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Localized Hydration in Lyophilized Myoglobin by Hydrogen-Deuterium Exchange Mass Spectrometry. 2. Exchange Kinetics

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Abstract

Solid-state hydrogen deuterium exchange with mass spectrometric analysis (ssHDX) is a promising method for characterizing proteins in amorphous solids. Though analysis of HDX kinetics is informative and well-established in solution, application of these methods to solid samples is complicated by possible heterogeneities in the solid. The studies reported here provide a detailed analysis of the kinetics of hydration and ssHDX for equine myoglobin (Mb) in solid matrices containing sucrose or mannitol. Water sorption was rapid relative to ssHDX, indicating that ssHDX kinetics was not limited by bulk water transport. Deuterium uptake in solids was well-characterized by a bi-exponential model; values for regression parameters provided insight into differences between the two solid matrices. Analysis of the widths of peptide mass envelopes revealed that in solution, an apparent EX2 mechanism prevails, consistent with native conformation of the protein. In contrast, in mannitol-containing samples, a smaller non-native subpopulation exchanges by an EX1-like mechanism. Together, the results indicate that the analysis of ssHDX kinetic data and the widths of peptide mass envelopes are useful in screening solid formulations of protein drugs for the presence of non-native species that cannot be detected by amide I FTIR.

Keywords

myoglobin; hydrogen-deuterium exchange; mass spectrometry; kinetics; amorphous; solid; sucrose; mannitol; protein

INTRODUCTION

Preserving the stability of protein drugs is of critical importance, as degradation reactions account for significant losses during manufacturing and storage of biopharmaceuticals. Many proteins that are susceptible to chemical and physical degradation processes, or are targeted for markets lacking a cold-chain, cannot be developed as liquid formulations and must be lyophilized. Degradation reactions can be slowed significantly in lyophilized solids. However, the freeze-drying process itself exposes proteins to several stresses (e.g. freezing, exposure to liquid-ice interfaces, high protein concentration, dehydration) that may compromise stability even in the solid state.

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Glass forming disaccharides such as sucrose and trehalose are well-known to stabilize proteins upon lyophilization and storage in the dry state. Other additives such as mannitol may also be added to provide a robust matrix, adjust tonicity and aid rehydration¹⁻³. Several mechanisms for the stabilizing effects of disaccharides on dried proteins have been proposed, including: (i) the replacement of hydrogen bonds lost upon dehydration (“water-replacement” hypothesis)^{4, 5}, (ii) preferential exclusion of the sugar, stabilizing the native state in the freeze-concentrated solution and trapping water at the protein surface (“preferential exclusion” or “water-entrapment” or hypothesis)⁶⁻⁸, and (iii) the formation of multiple hydrogen bonds between the protein and the rigid external matrix, which governs or “slaves” protein motions (“vitrification/solvent slaving” hypothesis)⁸⁻¹⁰.

Significant efforts are being made to relate formulation variables to protein stability at a mechanistic level. For example, several recent studies have identified key metrics that correlate well with stability, such as fast local dynamics by neutron scattering and free-volume from pycnometric density^{11, 12}. These methods generally provide a global measure of the coupling of the protein to the rigid external matrix (iii). Other analytical methods that characterize protein structure in the solid, such as FTIR, provide global measures of secondary structure. Our group has shown that solid-state hydrogen-deuterium exchange with mass spectrometric analysis (ssHDX) provides sufficient resolution to localize the effects of matrix environments to particular domains of the protein¹³⁻¹⁷. This method can support investigations of solid-state protein stability by providing higher-resolution information than other currently available methods.

In recent work, also presented in this issue of *Molecular Pharmaceutics*, our group has mapped the local effects of hydration on myoglobin (Mb) in solid matrices containing mannitol or sucrose (1:1 w/w, protein:additive) using ssHDX, and compared the results to solution controls¹⁷. A lyophilized powder containing the protein was exposed to D₂O in the vapor phase at controlled relative humidity. Deuterium uptake was measured using electrospray ionization mass spectrometry (ESI-MS) for both the intact protein and peptic fragments. The extent of exchange varied with matrix type, extent of hydration (i.e., water activity, a_w) and position in the protein sequence. HDX was less in sucrose matrices than in mannitol matrices at all a_w , while differences in HDX behavior in solutions containing the two additives were negligible. Protein structure in solid samples containing sucrose or mannitol was indistinguishable by Fourier-transform infrared spectroscopy (FTIR). In solids at low a_w , exchange occurred primarily in non-helical regions. With increasing a_w , a threshold was reached at which exchange increased markedly.

These results provided new insights into protein hydration in lyophilized solids, with unprecedented peptide-level structural resolution. However, while the kinetics of ssHDX were monitored in these studies, and in previous ssHDX studies in our labs, the data were not analyzed in detail. In solution, the kinetics of hydrogen/deuterium exchange are described by the Linderstrom-Lang scheme:

which relates the rates of conformational fluctuations in the protein’s structure (i.e., “opening” and “closing” events; k_{op} , k_{cl} , Eqn. 1) to the intrinsic chemical rate of exchange of an unstructured peptide (k_{int})¹⁸. Two limiting behaviors of this kinetic model for HDX have been identified, termed EX2 and EX1, and can be distinguished at intermediate levels of exchange. In the EX2 regime, the closing rate is rapid relative to the intrinsic rate of deuterium exchange (i.e., $k_{cl} \gg k_{int}$). EX2 kinetics are commonly observed for stable, natively folded proteins in solution HDX studies. In the EX1 regime, which is less common,

the intrinsic rate of deuterium exchange is greater than the rate of refolding ($k_{cl} \ll k_{int}$). EX1 kinetics is thought to occur when a portion of the protein molecule is unfolded in solution, undergoing simultaneous exchange at a rate much faster than its refolding rate¹⁹. Though the interpretation of solution HDX kinetic data using the Linderstrom-Lang model is well-accepted, it is unclear whether similar interpretations can be applied to ssHDX studies without modification or re-interpretation. For example, spatial heterogeneities in protein concentration and conformation and dynamics may occur in solid samples, and ssHDX studies may be influenced by rate processes that do not occur in solution HDX (e.g., D₂O vapor sorption and diffusion). These processes may contribute to the observed rate of exchange, complicating data analysis and interpretation.

In the studies reported here, we provide a detailed analysis of the kinetics of hydration and ssHDX for equine myoglobin (Mb) in solid matrices containing sucrose or mannitol at $a_w = 0.43$ and 5°C. The results demonstrate that water sorption is rapid relative to HDX, indicating that HDX measurements in the solid are not limited by bulk water transport. We also show that deuterium uptake in solids is well-characterized by a biexponential model and suggest interpretations for regression parameters. Finally, analysis of the widths of peptide mass envelopes reveals that in solution, the data are generally consistent with exchange by an apparent EX2 mechanism. In contrast, in mannitol-containing solids, Mb exchanges by a mixed EX2/EX1-like mechanism, suggestive of non-native species. We propose that the analysis of ssHDX kinetic data and of the widths of peptide mass envelopes are useful in screening solid formulations of protein drugs for the presence of non-native species that cannot be detected by amide I FTIR.

MATERIALS AND METHODS

Materials and sample preparation methods have been described previously¹⁷ and were used without modification for the analyses reported here.

Dynamic Vapor Sorption (DVS) Analysis

To measure the rate of water uptake and water content in the solid samples under the conditions of the ssHDX studies, water sorption was measured using a gravimetric analyzer (Q5000SA; TA Instruments, New Castle, DE). Approximately 3–4 mg of powder was loaded onto the sample pan, and the loosely bound water removed at 85°C, 0% RH until the signal was constant (<0.001% change in sample weight over 10 min). The samples were then equilibrated at 5°C, 0% RH prior to exposure to 43% RH.

Solid-State Hydrogen-Deuterium Exchange (ssHDX)

The ssHDX methods have been described previously¹⁷. For the data reported here, Mb powders were exposed to D₂O vapor in equilibrium with a saturated salt solution of K₂CO₃ in D₂O at 5°C. At equilibrium, the activity of water (a_w) in the solid samples is 0.43 (i.e., RH = 43%) according to published tables of RH over aqueous saturated salt solutions²⁰. This intermediate RH condition was selected because our previous studies showed the greatest differences in Mb ssHDX in sucrose and mannitol containing solids under these conditions¹⁷. Solution controls were labeled in D₂O buffer (2.5 μM Mb, 5 mM phosphate, pD 7.0). At eight time points over a 110 h period, solid samples were withdrawn, flash frozen and stored at –80°C prior to LC/MS analysis. Solution samples were treated similarly, except that six time points were used and samples were quenched with HCl to pH 2.5 prior to flash freezing. Analysis of Mb deuterium uptake was measured using ESI-MS as described previously¹⁷.

Data Analysis

Mass spectra were processed using HX-Express software²¹ to determine the width of the peptide mass envelope at 20% of the peak height and the average mass. In cases where mixed EX2/EX1 behavior was observed, the reported deuterium uptake is the average of the unresolved isotope distributions. The peptide average mass was corrected for deuterium loss during analysis by calibrating with a deuterated Mb standard. Normalized peak width increases were calculated by subtracting the average peak width of the deuterated solution control, which exchanges in the EX2 regime, from the peak width of the solid samples. Negative values of normalized peak widths occur at low levels of deuterium incorporation.

For the intact (i.e., undigested) protein and peptides produced by pepsin digestion, kinetic data for deuterium uptake was fitted to a biexponential model (Eqn 2):

$$D(t) = N_{fast} (1 - e^{-k_{fast} t}) + N_{slow} (1 - e^{-k_{slow} t}) \quad (2)$$

where $D(t)$ is the number of deuterium taken up at time t , N_{fast} is the number of amide groups undergoing 'fast' exchange, N_{slow} is the number of amide groups undergoing 'slow' exchange, and k_{fast} and k_{slow} are the apparent first-order rate constants for the amides in the 'fast' and 'slow' groups, respectively. Kinetic data for the intact protein and for each of the 38 peptic fragments were treated separately and subjected to independent regression analysis. Data were fitted to Eqn. (2) using the nonlinear regression feature in Kaleidagraph® (Synergy Software, Reading, PA) to obtain each of the four regression parameters (N_{fast} , N_{slow} , k_{fast} , k_{slow}) with their associated standard errors.

RESULTS AND DISCUSSION

Water Vapor Sorption Kinetics

In ssHDX studies, D₂O from the vapor phase must be absorbed onto the powder and diffuse through the solid matrix before exchange can occur. The rates of sorption and diffusion thus may influence the observed rate of exchange. Confounding rate processes may also occur for solution HDX studies, in which mixing (e.g., of D₂O and H₂O solutions) may limit the acquisition of reliable data at early time points. The rates of sorption/diffusion in solids are expected to be considerably slower than solution mixing times, however, and their relationship to ssHDX rates has not been well-characterized.

The rate of water uptake was measured to evaluate the timescale of sorption/diffusion relative to deuterium exchange in sucrose and mannitol matrices. In both matrices, water vapor sorption was essentially complete in ~1 h at $a_w = 0.43$ (Fig. 1). At equilibrium under these conditions, sucrose matrices sorb ~25% more water than those containing mannitol, though the mannitol system appears to reach equilibrium somewhat more rapidly. The time required to reach 50% of the water vapor sorption/diffusion plateau (t_{50}) was ~0.06 h in mannitol and ~0.09 h in sucrose. Samples were also pre-incubated in an H₂O vapor environment ($a_w = 0.43$, 0.5 h) prior to incubation in D₂O vapor ($a_w = 0.43$, an additional 0.5 h) to evaluate the effect of initial water content on D₂O vapor sorption. No significant difference ($p > 0.1$) in the number of deuterium taken up in pre-hydrated and non-prehydrated samples was observed (Table S1). At other a_w values, rates of water vapor sorption were similar to those reported for $a_w = 0.43$ though the plateau water content varied. This suggests that, beyond this time, ssHDX is insensitive to transient changes in the bulk level of hydration in this system.

Deuterium Exchange Kinetics of Intact Mb

Deuterium uptake for intact Mb in lyophilized samples containing mannitol or sucrose showed initial rapid uptake followed by a slower approach to a plateau, as reported in the accompanying manuscript¹⁷. Deuterium uptake increased with increasing a_w and was greater in mannitol solids than in sucrose solids, while exchange in solution was similar for the two excipients. Nonlinear regression parameters (Table 1) show that the number of amides in the rapidly exchanging pool (N_f) and the total number of exchangeable amides ($N_f + N_s$) increase with a_w in both solids, while the number of amides in the slowly exchanging pool (N_s) shows less variation with a_w . N_f and N_s are generally greater in mannitol samples than in sucrose samples at all a_w . Rate constants for the ‘fast’ phase of exchange (k_f) range from 0.4 to 1.7 h⁻¹, are generally greater in mannitol-containing solids than in sucrose-containing solids, and do not show a marked dependence on a_w . Rate constants for the ‘slow’ phase of exchange (k_s) vary from 0.01 to 0.03 h⁻¹, tend to increase with a_w , and are somewhat greater in mannitol samples.

A comparison of half-lives for the ‘fast’ and ‘slow’ phases of ssHDX (t_f , t_s) with water vapor sorption/diffusion times (t_{50}) at $a_w = 0.43$ provides an estimate of the contribution of water vapor sorption and diffusion to HDX kinetics. At $a_w = 0.43$, t_f is 0.41 h in mannitol samples and the t_{50} for sorption/diffusion is ~0.06 h, indicating that the ‘fast’ phase of exchange is ~6.8-fold slower than sorption/diffusion. In sucrose $a_w = 0.43$, t_f is 0.46 h while t_{50} is 0.09 h, a difference of ~5.1-fold. Values for t_s are > 30 h for both solids at $a_w = 0.43$, greater than t_{50} by more than 300-fold. Values of t_{50} , t_f and t_s are of the same order of magnitude at other a_w values. Therefore, while sorption/diffusion processes may influence HDX kinetics during the initial ‘fast’ phase, their contribution is minimal beyond 1 h. Since the time course of a typical ssHDX study is usually more than 100 h, the assumption that sorption/diffusion processes can be neglected is well-justified. In the sections below, we analyze ssHDX kinetic data with the assumption that sorption/diffusion can be neglected and the expectation that opening (k_{op} , k_{cl}) and exchange (k_{int}) events are the dominant rate processes (Eqn. 1). However, other relatively slow kinetic processes (e.g., matrix relaxation) may influence ssHDX kinetics and cannot be ruled out.

Peptide-level Deuterium Exchange Kinetics

As observed for intact Mb, exchange for the individual peptic fragments follows biexponential kinetics, as evidenced by plots of deuterium uptake vs. time for 38 peptic fragments in the two matrices. Figure 2 shows exchange kinetics for four peptides that represent the range of behavior observed; kinetic plots for all 38 peptides are given in Figure S2. For most peptic fragments, the data showed biexponential behavior consistent with subpopulations of amide groups undergoing ‘fast’ and ‘slow’ exchange. Exceptions to this trend were peptides Glu8-Val13 and Glu8-Trp14 in both sucrose and mannitol, and peptide Asp60-Leu69 in sucrose (Table S2). In these samples, the number of amide groups undergoing exchange is relatively small, due in part to the short fragment length, so that biexponential behavior was not observed. Kinetic data for the remaining 71 peptides are well-described by the biexponential model.

It should be noted that regression parameters for the peptic fragments cannot be simply related to those for the intact protein. The numbers of individual amides represented by the composite amplitudes (N_{fast} , N_{slow}) and rate constants (k_{fast} , k_{slow}) are significantly different in fragments and in the intact protein. While the ssHDX *rate* for the protein is a sum of the rates for individual amides, the fitted *rate constants* are not a simple average of the rate constants for individual amides. Thus, the observed ssHDX rate constant for the intact protein is not a linear combination of the rate constants for the peptic fragments. Analysis of

regression parameters at the peptide level reduces the homogenizing effect of averaging large numbers of exchangeable amides.

Because pepsin cleavage does not occur at fixed sites on the backbone, there is considerable overlap of the peptides in the complete data set. In the discussion that follows, a non-redundant subset of 48 peptides (i.e., 24 peptic fragments $\times$ 2 matrices) is presented for the sake of brevity and to avoid biasing averaged data (Fig. S1). Solution HDX typically occurs on a time scale of milliseconds-to-hours, and so is not captured fully by the methods and sampling schedule employed here. Thus, solution HDX data were not subjected to kinetic analysis. Regression parameters for the non-redundant peptides are presented in Figure 3; average values of the four regression parameters (N_{fast} , N_{slow} , k_{fast} , k_{slow}) provide a quantitative summary of the trends (Table 2). The number of amides undergoing exchange ($N_{fast} + N_{slow}$, Eqn. 2; N_e , Table S2) in solid samples is less than the theoretical maximum (N , Table S2) for all fragments. The remaining amides do not exchange during the timecourse of this study (168 h), presumably because dynamic constraints in solids limit opening events or because there is insufficient local water.

On average, the number of 'fast' exchanging amide groups (N_{fast}) is 34% greater in mannitol-containing solids than in sucrose-containing solids, while the number of 'slow' exchanging amide groups (N_{slow}) is 109% greater (Table 2). This suggests that greater exchange in mannitol occurs primarily through the recruitment of amide groups that do not exchange in sucrose matrices into the slowly exchanging pool. The relatively small increase of N_{fast} may reflect a barrier between the 'slow' and 'fast' groups, limiting the ability of 'slow' or non-exchanging amides in sucrose to become 'fast' exchangers in mannitol. At the molecular scale, it is interesting to note that the 'fast' group may include many of the 29 amides that are not engaged in intermolecular hydrogen bonds in the native structure. In fact, for most peptic fragments, there is a good correlation between N_{fast} and the number of non-intramolecularly hydrogen-bonded amides (Fig. S3).

For the peptic fragments, the average rate constants (k_{fast} , k_{slow} , Table 2) suggest that the greater overall exchange rate in mannitol matrices is largely due to a doubling of the rate for the 'fast' pool relative to that in sucrose (k_{fast} , Table 2). Rates for the 'slow' pool are similar in the two systems (k_{slow} , Table 2). The similarity of k_{slow} values in the two systems is consistent with a similar average level of constraint of protein dynamic motion for the 'slow' exchanging amides, which may be imposed by comparable higher order structure and dynamics and/or similar local water content for these amides. Similarly, the substantial increase in k_{fast} for mannitol may reflect greater mobility of a relatively constant number of 'fast' exchanging amides and/or an increase in local water content. However, in aqueous solutions containing protein and sucrose, the concentration of water is enriched in the immediate vicinity of the protein as a result of the preferential exclusion of disaccharides and polyols²²⁻²⁴. There is some evidence that this pattern is retained in solids^{7, 8}. Furthermore, sucrose samples sorb 25% more water than mannitol (Fig. 1). Thus, in the absence of significant phase separation, it is unlikely that the local water content is greater in mannitol than in sucrose.

Peak Width Analysis

Peak broadening is an indication of mixed EX2/EX1 behavior^{19, 25, 26}. EX2 kinetic behavior is typically observed in solution for proteins in their native conformation, for which peak broadening is small¹⁹. In contrast, EX1 kinetics often indicates partial unfolding, and show either a bimodal distribution or broadening of the peptide mass envelopes. A bimodal distribution is observed when the native and partially unfolded populations have distinct mass envelopes that are well-resolved during mass spectrometry, while broadening may occur when the envelopes are not well-resolved because the number of amides involved in

EX1 behavior is relatively small. The mass features indicative of EX1 kinetics are only observed when exchange is incomplete, since the population is homogenous if the protein is either fully protonated or fully deuterated. In solids, EX2/EX1-like peak broadening may be caused by: (i) a spatially homogenous, non-native Mb conformation throughout the solid, (ii) a spatially heterogenous matrix containing both non-native and native Mb subpopulations, and/or (iii) spatial gradients in local concentration of water. These possibilities cannot be distinguished on the basis of the ssHDX data.

In both solutions and in solids, peak widths at low levels of deuterium uptake are typically narrow relative to deuterated peak widths¹⁹. Some peak width broadening occurs as a result of deuterium uptake, regardless of EX2 or EX1 kinetic behavior. This is readily observed in solids because the timescale for exchange is significantly longer than in solution, where broadening due to deuteration often occurs at faster timescales than the HDX measurement. For example, the peak width broadening observed for the peptide Phe138-Gly153 from Mb in sucrose solids between 1–4 h occurs at low levels of deuterium incorporation (Fig. 4A, Fig. S5a). We thus present normalized peak width against deuterium uptake (Fig. 4B, Fig. S5) to quantify EX2/EX1-like peak broadening beyond the EX2 solution control and facilitate comparison of peptides with differing levels of deuterium uptake. Fig. 2 demonstrates that the condition of incomplete exchange is met, so that peak width analysis is valid.

Examination of the peak widths of the apparently unimodal peptide mass envelopes revealed broadening at intermediate levels of deuterium uptake, particularly in mannitol samples (Fig. 4, see also Figs. S4, S5). Specifically, mannitol samples showed normalized peak width increases of 1 to 3 Da, while peak widths of solution controls varied by less than 0.5 Da over the exchange period. As a result of low levels of deuterium exchange in sucrose, the widths of the peptide mass envelopes in the sucrose samples were typically less than those of the solution control. Thus, the extent of peak width broadening at intermediate levels of deuterium incorporation could not be fully characterized for most peptides from sucrose matrices. However, available data show peak broadening in sucrose matrices of 1.0 Da or less.

In mannitol matrices, the total number of Mb amides participating in EX1-like behavior is estimated to be ~16 from the sum of the peak maxima of the normalized peak width increases fit to a Gaussian model (Fig. S5a). The extent of peak broadening in sucrose is not clear because of the low level of HDX. However, available data indicate that peak broadening is consistently lower in sucrose than in mannitol at similar levels of deuterium uptake (Figs. 4, S5). This suggests that in sucrose, native structure and dynamics are retained to a greater extent and/or that local water content is less heterogeneous. Previously, we have shown that there is no difference in Mb secondary structure in mannitol and sucrose samples as measured by FTIR¹⁷. Thus, peak width analysis offers the potential to screen multiple formulations for the presence of non-native species that may go undetected in amide I FTIR spectra. HDX results suggesting multiple conformations of apomyoglobin in lyophilized solids have been reported previously²⁷. In-exchange in those studies occurred in solution, however, and multiple conformations were not observed for holomyoglobin, the protein studied here.

The studies presented in this two-manuscript series support the use of ssHDX to characterize proteins in amorphous solids. Using ssHDX with peptic digestion, we have mapped the effects of excipients on Mb hydration with peptide-level resolution. This level of structural resolution cannot be achieved for solid samples with vibrational spectroscopy methods such as FTIR, which report secondary structure at the intact protein level. As shown here, the analysis of ssHDX exchange kinetics and mass envelope peak broadening may provide

additional information on perturbations of protein structure that are not detected by FTIR. The sensitivity, structural resolution and wealth of data provided by ssHDX indicate that continued development of the method is warranted.

SUMMARY AND CONCLUSIONS

The studies reported here address the kinetics of ssHDX for Mb in mannitol and sucrose matrices. Water sorption was rapid (< 1 h) relative to ssHDX for most amides in both matrices, indicating that D₂O sorption and diffusion have little influence on ssHDX kinetics. Deuterium uptake kinetics in solids was well-characterized by a biexponential model, and values of the regression constants provided insight into differences in structure and dynamics in the two matrices. Peptide mass envelope width measurements revealed that, while Mb molecules in solution exchange by an EX2-like mechanism, a non-native subpopulation of roughly 16 amides exchanged by an EX1-like mechanism in mannitol-containing solids. Together, the results suggest that kinetic analysis of ssHDX data can be used to screen solid protein formulations for non-native subpopulations of amides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Water vapor sorption kinetics for Mb in lyophilized solids containing mannitol (1:1 w/w; dashed line) or sucrose (1:1 w/w; solid line) at 5°C and $a_w = 0.43$. Data was collected at intervals of 4 sec.

Figure 2.

Kinetics of deuterium uptake for the peptides (A) Met55-Leu69, (B) Glu8-Val13, (C) Ile30-Leu40, and (D) Glu41-Met55 the peptide from Mb in phosphate buffer (5 mM, pH 7.0, solid circles) and in lyophilized solids containing 1:1 w/w mannitol (closed diamonds) or sucrose (open diamonds) $a_w = 0.43$ and at 5°C ($n = 3 \pm \text{S.E.M.}$). Dashed lines are fits of data in solids to Eqn. 2 (see text). See Fig. S1 for data for all 38 peptic fragments.

Figure 3. Comparison of the regression parameters of the biexponential model for sucrose (white) and mannitol (black) containing samples. Regression parameters: (A) N_{fast} , (B) N_{slow} , (C) k_{fast} , and (D) k_{slow} .

4a

4b

Figure 4.

Peak width broadening of the Mb peptide Phe138-Gly153 at 20% peak height (**A**) raw data from solution control (closed squares) and from mannitol- (closed triangles) and sucrose-containing (open triangles) solids ($n=3$, $\pm$ S.E.M.). The dashed line is the average value of the peak width for the solution controls. (**B**) Normalized peak width broadening in mannitol- (circles) and sucrose-containing (crosses) solids. The solid line represents a fit of the data to a Gaussian model. Fig. S5 shows the peak width broadening curves for all 24 non-redundant peptic fragments.

Table 1

Regression parameters^{a,b} for deuterium uptake kinetics in lyophilized samples containing mannitol or sucrose, as a function of relative humidity (RH)

RH (%)	Mannitol				Sucrose			
	N_f	k_f (h ⁻¹)	N_s	k_s (h ⁻¹)	N_f	k_f (h ⁻¹)	N_s	k_s (h ⁻¹)
11	7 (1)	1.0 (0.5)	20 (6)	0.01 (0.01)	4 (1)	0.8 (0.4)	15 (8)	0.01 (0.01)
23	11 (1)	1.0 (0.1)	28 (1)	0.02 (0.01)	4 (1)	0.5 (0.2)	14 (1)	0.02 (0.01)
33	15 (2)	1.0 (0.3)	30 (2)	0.03 (0.01)	11 (3)	0.2 (0.1)	31 (119)	0.01 (0.02)
43	26 (1)	1.7 (0.3)	33 (1)	0.03 (0.01)	17 (1)	1.5 (0.2)	21 (1)	0.02 (0.01)
75	43 (2)	0.4 (0.1)	19 (2)	0.03 (0.01)	38 (4)	0.4 (0.1)	20 (4)	0.02 (0.01)

^a See Eqn. 2 for regression equation and parameter definitions.

^b Values in parentheses are standard errors of the regression parameters.

Table 2

Average kinetic parameters for Mb peptic peptides ssHDX in sucrose and mannitol matrices

	N_{fast}	N_{slow}	$k_{fast} \text{ (h}^{-1}\text{)}$	$k_{slow} \text{ (h}^{-1}\text{)}$
Sucrose ^a	2.6 ± 0.2	2.3 ± 0.2	0.65 ± 0.16	0.028 ± 0.008
Mannitol ^a	3.5 ± 0.3	4.8 ± 0.3	1.27 ± 0.29	0.033 ± 0.006
% Change ^b	+34%	+109%	+97%	+19%

^a Mean $\pm$ S.E. of parameters for 24 non-redundant fragments. See Eqn. 2 for regression equation and parameter definitions and Table S2 for individual values.

^b Calculated as $100 \times [(\text{value in mannitol}) - (\text{value in sucrose})] \div (\text{value in sucrose})$