region shape between spectra obtained by diffuse reflectance and ATR, it seems most conservative to compare the various samples based

Table 1
Secondary structure content of pure rh-DNase and rh-DNase/excipient freeze-dried mixture

Protein samples		β-sheet[a]		α-helix[a] (%)	Others (%)[a]
		Intermolecular (%)	Intramolecular (%)		
Pure	Solution	0	36	31	33
rh-DNase	Freeze-dried	44	5	8	43
15% excipient	Sucrose	41	20	10	29
	Trehalose	39	19	7	35
	Mannitol	51	12	11	26
	Dextran 69 kDa	49	12	6	32
	Dextran 503 kDa	53	13	5	29
50% excipient	Sucrose	5	27	23	37
	Trehalose	27	22	20	30
	Mannitol	24	17	13	47
	Dextran 69 kDa	40	17	6	36
	Dextran 503 kDa	51	16	5	27
90% excipient	Sucrose	1	37	30	32
	Trehalose	2	39	30	29
	Mannitol	7	24	19	50
	Dextran 69 kDa	15	33	16	35
	Dextran 503 kDa	5	43	13	39
Reconstituted rh-DNase		0	33	32	35

[a] The uncertainty of these secondary structural calculations is estimated at ±3%.

Table 2
Secondary structure content of pure rh-IGF1 and rh-IGF1/excipient freeze-dried mixture

Protein samples		β-sheet[a]		α-helix (%)[a]	Others (%)[a]
		Intermolecular (%)	Intramolecular (%)		
Pure	Solution	0	24	29	47
rh-IGF1	Freeze-dried	34	16	8	42
15% excipient	Sucrose	7	32	9	52
	Trehalose	7	30	13	50
	Mannitol	11	20	6	63
	Dextran 69 kDa	33	16	7	44
	Dextran 503 kDa	30	26	12	32
50% excipient	Sucrose	2	26	22	50
	Trehalose	2	28	24	46
	Mannitol	9	23	12	56
	Dextran 69 kDa	32	28	11	29
	Dextran 503 kDa	37	21	10	32
90% excipient	Sucrose	0	20	26	54
	Trehalose	0	23	32	45
	Mannitol	0	15	36	49
	Dextran 69 kDa	0	29	28	43
	Dextran 503 kDa	6	24	18	52
Reconstituted rh-IGF1		0	24	26	50

[a] The uncertainty of these secondary structural calculations is estimated at $\pm 3\%$.

on curve fitting rather than on the second derivative spectra which involves a band narrowing procedure. Major peaks obtained from curve fitting of the amide I region were observed at 1698, 1683, 1669, 1656, 1646, and 1634 cm^{-1}. The peak observed at 1698 cm^{-1} can be attributed to the formation of intermolecular β-sheets arising from aggregation of rh-DNase upon freeze-drying. This peak was consistent with the previous observation of a band at 1691 cm^{-1} for freeze-dried rh-DNase dispersed in a KBr pellet (Costantino et al., 1998). Conformational changes upon desiccation have also been observed by X-ray crystallography for bovine ribonuclease A (Bell, 1999). The peaks at 1683 and 1669 cm^{-1} are assigned to β-turns. The peaks at 1656, 1646, and 1634 cm^{-1} can be attributed to α-helices, unordered polypeptides, and intramolecular β-sheets, respectively. Quantitative analysis of the amide I band shows that the secondary structure of rh-DNase changed significantly upon freeze-drying (Table 1). A major decrease in α-helix content and an increase in β-sheet content were observed. Similar observations were previously reported for rh-DNase (Costantino et al., 1998) and other proteins containing α-helices (Carpenter and Crowe, 1989; Griebenow and Klibanov, 1995).

The FTIR spectra of various rh-DNase/sucrose freeze-dried mixtures were also examined by diffuse reflectance spectroscopy. Freeze-dried mixtures containing 15, 50 and 90% sucrose were studied. No significant differences in moisture content (3–5%) were observed among the samples containing various sucrose/rh-DNase ratios. A progressive shift of the amide I region towards lower wavenumbers was observed as the percentage of sucrose increased in the formulations (Fig. 2). Upon Gaussian/Lorentzian curve fitting, the relative contribution of peaks characteristic of intermolecular β-sheets (at about 1695 cm^{-1}) progressively decreased as the amount of sucrose increased. Simultaneously, the peaks from α-helices (at about 1655 and 1665 cm^{-1}) and intramolecular β-sheets (at 1675 and below 1635 cm^{-1}) increased with sucrose content. Even at the lowest sucrose concentration tested, 15%, the alteration of the secondary structure of the protein

upon freeze-drying was significantly reduced. The secondary structure content for mixtures containing 90% sucrose and 10% rh-DNase was similar to that of the protein in solution (Table 1).

DRIFTS were also measured for rh-DNase/sucrose physical mixtures. The amide I region obtained for physical mixtures of the protein and sucrose was not significantly different than that of the pure freeze-dried rh-DNase. This lack of changes compared with the previous result is suggestive of protein/sucrose interactions that require intimate molecular contacts provided by co-freeze-drying.

We also examined the effect of trehalose which, like sucrose, tends to remain amorphous upon freeze-drying. Again, no significant differences in moisture content (3–5%) were observed among the samples containing various trehalose/rh-DNase ratios. A similar shift toward lower wavenumbers was observed for the rh-DNase/trehalose freeze-dried mixtures as the amount of trehalose increased (Table 1). Peaks assigned to intermolecular β-sheets decreased, while peaks corresponding to α-helices and intramolecular β-sheets increased as the amount of trehalose in the formulation increased. When 90% trehalose was present, the secondary structure content of rh-DNase was similar to that of the native protein in solution (Table 1). Similar to the result for sucrose-containing physical mixtures, no significant spectral shifts (compared with the pure freeze-dried protein) were observed for the physical mixture containing 90% trehalose. These results suggest that trehalose is as efficient as sucrose in minimizing secondary structure changes of rh-DNase upon freeze-drying.

Mannitol represents another potential excipient for proteins which, unlike sucrose and trehalose, is prone to crystallization when freeze-dried alone. A shift of the amide I band toward lower wavenumbers was also observed in going from 15

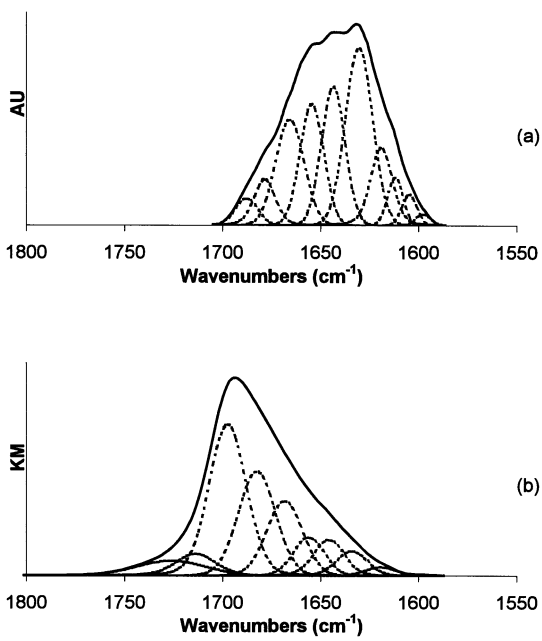

Fig. 1. Infrared spectra of pure rh-DNase in the amide I region. (a) Aqueous solution. (b) Freeze-dried.

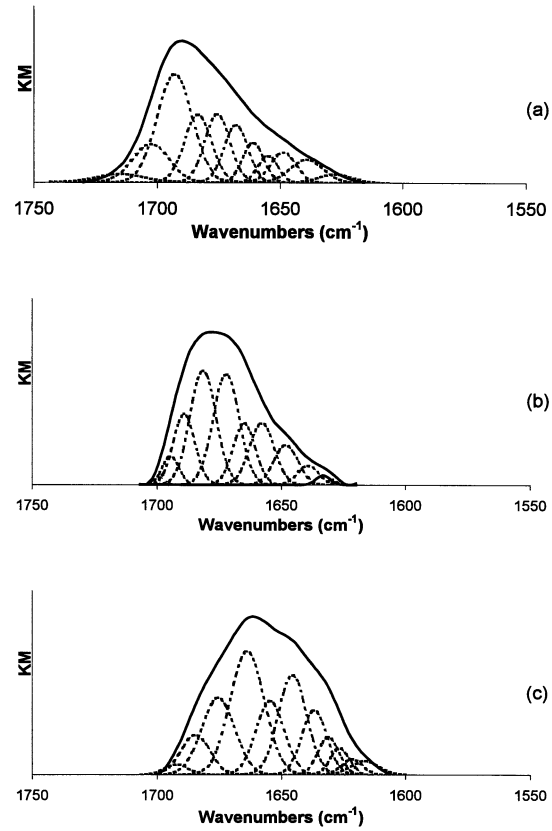

Fig. 2. Infrared spectra of rh-DNase/sucrose freeze-dried mixtures in the amide I region. (a) 15% sucrose. (b) 50% sucrose. (c) 90% sucrose.

to 90% mannitol with the freeze-dried mannitol-containing mixtures. Curve fitting of the original spectra revealed the disappearance of intermolecular β-sheets as the percentage of mannitol increased. Aggregates (i.e. intermolecular β-sheets) were still present, however, in the sample containing 90% mannitol (Table 1). The presence of interactions between mannitol and rh-DNase was previously demonstrated by DSC (Souillac et al., 2002). Also, the partial amorphization of mannitol when mixed with rh-DNase was demonstrated by solution calorimetry and DSC (Souillac et al., 2002). The interaction of DNase and mannitol is confirmed by these FTIR measurements. In contrast, however, the secondary structure of rh-DNase in the sample containing 90% mannitol was significantly different than that of the protein in solution. Therefore, despite providing significant protection against the unfolding of rh-DNase upon freeze-drying, mannitol seems to be a less efficient protective agent than sucrose or trehalose.

We also tested the effect of adding the polysaccharide dextran. The FTIR spectra of freeze-dried mixtures containing different ratios of rh-DNase and dextran were separately obtained using two forms of dextran (69 and 503 kDa). No significant shift of the amide I band toward lower wavenumbers was observed for the 15 and 50% dextran-containing mixtures (Fig. 3). A detectable shift was only observed for the mixtures containing 90% dextran. The same trend was observed with both dextrans. Assignments of the peaks obtained from curve fitting the amide I region confirmed the absence of protective effects from either type of dextran when used at a concentration of up to 50% (Table 1). For mixtures containing 90% dextran, a significant amount of aggregate formation was still detected in the samples. The secondary structure content of rh-DNase when freeze-dried with 90% of either of the dextrans was still significantly different than that of the protein in solution. Thus, among all additives tested, dextrans seemed to be the least effective in preventing secondary structure changes upon freeze-drying. The lack of a protective effect by dextrans on the dehydration-induced denaturation of proteins may be due at least in part to their larger size (Pikal et al., 1991; Kreilgaard et al., 1999).

The ATR-FTIR spectrum of rh-DNase was obtained after reconstitution in distilled water of a freeze-dried sample of pure protein. The amide I region of the reconstituted rh-DNase spectrum was not significantly different than that of the initial solution. Upon curve-fitting of the amide I region of the reconstituted sample, no significant secondary structure differences were observed (Table 1). This indicates reversibility of the secondary structure changes upon rehydration of the freeze-dried samples. Therefore, although the presence of carbohydrates significantly decreased the extent of secondary structure changes upon freeze-drying, their presence was not necessary to prevent irreversible changes. Similarly, Chan et al. (Chan et al., 1996), using size exclusion chromatography, did not observe protein aggregation

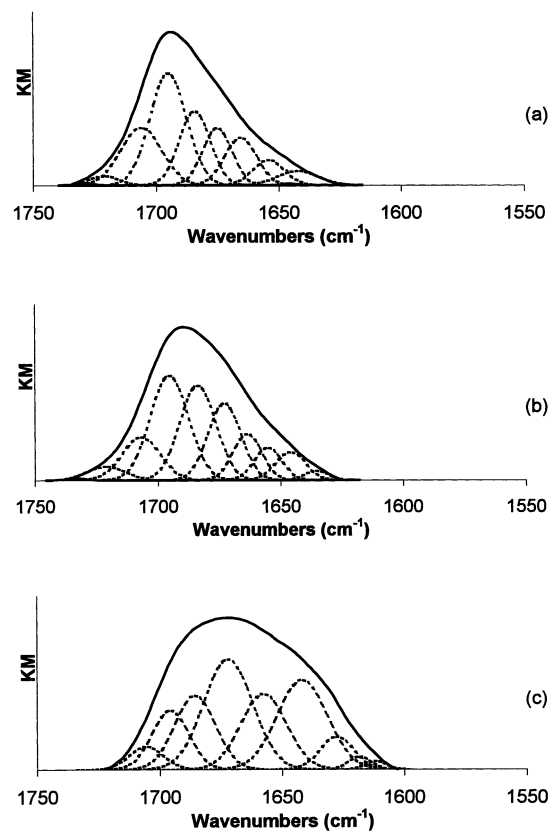

Fig. 3. Infrared spectra of rh-DNase/dextran 69 kDa freeze-dried mixtures in the amide I region. (a) 15% 69 kDa dextran. (b) 50% 69 kDa dextran. (c) 90% 69 kDa dextran.

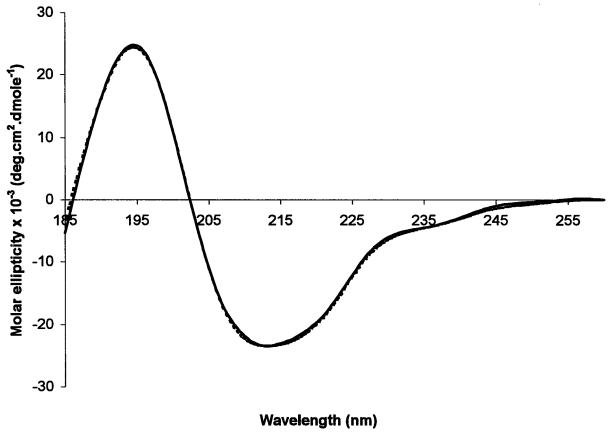

Fig. 4. Circular dichroism spectra of initial (full line) and reconstituted (dotted line) rh-DNase samples.

after reconstituting freeze-dried samples of rh-DNase at 20 mg/ml (four times the protein concentration used in this study). The presence of carbohydrates in the formulation could, however, play a significant role in storage stability. All of the studies presented here were, in fact, performed immediately after freeze-drying.

Far-UV CD Spectra for rh-DNase in solution were also obtained before and after freeze-drying and reconstitution (Fig. 4). The spectra manifest a small minimum of small amplitude at about 237 nm followed by a markedly broader minimum at about 213 nm. A maximum was observed at about 194 nm. No significant difference was observed between the two spectra, confirming the reversibility of the secondary structure changes upon rehydration.

3.2. rh-IGF-I-containing mixtures

The FTIR of a second protein, rh-IGF-I, was obtained in aqueous solution using ATR spectroscopy (Fig. 5). Deconvolution found peaks at 1690, 1680, 1669, 1656, 1644, 1635, 1626, and 1613 cm^{-1}. The first three peaks were assigned to β-turns and the peaks at 1656 and 1644 cm^{-1} to α-helices and disordered regions, respectively. The assignment of the other three peaks was more ambiguous. Peaks below 1637 cm^{-1} are usually attributed to β-sheets (Markovich and Pidgeon, 1991; Dong et al., 1995). The crystal structure of rh-IGF-I does not demonstrate the presence of β-sheets, although some residues have been shown to adopt a β-strand-like structure in solution (Cooke et al. 1991). Furthermore, extended structures have been observed by NMR (Sato et al., 1993). The β-sheet content of rh-IGF-I (Table 2) could be overestimated due to a misassignement of these peaks. Indeed, peaks at 1613 cm^{-1} and between 1620 and 1640 cm^{-1} have also been assigned to side chain vibrations (Kalnin et al., 1990).

The freeze-dried protein displays a significant shift toward higher wavenumbers (Fig. 5), probably reflecting intermolecular protein contacts upon freeze-drying. The most significant peaks observed after curve fitting the amide I region were positioned at 1702, 1692, 1682, 1673, 1668, 1657, and 1648 cm^{-1}. The first two peaks are assigned to intermolecular β-sheets, while the peaks at 1682 and 1668 cm^{-1} can be attributed to β-turns. The peaks at 1657 and 1648 cm^{-1} were assigned to α-helices and unordered structure, respectively. The secondary structure of rh-IGF-I

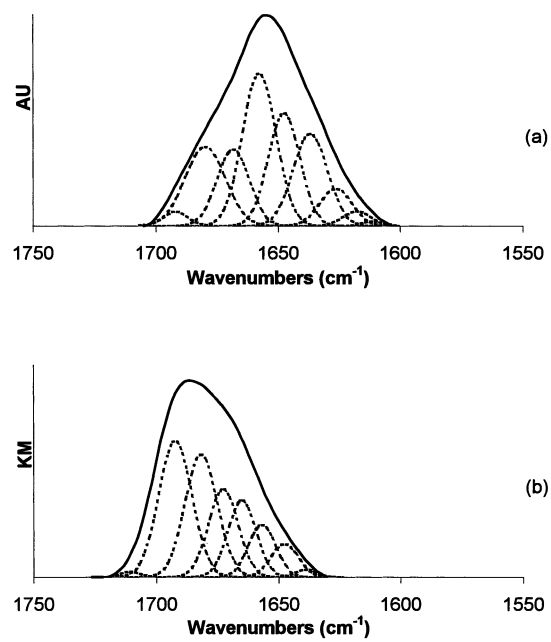

Fig. 5. Infrared spectra of pure rh-IGF1 in the amide I region. (a) Aqueous solution. (b) Freeze-dried.

was significantly altered upon freeze-drying (Table 2). A major decrease in α-helix content with a simultaneous increase in β-structure was observed.

Freeze-dried mixtures containing 15, 50, and 90% sucrose in the presence of rh-IGF-I were also obtained (spectra not shown). No significant differences in moisture content (3–5%) were observed among the samples containing various sucrose/ rh-IGF-I ratios. A progressive shift of the amide I region toward lower wavenumbers and the original solution spectrum was observed as the percentage of sucrose increased in the formulations. The estimated secondary structure content of rh-IGF-I in the different formulations is shown in Table 2. The relative contribution of peaks characteristic of intermolecular β-sheets (at about 1700 cm^{-1}) progressively diminished as the amount of sucrose increased in the formulation. The presence of as little as 15% sucrose decreased the intermolecular β-sheet content from about 34 to 7%. The relative importance of the peaks assigned to α-helices (at about 1955 cm^{-1}) also increased with sucrose content. The secondary structure content of rh-IGF-I of the mixtures containing 90% sucrose was similar to that of the protein in solution. The amide I region of rh-IGF-I obtained for physical mixtures containing-sucrose was not significantly different than that of the pure freeze-dried protein.

A shift toward lower frequency of the amide I regions of rh-IGF-I/trehalose freeze-dried mixtures was also observed. Peaks assigned to intermolecular β-sheets decreased while peaks corresponding to α-helices increased as the amount of trehalose in the formulation increased (Table 2). When 90% trehalose was present in the formulation, the secondary structure content of rh-IGF-I was similar to that of the protein in solution. No significant shift toward lower wavenumbers of the amide I region (as compared with the pure freeze-dried protein) was observed for physical mixtures. Thus, trehalose seemed to be as efficient as sucrose in preventing secondary structure changes in rh-IGF-I upon freeze-drying.

A significant shift of the amide I region band toward lower wavenumbers was again observed in increasing mannitol from 15 to 90% in the formulation. A progressive disappearance of intermolecular β-sheets as the percentage of mannitol increased was seen (Table 2). The relative content of α-helices in the freeze-dried mixture containing 90% mannitol appeared to be larger than that of the protein in solution. This result could be explained by the presence of particularly strong interactions between mannitol and rh-IGF-I as demonstrated by DSC experiments (Souillac et al., 2002). The disappearance of the melting endotherm of mannitol as its amount reached 50% indicated the presence of stronger rh-IGF-I/mannitol interactions than those observed with other proteins previously studied (rh-DNase, rh-GH, and rh-MetGH). The partial amorphization of mannitol observed with solution calorimetry is confirmed by its protective effect against secondary structure changes upon freeze-drying.

The FTIR spectra of freeze-dried mixtures of rh-IGF-I and two different molecular weight dextrans (69 and 503 kDa) were also separately obtained. No significant shift of the amide I band toward lower wavenumbers was observed for the 15 and 50% dextran-containing mixtures. A shift was only observed for the mixtures containing 90% dextrans. The same trend was observed with both types of dextran. Assignments of the peaks obtained from curve fitting the amide I regions confirmed the absence of a protective effect from either type of dextran when used at concentrations up to 50% (Table 2). For the mixture containing 90% 69 kDa dextran, no intermolecular β-sheets were detected and the secondary structure content was similar to that of the protein in solution. On the other hand, intermolecular β-sheets were detected in the presence of 90% 503 kDa dextran. Therefore, the dextrans again seemed to be the least efficient of all of the excipients examined in preventing freeze-drying induced secondary structure changes.

To evaluate the reversibility of the secondary structure changes observed upon freeze-drying of rh-IGF-I, its FTIR spectrum was measured after reconstitution in distilled water. The amide I region of the reconstituted sample was not significantly different than that of the initial solution and no significant differences were observed in the calculated secondary structure (Table 2). Thus,

the secondary structure changes seen in the lyophilized protein were reversible upon rehydration. Therefore, although carbohydrates significantly decrease the extent of secondary structure alterations upon freeze-drying, their presence was not required to prevent irreversible changes.

Far-UV CD spectra of pure rh-IGF-I in solution were also obtained before freeze-drying and after reconstitution in distilled water (data not shown). The spectra presented a double minimum at 207 and 223 nm and a strong positive peak at 191 nm. These results are consistent with the presence of the expected helical content. No significant difference could be observed between the spectra of rh-IGF-I obtained before and after freeze-drying, supporting the reversibility of the secondary structure changes observed in the solid state.

4. Conclusions

Diffuse reflectance infrared spectroscopy provides an attractive alternative method for examination of solid proteins compared with the more commonly used KBr pellet method. The therapeutic proteins rh-DNase and rh-IGF-I undergo significant reversible changes in their secondary structure upon freeze-drying. For both proteins, the presence of intermolecular β-sheets was detected and α-helix content decreased significantly. The addition of carbohydrates inhibited protein secondary structure rearrangement in a concentration-dependent manner. Sucrose and trehalose appear to be the most efficient additives in preventing these secondary structure changes. These results support interaction between protein and excipients in the solid state, consistent with data provided by previous calorimetric measurements. Furthermore, diffuse reflectance spectroscopy appears to be a valuable alternative to the KBr pellet method for examining dried proteins.

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