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Research Paper

Effects of Excipients on Protein Conformation in Lyophilized Solids by Hydrogen/Deuterium Exchange Mass Spectrometry

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Purpose. Excipients are added to lyophilized protein drug formulations to protect the protein during processing and storage, but the mechanisms are poorly understood. Here, hydrogen/deuterium (H/D) exchange with mass spectrometry was used to assess protein conformation and excipient interactions in lyophilized solids.
Methods. Calmodulin (CaM, 17 kD) was co-lyophilized with carbohydrate excipients (sucrose, mannitol, trehalose, raffinose, dextran 5,000, dextran 12,000) or guanidine hydrochloride (negative control) and exposed to D_2O vapor at 33% RH and RT. Samples were then dissolved and analyzed by mass spectrometry (+ESI/MS). Peptic digestion provided additional, site-specific information on H/D exchange. Solids were further characterized by powder x-ray diffraction (PXRD), differential scanning calorimetry (DSC), infrared spectroscopy (FTIR) and water vapor sorption.
Results. Excipients protected CaM from H/D exchange, increasing in the order guanidine hydrochloride < no excipient, mannitol < dextran 5,000, dextran 12,000 < sucrose < raffinose < trehalose. Effects were exerted primarily in the protein's α-helical segments.
Conclusions. The effects of carbohydrate excipients on protein conformation in lyophilized solids are not exhibited uniformly along the protein sequence, but instead are exerted in a site-specific manner. The results also demonstrate the utility of H/D exchange with ESI/MS for protein structure characterization in lyophilized samples.

KEY WORDS: ESI mass spectrometry; excipient; FTIR; hydrogen/deuterium exchange; lyophilization; protein.

INTRODUCTION

Because of the intrinsic chemical and physical instability of protein molecules, many protein drugs are formulated as lyophilized solids. As of December 2003, almost half of the newly developed biopharmaceutical products were in lyophilized form (1). To protect the protein from various stresses during lyophilization and to preserve its stability during storage, excipients are often added. The most widely used excipients are carbohydrate-based excipients such as sucrose, trehalose and dextrans. The rational development of lyophilized protein drug products requires an understanding of the properties of those excipients and of their ability to preserve protein native structure during lyophilization and storage. Moreover, an appreciation of the mechanism by which excipients exert their protective effects would improve formulation design. A detailed mechanistic understanding of protein/excipient interactions in the solid state and their effects on protein drug stability is lacking at present. Fourier transform infrared spectroscopy (FTIR) is widely used to study protein secondary structure in lyophilized solids (2,3). By comparing the FTIR spectrum of the solid protein formulation to the spectrum recorded under comparable solution conditions, an excipient's ability to protect the protein's native structure can be evaluated (4–7). On the basis of such studies, several groups have suggested that the stabilization of the protein by the excipients is the result of protein-excipient interactions (4,6). Izutsu et al. (6) reported that higher molecular weight carbohydrates had less protective effect than smaller carbohydrates such as sugars due to a reduction in the number of free hydroxyl groups available to interact with the protein. Costantino et al. (4) reported that the molar ratio of excipient to protein is important for stabilizing the protein during lyophilization, and also noted that excipients that remain amorphous in the solid state have better ability to prevent protein aggregation. Carpenter et al. provided FTIR evidence that the interactions between proteins and sugars in lyophilized solids involve hydrogen bonding (8). These and other FTIR studies have provided valuable information on proteins in lyophilized solids, but are limited in that the FTIR method cannot provide site-specific information on specific portions of the protein sequence and their interactions with excipients. It is the local, specific interactions that are most likely to determine the reactivity of labile amino acids and the conformational stability of structural domains in

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the spatially heterogeneous environment of the amorphous solid.

We previously reported that hydrogen/deuterium (H/D) exchange with mass spectrometry can provide useful information on protein structure in amorphous solids and, with peptic digestion of the samples, can provide site-specific information on interactions between the protein and excipients in the solid phase (9). Here, we apply the solid-state H/D exchange method to protein formulations containing different excipients in an effort to obtained detailed information on local protein/excipient interactions. We have used a small, largely α-helical protein as a model (calmodulin, CaM, 17 kD) and various carbohydrates (di- and trisaccharides, dextrans) as representative excipients. The results demonstrate that the excipients provide varying degrees of protection from H/D exchange in the solid samples and that the differences are primarily localized in the non-terminal α-helical segments.

MATERIALS AND METHODS

Materials

Mannitol, sucrose, trehalose, raffinose and all of the chemicals used in CaM expression and purification were obtained from Sigma-Aldrich Co. (St. Louis, MO). Dextrans (molecular weight standard 5,000 and 12,000) were obtained from Fluka (Milwaukee, WI), formic acid and chloroacetic acid were obtained from Acros Organics (Morris Plains, NJ) and pepsin was obtained from Worthington Biochemical Corp. (Lakewood, NJ). All other chemicals were obtained from Fisher Scientific (Hampton, NH). Milli-Q water was used to prepare all solutions.

Calmodulin Expression and Purification

Calmodulin (CaM, 17 kDa) was overexpressed in *Escherichia coli* by transforming the recombinant plasmid (kindly provided by Dr. Jeffrey Urbauer, University of Georgia) containing the gene encoding the protein into BL21(DE3)-T1^{R} competent cells. The cell culture and protein purification procedures have been described previously (9). The purified protein was dialysed with water and the final protein concentration determined by UV absorption at 280 nm.

Lyophilization

A 100 μl aliquot of a CaM solution (4 mg/ml, 0.24 mM) was co-lyophilized with different excipients (1:1 weight ratio to CaM) using a VirTis AdVantage bench top freeze dryer (Gardiner, NY). The sample was first frozen at −35°C for 2 h. Drying was then conducted with a vacuum of 15 mT at shelf temperature of −35°C for 2 h, −5°C for 8 h, 5°C for 6 h, 15°C for 6 h and 25°C for 10 h.

Characterization of Solid Samples

Powder X-ray Diffraction (PXRD)

Powder x-ray diffraction was use to evaluate the crystallinity of the solids. The solid sample was packed into the shallow cell of a plastic sample holder and a glass slide was mounted on top of the sample holder with adhesive tape. The measurement was carried out using a powder x-ray diffractometer (Bruker D8 Discover powder diffractometer with a solid state detector, Bruker, Madison, WI) using Cu–Kα at a scan rate at 1.2° 2θ per min from 10° to 40° 2θ.

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was used to measure the total water content of the lyophilized solids. The lyophilized solids were stored in a 33% RH chamber up to 72 h and samples were withdrawn at designated times. The sample was then analyzed by a Q50 TGA (TA Instruments, New Castle, DE) with a thermal scan from ambient (~22°C) to 200°C at a scan rate of 10°C/min in an open platinum pan with nitrogen purge.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was used to measure the Tg of the lyophilized solids. Amorphous samples were sealed in hermetic aluminum pans immediately after lyophilization. To differentiate glass transition events from kinetic thermal events such as dehydration and degradation, thermally modulated DSC (MTDSC) was performed. MTDSC experiments were carried out at a scan rate of 1°C /min from 25 to 200°C with modulation amplitudes of ±0.32°C and a modulation period of 60 s. The resulting thermograms were analyzed using TA's universal analysis software.

FTIR Spectroscopy

Fourier-Transform infrared (FTIR) spectra of lyophilized CaM formulations were measured to detect any gross changes in secondary structure during lyophilization or H/D exchange. The spectra were acquired on a PerkinElmer FTIR One spectrometer with universal ATR (UATR) accessory (Wellesley, MA). The lyophilized powder was placed onto the diamond crystal surface and a stainless steel slide was used to cover the powder. To ensure good contact between the powder and the crystal surface, 90 T pressure was applied to the stainless steel cover. Each sample was subjected to 128 scans at a 4 cm^{-1} resolution. The spectra were first smoothed with the 13-point Savitzky–Golay smoothing function and then second derivatives of the spectra were calculated using GRAM AI software (Thermo Electron Corp., Waltham, MA). The second derivative spectra were further normalized to the same area after baseline and offset correction (2,3) and were overlaid for comparison (see Fig. 3). Correlation coefficients were calculated using the method of Kendrick et al. (3).

Solid-state H/D Exchange with D_2O Vapor

Samples of lyophilized CaM were placed into sealed desiccators at room temperature (~23°C) at a controlled relative humidity (RH) of 33% over D_2O vapor. A controlled RH environment was produced using saturated solutions of $MgCl_2$ (33% RH) in D_2O (10). The RH values correspond to the relative humidity over aqueous solutions of these salts at

room temperature, and have not been corrected for differences in the vapor pressure of D_2O vs. H_2O. Triplicate samples were withdrawn at designated times for immediate analysis or sealed and stored at −80°C before analysis.

LC +ESI/MS Analysis of Intact Protein

The method for LC+ESI/MS analysis of intact and peptic digested protein has been described previously (9). Briefly, samples exposed to D_2O vapor in the solid state were reconstituted with low pH and cold buffer and then desalted on a short desalting column before eluting into a Micromass® Q-Tof II mass spectrometer (Waters, Inc., Milford, MA) operated in the positive ion mode ($^+$ESI). After deconvoluting the raw mass spectrum, protein molecular weights were taken as the centroid of the deconvoluted peak. Back exchange control and correction was performed by the method of Zhang et al. Deuterium incorporation was then fitted to the following biexponential equation to obtain the kinetic parameters for exchange:

$$D = N_{\text{fast}}\left(1 - e^{-k_{\text{fast}}}\right) + N_{\text{slow}}\left(1 - e^{-k_{\text{slow}}}\right) \quad (1)$$

Here, D is the total number of deuterium incorporated, k_{fast} and k_{slow} are the apparent rate constants, and N_{fast} and N_{slow} are the number of sites associated with the "fast" and "slow" rates. We have selected a bi-exponential equation because exponential forms have been used to fit CaM H/D exchange in solution (11) and because this is the simplest equation that provides a reasonable fit to the data. N_{fast} and N_{slow} were subject to the additional constraint that $N_{\text{fast}}+N_{\text{slow}} = 145$, the total number of exchangeable backbone amide protons in CaM. Kinetic data were fitted using non-linear regression in Origin 7.0 (Microcal Software, Inc., Northampton, MA).

LC $^+$ESI/MS Analysis of Peptic Digestion

To obtain local information on protein H/D exchange, the deuterium labeled protein was digested with pepsin (pepsin to CaM molar ratio 1.2:1). The detailed procedure has been described previously (9). In brief, pepsin solution was freshly prepared before each digestion and activated by adding pH 2.3 chloroacetic acid. A CaM sample was added and digested for 3 min. The mixture of peptic fragments was then subjected to LC $^+$ESI/MS on C4 reverse phase column (Grace Vydac, Hesperia, CA) using a gradient elution. For each fragment, the MS scan with the highest ion count was selected. The peak cluster in this scan was centroided and the value used to calculate the back exchange correction and deuterium incorporation (12). Low pH and submersion of the chromatographic system in an ice bath were used to minimize back exchange.

RESULTS

Characterization of Solid Samples

The solid samples were characterized by traditional techniques such as PXRD, TGA and DSC to establish the fundamental properties of the lyophilized solids. Figure 1 shows the PXRD patterns for formulations containing mannitol (Fig. 1a) or guanidine hydrochloride (Fig. 1b) before and after storage at 33% RH for 72 h. PXRD patterns for formulations containing sucrose, trehalose, raffinose, dextran 5,000 and dextran 12,000 were featureless and consistent with a fully amorphous solid, and so are not shown. The amorphous nature of formulations containing the low molecular weight sugars is consistent with a previous report by Hancock et al., in which lyophilized powders containing sucrose, trehalose or raffinose maintained their amorphous character for three months at 30°C and 33% RH (13). The dextran formulations also remain amorphous; the recrystallization of these polymeric excipients is unlikely. In contrast, the PXRD pattern for formulations containing guanidine hydrochloride is largely crystalline before and after storage (Fig. 1b). Because the mass ratio of guanidine hydrochloride to protein is 72:1 and the molar ratio is 12,500:1, there is much more guanidine hydrochloride than protein in the formulation. Thus, it is likely that the PXRD pattern reflects the crystallinity of the excipient (GdnHCl) and that any crystallinity of the protein may not be detected. The mannitol formulation (Fig. 1a) showed some crystalline character before storage and a more distinct crystalline pattern after storage. The partial recrystallization of mannitol in solid protein formulations has been reported previously (14,15). Since it is expected that only the excipients can recrystallize, detection of crystallinity in the protein formulation suggests that phase separation has occurred, producing an excipient-rich crystalline phase and a protein-rich amorphous phase.

Water sorption kinetics was measured by TGA for the various formulations. As shown in Fig. 2, the water content for all of the formulations studied did not exceed 8% during the 72-h storage period at 33% RH. The trehalose-containing formulation showed rapid water sorption, reaching a plateau value of ~6.5% in less than 5 h. The remaining formulations showed two-phase water sorption kinetics, with a growth phase at $t < 24$ h followed by an approach to a plateau. The plateau values are reported as the pseudo-equilibrium water content in Table I. The values increase in the order mannitol < sucrose < raffinose < trehalose, dextran 5,000, dextran 12,000. The low water content for the mannitol formulation could be due to partial recrystallization of the excipient during lyophilization with exclusion of water from the crystal, which facilitates drying. Formulations containing the polymeric excipients or trehalose had slightly higher water levels than the formulation without excipients (Table I). DSC was only performed for the amorphous solids; the Tg values of the partially crystallized mannitol formulation and crystalline guanidine hydrochloride formulation were not measured. The Tg for the formulation with no excipients was not detected. For the low molecular weight excipient formulations, the Tg values increase in the order sucrose < trehalose< raffinose, which can be taken as a rough indication of decreasing mobility in the formulation. The two polymeric formulations have similar Tg values that are much higher than the formulations containing the small molecules, as expected.

FTIR Spectroscopy

FTIR analysis was performed on lyophilized CaM solids immediately after lyophilization to examine the secondary structure of CaM in the solid state. The normalized second

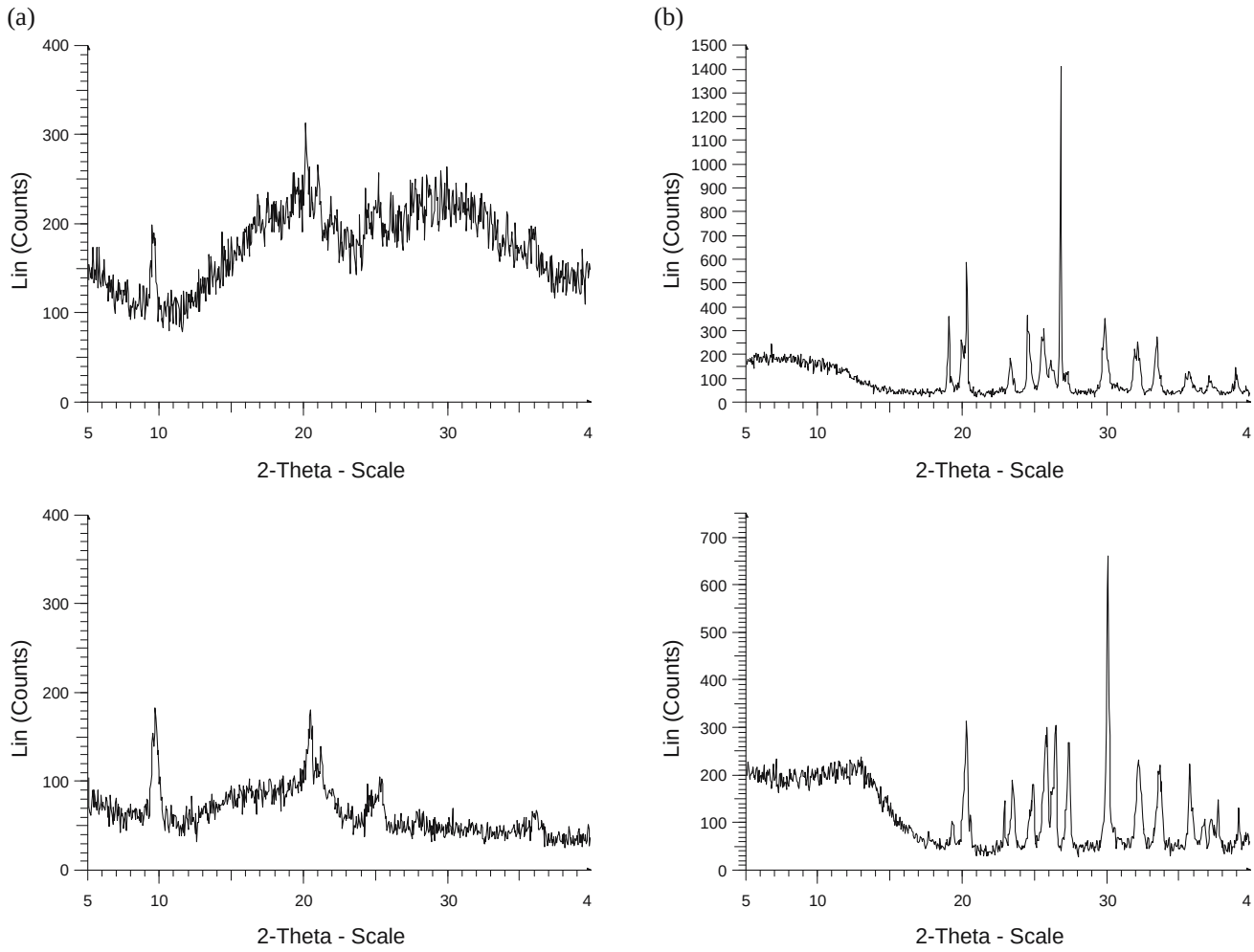

Fig. 1. PXRD pattern for lyophilized calmodulin solids with different excipients. (**a**) Mannitol and (**b**) guanidine hydrochloride before (Top) and after (Bottom) storage at 33% RH for 72 h.

derivatives of the amide I band are shown in Fig. 3. The spectra of protein formulations containing no excipient ("None", Fig. 3) raffinose, dextran 5,000 or dextran 12,000 show the strongest absorption bands at 1,651 cm^{-1}, with other major absorption bands at 1,640 and 1,658 cm^{-1}. Based on previous FTIR studies of lyophilized proteins (4,5), the bands at 1,658 and 1,651 cm^{-1} can be tentatively assigned to the α-helical structure and the band at 1,640 cm^{-1} assigned to unordered structure. The smaller band at 1,680 cm^{-1} also represents unordered structure and the band at 1,630 cm^{-1} typically corresponds to β-sheet structure (4,5), expected to be absent in native CaM. The spectra of CaM in formulations containing raffinose or dextrans, or without excipient ("None", Fig. 3), are very similar and are consistent with a predominantly α-helical structure in the solid samples. Since previous studies of CaM in solution have shown that the protein is largely helical (16–18), this result suggests that the helical structure is largely maintained in solid formulations containing raffinose or dextrans, and also in the absence of excipients. Formulations containing trehalose show a strong band at 1,658 cm^{-1} and a somewhat weaker band at 1,651 cm^{-1}, again consistent with a predominantly α-helical structure. Formulations containing mannitol and sucrose show more distinct bands at 1,640 and 1,680 cm^{-1}, suggesting that the protein has higher unordered content than the other formulations. Interestingly, the spectrum of the sucrose formulation also shows higher intensity for the bands at 1,658 and 1,651 cm^{-1}, suggesting increased α-helical structure. The reasons for the increase in intensity of the both

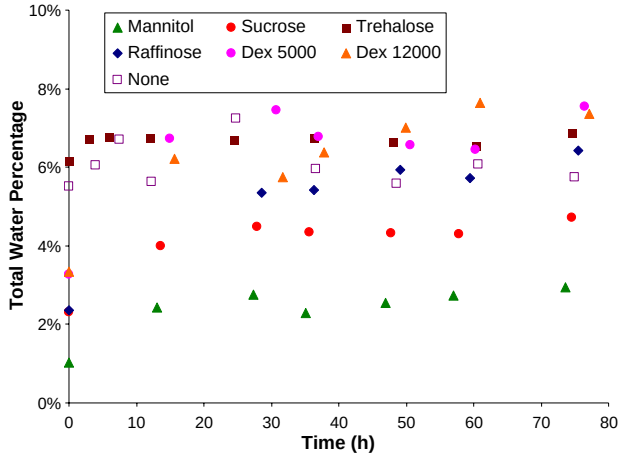

Fig. 2. Water sorption kinetics at 33% RH for lyophilized CaM solids with or without an equal mass of various excipients.

Table I. Physical Properties of Lyophilized CaM Formulations Before and After H/D Exchange with D_2O Vapor at 33% RH for 72 h

	None	Mannitol	Sucrose	Trehalose	Raffinose	Dextran 5,000	Dextran 12,000	Guanidine Hydrochloride
Tg (°C), post-lyophilization	N/A	N/A	16 ± 4	27 ± 8	76 ± 3	110 ± 13	104 ± 1	N/A
Tg (°C), after storage[a]	N/A	N/A	34 ± 4	42 ± 2	49 ± 2	82 ± 2	78 ± 5	N/A
Moisture content	6.13 ± 0.29%	2.65 ± 0.11%	4.44 ± 0.08%	6.70 ± 0.06%	5.77 ± 0.20%	6.96 ± 0.23%	6.82 ± 0.35%	N/A
Crystallinity before H/D exchange	Amorphous	Partial crystalline	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Crystalline
Crystallinity after H/D exchange	Amorphous	Partial crystalline	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Crystalline

[a] *Storage at 33% RH and room temperature for more than 24 h*

the α-helical and unordered bands in the sucrose formulation is not clear. A similar result is also observed for the mannitol formulation except that the bands at 1,658 and 1,651 cm^{-1} have apparently merged into one band at 1,654 cm^{-1}. Taken together, the FTIR results suggest that the carbohydrate-based excipients influence CaM secondary structure in the lyophilized solids, but local, site-specific information is not provided. For example, CaM has eight α-helical segments (see Fig. 5); the FTIR results do not indicate which of these helical regions are affected by changes in the excipient.

The correlation coefficients for normalized second derivative FTIR spectra enable the spectra to be compared quantitatively (Table II). All the entries in the table are greater than 0.92, suggesting that the spectra are fairly similar. The highest correlation coefficients indicate the most similar spectra, and are reported for dextran 5,000 and dextran 12,000, for raffinose and dextran 5,000, and for dextran 5,000 and the excipient-free formulation ("None", Table II). It is reasonable to expect the FTIR spectra for the two polymeric dextran formulations to be similar. The lowest correlation coefficients generally involve comparisons with the mannitol formulation, which, unlike the other formulations, shows partial crystallization (Table I).

Solid State Hydrogen/Deuterium Exchange—Intact Protein

Hydrogen/deuterium exchange with ESI-MS analysis can be used to study protein conformation and interactions with excipients in the solid state (9). The kinetics of solid-state hydrogen/deuterium exchange over D_2O vapor for formulations containing different excipients are shown in Fig. 4. The type of excipient significantly affected the exchange process. For all the excipients studied, the extent of exchange increased rapidly during the first 24 h of storage. After this time, H/D exchange approached or reached a plateau, with the extent of exchange at longer time ($t > 24$ h) varying markedly among the excipients studied. The greatest exchange is observed for samples containing guanidine hydrochloride, an excipient expected to denature the protein in solution and that crystallizes on lyophilization and storage (see Fig. 1b). In samples containing guanidine hydrochloride, ~120 of the 145 theoretically exchangeable backbone amide protons are deuterated, more than 85% of the theoretical maximum. The high level of exchange for this sample is consistent with a protein that is denatured in the solid sample, and therefore having highly exposed and exchangeable backbone amide proteins. The formulation without excipients (Fig. 4, "None") reached a level of ~70 deuterium incorporated after 24 h exchange, almost half of the exchangeable sites on the backbone of the CaM (145 total). That this value is less than for the guanidine hydrochloride formulation suggests that the protein in the excipient-free formulation retains some structure, and/or that intramolecular interactions between protein molecules in the solid provide some protection from exchange. The formulation containing mannitol (Fig. 4) has a similar level of deuterium uptake as that without excipients, indicating that the presence of mannitol failed to protect the protein from exchange. This is consistent with the mannitol crystallization observed by PXRD (Fig. 1a); formation of a crystalline mannitol phase, probably phase-separated from the protein, provides little opportunity for protein-excipient interaction and little protection from exchange. Dextran 5,000 and dextran 12,000, polymeric carbohydrates, show somewhat greater protection from exchange than the excipient-free and mannitol-containing samples. The greatest protection from exchange is observed for the disaccharides sucrose and trehalose, and the trisaccharide raffinose. The reasons for the differences among the excipients that protect from exchange are not clear from data on the intact protein, but additional mechanistic information is provided by peptic digests, as described below.

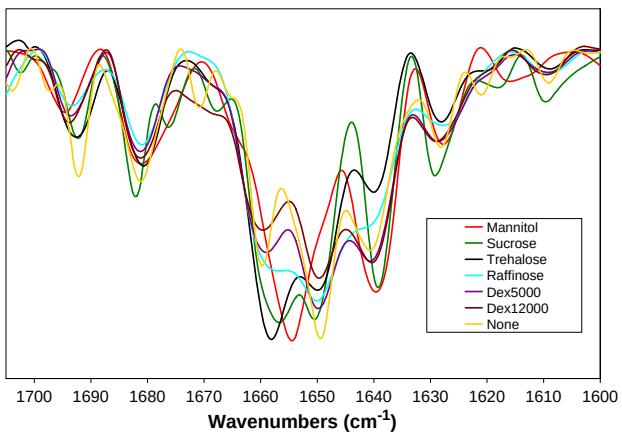

Fig. 3. Normalized second-derivative FTIR spectra for lyophilized CaM in the presence or absence of an equal mass of various excipients.

To quantitatively analyze the kinetics of H/D exchange, the data in Fig. 4 were fitted to biexponential equations to

Table II. Correlation Coefficients for Normalized Second Derivative FTIR Spectra (Fig. 3) for Lyophilized CaM Solids in the Presence or Absence (−Ca) of Various Equal Mass of Excipients

	None	Mannitol	Sucrose	Trehalose	Raffinose	Dextran 5,000	Dextran 12,000
None	1	0.926	0.945	0.946	0.971	0.981	0.975
Mannitol	N/A	1	0.961	0.938	0.955	0.954	0.950
Sucrose	N/A	N/A	1	0.966	0.955	0.952	0.943
Trehalose	N/A	N/A	N/A	1	0.971	0.955	0.937
Raffinose	N/A	N/A	N/A	N/A	1	0.992	0.975
Dextran 5,000	N/A	N/A	N/A	N/A	N/A	1	0.992
Dextran 12,000	N/A	N/A	N/A	N/A	N/A	N/A	1

Correlation coefficients were calculated using the method of Kendrick et al. (3)

obtain the kinetic parameters (Table III). The regression effectively divides the 145 exchangeable sites on the CaM backbone into two groups: a rapidly exchanging group ("fast") and a more slowly exchanging group ("slow"). As expected, excipients providing greater protection from exchange showed smaller values for the number of rapidly exchanging sites (N_{fast}) and correspondingly greater values for the number of slowly exchanging sites (N_{slow}) (Table II). These two parameters co-vary since their sum is constrained to equal 145, the total number of exchangeable sites. Excipients showing a greater degree of protection also tended to show smaller values of both rate constants (k_{fast}, k_{slow}), though there were exceptions to this trend (see e.g. mannitol, Table III). It should be noted that kinetic processes such as D_2O vapor sorption and diffusion into the solid may influence the observed kinetic profiles. As a result, the "fast" and "slow" processes (and the parameters associated with them) may describe not only the H/D exchange reaction, but also these mass transport processes. The water sorption kinetics (Fig. 2) indicate that, with the exception of trehalose-containing and excipient-free formulations, water sorption does not reach its equilibrium value until after 24 h. This corresponds closely to the "fast" phase of the H/D exchange kinetics (Fig. 4 and Table III), suggesting that this early phase of H/D may at least partly reflect the kinetics of D_2O vapor sorption.

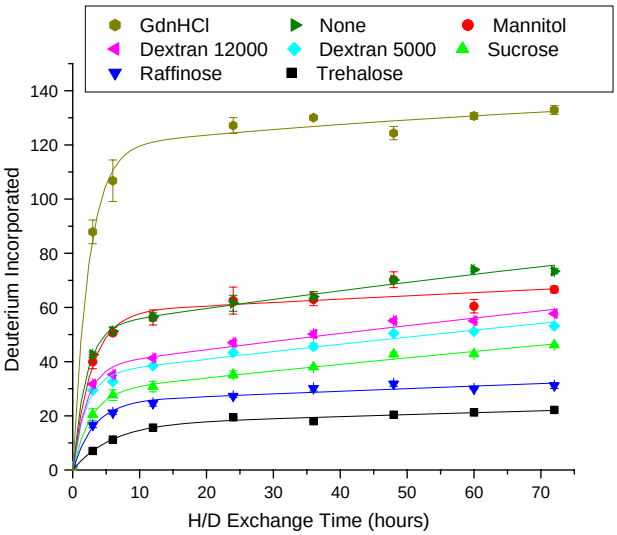

Fig. 4. Kinetics of hydrogen/deuterium (H/D) exchange for solid samples of CaM stored over D_2O vapor at relative humidities (RH) of 33%, lyophilized in the presence or absence of an equal mass of excipients, $n = 3 \pm SD$.

Solid State Hydrogen/Deuterium Exchange—Peptic Digests

The various excipients show marked differences in their ability to protect CaM from exchange in the solid state. Hypothetically, these differences may be exerted uniformly along the protein backbone, or may influence particular regions of the protein in a more site-specific manner. Analysis of peptic digests of CaM following solid-state H/D exchange was used to discriminate between these two possibilities. Twenty-six fragments were generated following pepsin digestion. Thirteen of these were selected to provide near-complete coverage of the sequence (Fig. 5). As previously reported (9), Fragments 2, 5, 7, 9 and 11 are from the α-helical regions, Fragments 3, 6, 8 and 12 are from the calcium binding loops and Fragments 4 and 10 are from non-calcium binding loops. In the discussion that follows, the helical N- and C-terminal fragments (Fragments 1 and 13) are grouped together with the non-calcium binding loops.

The deuteration level of the various fragments (Fig. 5) following exposure to D_2O vapor at 33% RH for 48 h is shown in Fig. 6. For the intact protein, low molecular weight di- and trisaccharides showed the greatest ability to protect the protein from deuteration (Fig. 4). Figure 6a shows that the protection from exchange by these low molecular weight sugars is not uniform, but rather is greatest in the α-helical sections of the protein (Fragments 2, 5, 7, 9 and 11). Trehalose and raffinose also show some protective effects in other fragments (Fig. 6a, Fragments 3, 8, 12, 1, 10, 13). In contrast, sucrose is less protective or not protective in these non-helical fragments. Recall that data for the intact protein show that sucrose-containing formulations show less protection from exchange than those containing raffinose or

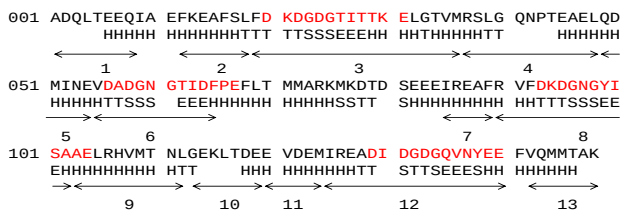

Fig. 5. Sequence and predicted secondary structure of CaM obtained from Protein Data Bank (PDB) entry 1CLL. The selected peptic fragments are underlined with arrows and calcium binding loops for CaM are highlighted in red.

Table III. Kinetic Parameters for Intact CaM Solid State Hydrogen/Deuterium Exchange

Excipient	Fast Exchange		Slow Exchange	
	N_{fast}	k_{fast} (h^{-1})	N_{slow}	k_{slow} (h^{-1})
None	51 ± 1	0.54 ± 0.06	94 ± 1	0.0094 ± 0.0008
Mannitol	58 ± 4	0.36 ± 0.07	88 ± 4	0.0034 ± 0.0020
Sucrose	28 ± 1	0.39 ± 0.04	117 ± 1	0.0040 ± 0.0003
Trehalose	17 ± 1	0.17 ± 0.02	128 ± 1	0.0009 ± 0.0003
Raffinose	25 ± 1	0.32 ± 0.05	120 ± 1	0.0014 ± 0.0004
Dextran 5,000	34 ± 2	0.53 ± 0.1	110 ± 2	0.0051 ± 0.0006
Dextran 12,000	37 ± 1	0.53 ± 0.10	108 ± 2	0.0060 ± 0.0007
Gdn·HCl	119 ± 6	0.42 ± 0.05	26 ± 6	0.010 ± 0.005

Kinetic data (Fig. 4) were fitted to the biexponential equation $D = N_{fast}(1 - e^{-k_{fast}}) + N_{slow}(1 - e^{-k_{slow}})$ using nonlinear regression, with $N_{fast}+N_{slow} = 145$

trehalose (Fig. 4). Data for the peptic fragments suggest that this difference is related to the restriction of the protective effects of sucrose to α-helices, while trehalose and raffinose also show protective effects in other regions of the protein.

The formulations containing the polymeric dextrans showed modest protection from exchange for the intact protein (Fig. 4). Figure 6b shows that these effects are also exerted primarily on the α-helical fragments, with little to no effect in other regions of the protein. Some segments (e.g., Fig. 6b, Fragments 1 and 10) show greater exchange in the presence of these excipients than the excipient-free control, though the increase is not large. Because the dextrans are polymeric glucose and have functional groups similar to those in the low molecular weight di- and trisaccharides, the difference in the extent of exchange for the small and polymeric carbohydrates is probably due to the size of the molecule and/or the loss of alcohol functional groups on polymerization.

For the formulation containing mannitol, most fragments have slightly greater uptake of deuterium than the excipient-free control (Fig. 6a). This is consistent with the intact protein results (Fig. 4), and also indicates that the deprotecting effects of mannitol occur in nearly every fragment. Exceptions are Fragments 2 and 5, which are α-helical. The failure of the mannitol to protect the protein from exchange can be attributed to mannitol recrystallization and phase separation. Finally, for the formulation containing the denaturing agent guanidine hydrochloride, all of the fragments have very similar and very high (80%) deuterium uptake (Fig. 6c). This suggests that in the presence of the denaturing agent, the protein maintained the denatured conformation in the solid state following lyophilization. The facile exchange for all the peptic fragments suggests that the α-helices are denatured and that exchange in these sequences occurred as readily as in the loop regions of the structured protein. Since guanidine hydrochloride has a strong absorption band at 1,640 cm^{-1} which overlaps with the amide I bands, the protein secondary structure in solid samples containing guanidine hydrochloride could not be assessed by FTIR. The solid state H/D exchange experiment provides evidence that the protein remains denatured in the solid state when colyophilized with this denaturing agent.

DISCUSSION

An interpretation of solid-state H/D exchange results requires an understanding of the kinetic processes that may occur following exposure of the solid sample to D_2O vapor. Although the detailed mechanism of solid-state exchange has not been established, we postulate the following sequence of steps. D_2O vapor is first adsorbed onto the lyophilized solids, a process that may involve a D_2O phase transition from the vapor to the liquid phase. The absorbed D_2O then diffuses through the solid particles and comes into intimate contact with individual protein molecules in the solid. As in solution, the H/D exchange reaction then occurs at exchangeable protons on the protein backbone and side chains. Because side chain sites readily undergo back exchange when the solid is dissolved, it is likely that only the backbone amide protons

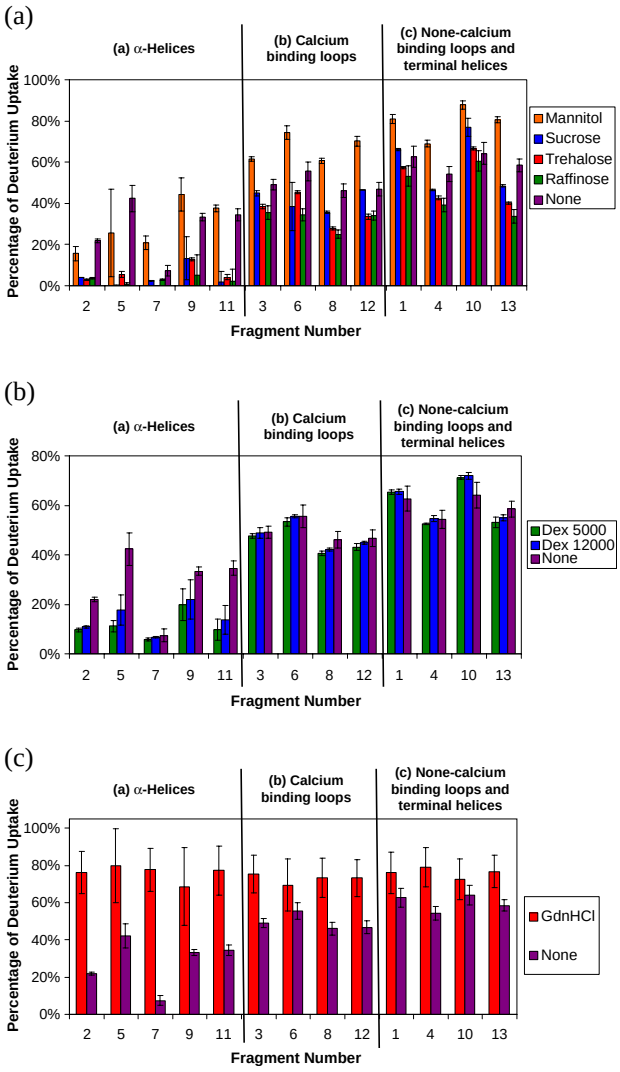

Fig. 6. Effect of excipients on hydrogen/deuterium (H/D) exchange in the peptic fragments of CaM. Solid samples were stored over D_2O vapor at relative humidities (RH) of 33%, and were lyophilized in the presence or absence of an equal mass of excipients, $n = 3 \pm SD$. Excipient groupings: (**a**) low molecular weight sugars, (**b**) dextrans, (**c**) guanidine hydrochloride (GdnHCl).

remain deuterated for ESI/MS analysis, as in solution H/D exchange studies.

The observed effects of the various excipients on solid-state H/D exchange can be explained in view of the proposed sequence of steps. For example, the excipients may influence the global structure of CaM in the solid state and hence change the H/D exchange rates by changing the exposure of the protein molecule. Guanidine hydrochloride may act in this manner by denaturing the protein in solution and allowing retention of the unfolded form on lyophilization.

The excipients may also serve as water scavengers, sequestering absorbed D_2O away from the protein in the solid phase, reducing exposure of the protein to D_2O. This explanation is countered to some extent by the total water content data (Table I), which do not correlate well with levels of exchange at longer time ($t > 24$ h). For example, the mannitol formulation has the lowest water content (~2.7%) but the protein H/D exchange level is greater than any other formulation except the one containing guanidine hydrochloride. However, it is likely that the global measure of moisture content does not indicate the local moisture levels in contact with the protein, which cannot be measured by current techniques. It is the local water content in close contact with the protein that determines the H/D exchange level.

The excipients may also interact directly with the protein molecules through hydrogen bonds. These hydrogen bonding interactions may involve the polar side chains or the protein backbone. Hydrogen bonds involving the protein side chains such as carboxylate have been implicated in previous work (19). The involvement of the side chains cannot be assessed by H/D exchange, however, due to rapid back exchange of these sites when the sample is dissolved for analysis. Any hydrogen bonding between the protein backbone and carbohydrate excipients probably involves the imino hydrogens of the peptide bond as a proton donor and the oxygen on the carbohydrate ring or the carbohydrate hydroxyl oxygens as H-bond acceptors. When the protein's imino hydrogens are located in unstructured or loop regions, their ability to participate in hydrogen bonds with neighboring excipient molecules is unhindered by intramolecular hydrogen bonds within the protein. Protein-excipient hydrogen bonds in these relatively unstructured regions are expected to provide protection from H/D exchange, since the backbone hydrogens are no longer free for exchange. This explains the ability of sucrose, trehalose and raffinose to provide partial protection from exchange in the calcium binding loops and unstructured regions [Fig. 6a, (b) and (c)]. When the protein's imino hydrogens are located in intact α-helices, however, any hydrogen bonding to the excipient must compete with the *i→i+4* intramolecular hydrogen bonds that form the helix. In this case, the only possible hydrogen bond configuration that allows for both intra- and intermolecular interactions is a bifurcated, three-center hydrogen bond. In this configuration, the backbone imino hydrogen serves as the H-donor and both the *i+4* carbonyl and the excipient carbohydrate oxygen or hydroxyl are acceptors. Bifurcated, three-center hydrogen bonds have been observed in carbohydrates and proteins crystals (20,21) and are generally weaker than typical two-center hydrogen bonds (22). This suggests that the formation of intermolecular hydrogen bonds between excipients and α-helical regions of the protein backbone will weaken the original intramolecular hydrogen bonds in the α-helix, enhancing their ability to undergo H/D exchange. This type of protein-excipient hydrogen bonding thus cannot explain the protection from exchange exerted by the carbohydrate-based excipients in the α-helical regions of CaM [Fig. 6a, (a) and b, (a)].

Finally, the protective effects of the excipients may be due to an effect on protein dynamics in the amorphous solid. In solution, the "breathing" of protein secondary structural elements is related to their ability to undergo H/D exchange. In amorphous solids, protein dynamics may be strongly influenced by the rigidity of the surrounding solid matrix and by hydrogen bonding interactions between the matrix and the protein. The effects of matrix rigidity and hydrogen bonding to excipients on protein stability in the amorphous solid state have been presented as the "vitrification" and "water replacement" hypotheses, respectively (23). There is some indication of an effect of matrix rigidity in the H/D exchange data reported here. For example, in Fig. 6a, protection from exchange within a particular secondary structural grouping scales with the Tg of the sugar. In the calcium binding loops, protection from exchange generally increases in the order sucrose < trehalose < raffinose, a ranking that corresponds to their increasing Tg values (Table I). A similar trend is observed in the non-calcium binding loops and terminal helices (Fig. 6c). Matrix rigidity cannot account for the differences observed among the various secondary structural elements of the protein, however (e.g., Fig. 6a vs. b, c), nor can it account for the performance of the phase-separated (e.g., mannitol) or denaturing excipients (e.g., guanidine hydrochloride). To a degree, the distinction between hydrogen-bonding (i.e., "water replacement") and matrix rigidity (i.e., "vitrification") effects (23) is a false dichotomy, since both ultimately involve hydrogen bonding networks in the amorphous solid. Amorphous solids are thought to contain networks of intra- and intermolecular hydrogen bonds (20). In an amorphous solid containing both protein and excipient, the latter in molar excess, intermolecular hydrogen bonds may "anchor" the protein in the excipient's hydrogen-bond network.

Interestingly, FTIR and H/D exchange give somewhat different results with regard to the ability of the excipients to preserve CaM structure. The FTIR studies suggest that the excipients should be ranked in the following order with regard to their ability to preserve secondary structure: raffinose ~ dextran ~ trehalose > mannitol ~ sucrose. In contrast, the solid state H/D exchange results suggest that they should be ranked in the following order with regard to the ability to protect from exchange: raffinose ~ trehalose > sucrose > dextran > mannitol. There are several possible reasons for this inconsistency. First, FTIR absorption in the Amide I region is related to the stretching of the C=O group on the protein backbone. H/D exchange studies measure the ability of the backbone imino hydrogen to undergo exchange in the presence of D_2O, a simple chemical reaction. Thus, the two techniques observe different parts of the peptide bond and are concerned with different properties. As applied here, the FTIR studies were performed on samples immediately following lyophilization, while H/D exchange studies were performed during three days of storage at 33% RH, so that differences in sample moisture content may also contribute to

the discrepancy between the two measurements. Performing H/D exchange studies at a range of RH values, from nearly dry solids through deliquescence to true solutions, is expected to provide additional information on the protein/excipient interactions in amorphous solids. Such studies are planned.

CONCLUSIONS

The results show that carbohydrate excipients offer varying degrees of protection from H/D exchange in the solid state, as measured by ESI-MS with and without peptic digestion. The small di- and trisaccharides showed the greatest protection from exchange, an effect that was primarily exerted in the α-helical sections of the protein. Highly protective excipients such as raffinose and trehalose also showed some protection in non-helical fragments. The effect of the small molecule excipients can be attributed to their ability to maintain the secondary structure of the protein during lyophilization, and/or to the formation of H-bonding network in the solid that protects the protein from exchange. The polymeric dextrans showed less protective effect, presumably due to the increased molecular size. Mannitol showed recrystallization and little protection from exchange, which can be attributed to limited interactions to the protein. Solid-state H/D exchange with ESI-MS may provide a rapid method for screening excipients for solid protein formulation. Studies relating solid-state H/D exchange to protein stability in solid formulations are in progress.

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