Published in final edited form as:

Mol Pharm. 2012 April 2; 9(4): 718–726. doi:10.1021/mp3000088.

Localized Hydration in Lyophilized Myoglobin by Hydrogen-Deuterium Exchange Mass Spectrometry. 1. Exchange Mapping

Andreas M. Sophocleous^{*}, Jun Zhang^{*}, and Elizabeth M. Topp^{*,‡}

^{*}Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN

Abstract

The local effects of hydration on myoglobin (Mb) in solid matrices containing mannitol or sucrose (1:1 w/w, protein:additive) were mapped using hydrogen-deuterium exchange with mass spectrometric analysis (HDX-MS) at 5°C and compared to solution controls. Solid powders were exposed to D₂O(g) at controlled activity (a_w) followed by reconstitution and analysis of the intact protein and peptides produced by pepsin digestion. HDX varied with matrix type, a_w , and position along the protein backbone. HDX was less in sucrose matrices than in mannitol matrices at all a_w while the difference in solution was negligible. Differences in HDX in the two matrices were detectable despite similarities in their bulk water content. The extent of exchange in solids is proposed as a measure of the hydration of exchangeable amide groups, as well as protein conformation and dynamics; pepsin digestion allows these effects to be mapped with peptide-level resolution.

Keywords

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

INTRODUCTION

Understanding the effects of hydration on proteins in dried solids is critical to the development of solid formulations of protein drugs, to the desiccation and preservation of cells and microorganisms, and in the food sciences. In these applications, dried proteins are often amorphous solid powders, and contain residual water not removed during drying or sorbed from the surroundings. Though proteins are generally more stable in amorphous solids than in solution, various chemical and physical degradation processes still occur at rates relevant to food and pharmaceutical systems. Water can influence degradation by (e.g.): (i) participating in chemical reactions as a reactant, catalyst or local solvent, (ii) interacting with the protein to affect local conformation and dynamics and/or (iii) acting as a plasticizer. Unlike aqueous solutions, amorphous solids can be spatially and dynamically heterogeneous¹, further complicating their characterization as a reactive environment.

A considerable literature addresses protein-water interactions in amorphous solids. Though controversy remains, several critical hypotheses have been advanced. Early reports proposed that water does not interact uniformly with the protein surface, but occurs first at the side chains of ionizable and polar residues, proceeding to more hydrophobic regions as hydration increases^{2, 3}. The water replacement hypothesis asserts that, in the solid state, stabilizing co-

[‡]To whom correspondence should be addressed: Department of Industrial and Physical Pharmacy Purdue University 575 Stadium Mall Drive, Room 124D West Lafayette, IN 47901-2091 Phone: 765-494-1450 Fax: 765-494-6545 topp@purdue.edu.

solutes replace hydrogen bonds to water to preserve protein structure⁴. The unified model of protein dynamics states that protein dynamic motions are coupled (“slaved”) to α - and β -relaxation processes of the surrounding medium, with α -relaxation suppressed or absent in solids^{5, 6}. The coupling of protein dynamics to β -relaxation of the hydration shell (β_h) has also been proposed^{5, 6}. The idea of a protein dynamical transition has been set forth⁷ and challenged⁸. The protein dynamical transition is characterized by a sharp onset of protein motion with increasing temperature and is coupled to the glass transition of the solvent in the hydration shell.

These hypotheses have been supported by physical and chemical measurements, including neutron scattering^{9–12}, broadband dielectric spectroscopy^{12, 13}, Fourier transform infrared spectroscopy (FTIR)^{14, 15}, Raman spectroscopy¹⁶, differential scanning calorimetry (DSC)^{5, 17} and solid-state nuclear magnetic resonance spectroscopy (ssNMR)^{17, 18}. While these methods have improved our understanding of the hydration and structure of proteins in amorphous solids, most lack sufficient resolution to localize observed effects to particular protein domains. For example, neutron scattering and broadband dielectric spectroscopy have provided important insights into the dynamic modes of protein and water molecules, but cannot identify the particular protein domains involved. Methods such as FTIR and Raman spectroscopy can detect hydration-induced changes in secondary structure at the whole-protein level, but cannot localize changes to particular structural domains. Though ssNMR has been used to measure relaxation times in amorphous protein-saccharide matrices^{19, 20} and to provide site-specific information on protein dynamics^{17, 18} current ssNMR methods generally require that samples possess a degree of microscopic order (e.g., crystallinity, membrane incorporation), have low molecular weight and be isotopically labeled, and so are often impractical for food and pharmaceutical systems. As a result, the distribution of water within the solid, the local protein structure and the environment governing the reactivity of particular amino acids remain poorly understood.

In recent studies, our group has adapted hydrogen-deuterium exchange with mass spectrometric analysis (HDX-MS) to proteins in the solid state^{21–24}, providing higher resolution information on protein structure and matrix interactions. HDX-MS has been widely used to assess solvent exposure and dynamics for proteins in solution^{25–27}. Despite a seminal study on powder samples of lysozyme in the early 1980s²⁸, few investigators have explored the potential of solid-state HDX^{29, 30}, though there has been rapidly expanding interest in solution HDX. Our HDX-MS method exposes lyophilized samples of a protein to D₂O in the vapor phase at controlled activity (a_w ; equivalent to relative humidity, RH), followed by reconstitution under quenched conditions and LC/MS analysis. Pepsin digestion prior to LC/MS provides information on deuterium uptake with peptide-level resolution, an approach often used in solution HDX. Our results have shown that proteins in solid powders are protected from exchange in a manner that depends on both composition and the local site within the protein^{23, 24}. However, the studies have generally been conducted at a single RH and the effects of hydration have not been explored in detail.

In the current study, we investigate the effects of hydration on the HDX of equine myoglobin (Mb) in amorphous solids containing sucrose or mannitol. The results show that HDX in Mb varies with a_w , and is influenced by position in the protein sequence and matrix composition. We propose that the extent of exchange in solids is a measure of the hydration of exchangeable amide groups, as well as protein conformation and dynamics; pepsin digestion allows these effects to be mapped with peptide-level resolution. A detailed analysis of the kinetics of HDX in this system is presented in a second manuscript, also published in this issue of *Molecular Pharmaceutics*³¹.

MATERIALS AND METHODS

Materials

Mass spectrometry grade water, acetonitrile and formic acid (FA) were purchased from ThermoFisher Scientific (Waltham, MA). D₂O was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Equine myoglobin (Mb) and porcine pepsin were purchased from Sigma-Aldrich (St. Louis, MO). Pepsin was immobilized on Poros AL (Applied Biosystems, Foster City, CA) and packed into a high performance liquid chromatography (HPLC) column (50 × 2.1 mm, Grace Davison Discovery Sciences, Deerfield, IL) following a method described by Wang *et al.*³². All other chemicals used were of reagent grade or higher and used as received.

Sample Preparation

Mb was exhaustively dialyzed with potassium phosphate buffer (2.5 mM, pH 7.0) at 4°C and the dialysate used as a stock solution. Mb solutions for lyophilization were prepared by diluting the stock solution with phosphate buffer, adding sucrose or mannitol (1:1 w/w), and filtering (Millex-GV 0.22 µm PVDF; Millipore, Billerica, MA). Sucrose is a glass-forming disaccharide often used as a lyoprotectant³³, while mannitol is a common bulking agent in lyophilized pharmaceuticals³⁴. Samples were placed in 2 mL vials (0.1 mL for LC/MS or 0.45 mL for PXRD and TGA) and lyophilized using a VirTis Advantage Plus freeze-dryer (SP Scientific, Gardiner, NY). The lyophilization procedure consisted of pre-cooling the shelves to -2°C, freezing at -40°C, then drying under vacuum (70 mTorr) at -35°C for 10 h, -20°C for 8 h, -5°C for 8 h, and 20°C for 8 h. Vials were backfilled with nitrogen prior to sealing. After lyophilization, none of the samples showed evidence of collapse on visual inspection. The powder in each vial contained 10 wt% phosphate buffer, 45 wt% Mb and 45 wt% sucrose or mannitol. Samples for mass spectrometry contained 10 nmol Mb/vial (170 µg Mb, 378 µg total), while samples for all other analyses contained 45 nmol Mb/vial (763 µg Mb, 1700 µg total).

In HDX studies, Mb powders were exposed to D₂O vapor in equilibrium with saturated salt solutions of LiCl, KC₂H₃O₂, MgCl₂, K₂CO₃, and NaCl in D₂O at 5°C and atmospheric pressure. At equilibrium, the activities of water (a_w) in the solid samples are 0.11, 0.23, 0.33, 0.43, and 0.75, respectively³⁵. Activities of D₂O are assumed to be identical to those of H₂O, so that " a_w " refers to both D₂O and H₂O activity.

Physical Characterization of Solid Samples

Powder X-Ray Diffraction (PXRD)—To confirm that lyophilized Mb samples were amorphous, X-ray diffractograms were collected using a Scintag X2 θ - θ diffractometer (Scintag, Inc., Cupertino, CA) equipped with a Cu K α anode operating at a wavelength of 1.5406 Å. Prior to analysis, samples were stored for 48 h at controlled a_w of 0.11, 0.23, 0.33, 0.43, and 0.75. Diffraction patterns were collected from 5–40° 2 θ at a scan rate of 4° 2 θ /min and a step size of 0.4°.

Thermogravimetric Analysis (TGA)—To relate RH to water content in the solids, water sorption was measured using a gravimetric analyzer (Q5000SA; TA Instruments). The Mb powder (3–4 mg) was loaded onto the sample pan and loosely bound water removed at 358K, 0% RH until the signal was constant (<0.001% change in weight over 10 min). The samples were then equilibrated at 5°C, 0% RH prior to exposure to humidity levels of 11, 23, 33, 43, 59, and 75% RH over H₂O. Previous studies have shown no significant isotope effect on water sorption in lyophilized proteins³⁶. Each humidity level was maintained until the signal was constant, requiring ~6.5 h at low RH and ~2 h at higher RH. At equilibrium, the activity of water in the solid and in the vapor phase are equal, so that the conditions

above correspond to solid-phase $a_w = 0.11, 0.23, 0.33, 0.43, 0.59$ and 0.75 , respectively. It has been reported that the method is unable to remove tightly bound water². If so, the reported hydration levels are slightly underestimated, as in other studies of hydrated proteins.

FTIR Spectroscopic analysis—FTIR spectra were acquired for Mb samples after lyophilization to assess whether freeze-drying induced gross changes in secondary structure. Data were collected using a Tensor 37 FTIR spectrometer (Bruker Optics, Billerica, MA) with a single reflection, germanium crystal ATR accessory (MIRacle, PIKE Technologies, Madison, WI) purged under nitrogen to remove atmospheric water vapor. Background spectra of 128 scans at a resolution of 4 cm^{-1} were subtracted from sample spectra, baseline corrected, area-normalized, and the second derivative obtained using Opus software (Bruker Optics).

Hydrogen-Deuterium Exchange-Mass Spectrometry

The deuterium uptake of Mb was measured using a high-performance liquid chromatography-mass spectrometer (LC/MS) (1200 series LC, 6520 qTOF; Agilent Technologies, Santa Clara, CA), equipped with a custom-built refrigeration unit³⁷. Solution controls were prepared by diluting the stock Mb solution to $100\text{ }\mu\text{M}$ (5 mM potassium phosphate, pH 7.0). Deuterium labeling was initiated by further diluting the Mb solutions ($40:1\text{ v/v}$) with buffered D_2O (5 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pD 7.0) containing mannitol or sucrose ($1:1\text{ w/w}$) at 5°C . At various times, samples were quenched to pH 2.5 with HCl, immediately flash frozen in liquid nitrogen, and stored at -80°C until analysis.

Lyophilized powder samples were reconstituted with chilled ($\sim 0^\circ\text{C}$) 0.2% formic acid (FA) and immediately injected into a sample loop within the refrigeration unit held at 0°C to begin analysis. In the refrigeration unit, the sample flowed at 0.2 mL/min through an immobilized pepsin column and then was desalted on a peptide microtrap (Michrom Bioresources, Inc., Auburn, CA) for 2.5 min . Subsequently, flow through the microtrap was switched to 5% B ($\text{B} = 0.1\%$ FA in acetonitrile, $\text{A} = 0.1\%$ FA in water, $50\text{ }\mu\text{L/min}$) using a two-position valve (EPC12CW, VICI Valco Instruments Co., Inc., Houston, TX) to elute the trapped peptides onto an analytical column (Zorbax 300SB-C18; Agilent Technologies) for separation prior to mass analysis. Proteolytic fragments were identified by MS/MS analysis (CID fragmentation; MassHunter Software; Agilent Technologies). The time elapsed between sample reconstitution and MS analysis was $4\text{--}9\text{ min}$.

Mass spectra were processed using HX-Express software³⁸ to determine the peptide average mass. Measured deuterium uptake was corrected for deuterium loss during analysis by calibrating with a deuterated Mb standard. Deuterated Mb was obtained by incubating Mb at 70°C for 48 h in D_2O (97.5% D_2O , 10 mM potassium phosphate, pD 7.0). The average deuterium loss due to back-exchange during analysis was 20% (Table S1; figures and tables designated “S” can be found in Supplemental Information available on-line). For both intact protein and peptic fragments, deuterium incorporation is expressed as a percentage of the maximum theoretically attainable. The theoretical maximum is taken as N-P-2 , where N is the total number of amino acids in the protein or fragment, P is the number of Pro-residues and “2” accounts for the N-terminal amino group and amide hydrogen, which undergo back-exchange rapidly³⁹. To obtain higher resolution information, deuterium incorporation in “derived” peptides and in several single amide hydrogens was calculated by subtraction of measured values for overlapping peptic fragments (Table S2). When more than one pair of peptic peptides could be used, deuterium uptake was calculated as an average of all subtracted values. Both peptic and derived peptides are numbered sequentially from the N-

terminus; peptic peptides are identified by a “P” preceding the number, while derived peptides are identified using a “D”.

RESULTS

Physical Characterization of Solid Samples

Crystallinity—PXRD patterns for all samples showed broad, featureless bands consistent with amorphous materials. No apparent crystallinity was observed following 48 h of storage at any hydration level (Fig. S1).

Water Content—Both sucrose- and mannitol-containing samples sorbed water rapidly, as is typical of amorphous solids⁴⁰. At each RH, 90% of the plateau water content was attained in less than 1 h (not shown)³¹. Sorption isotherms show that the water content at $a_w \leq 0.43$ is similar for both mannitol and sucrose matrices (Fig. 1). At $a_w = 0.75$, the sucrose matrix sorbed ~35% more water than the mannitol matrix. Thus, it is unlikely that differences in the bulk water content of the mannitol and sucrose matrices play a role in HDX, perhaps with the exception of $a_w = 0.75$.

Mb Secondary Structure—Second-derivative FTIR spectra for Mb after co-lyophilization with either mannitol or sucrose showed an intense peak at $\sim 1654 \text{ cm}^{-1}$, assigned to α -helices, and other low intensity peaks which can be attributed to β -turns and side-chains⁴¹ (Fig. 2). This result indicates that at least some native Mb secondary-structure is retained in the solid powders, and that the two solid formulations have very similar secondary structure as measured by FTIR. UV absorption spectra of initial Mb solutions and reconstituted powders were superimposable with a maximum at 410 nm (not shown).

Hydration Effects on Deuterium Uptake

The results of solid-state HDX studies are presented below in the order of increasing structural resolution. Results are first presented for the intact protein, then for peptic digests and finally for the shorter derived peptides.

Intact Protein—Deuterium uptake for intact Mb in lyophilized samples containing mannitol or sucrose (1:1 w/w) at 5°C showed an initial period of rapid uptake over the first ~8 h, followed by a slower approach to a plateau (Fig. 3). The plateau increased with increasing a_w and was greatest in solution. For all hydration values studied and at all time points, deuterium uptake was less in sucrose samples than in mannitol samples, while exchange in solution was similar for the two additives. Though the extent of exchange in sucrose samples is *less* than in mannitol samples, the water content of the two solids is *similar* at $a_w < 0.4$ and *greater* in sucrose samples at $a_w > 0.4$ (Fig. 1). This suggests that factors other than water content contribute to solid-state HDX. These factors may include differences in protein structure or dynamics, or spatial heterogeneities in the distribution of water in the solid. Our FTIR data indicate that Mb secondary structure is similar in both matrices (Fig. 2). Thus, it is unlikely that gross differences in secondary structure are responsible for the greater exchange in mannitol, though subtle structural differences cannot be ruled out. Dynamic opening events may also be more restricted in sucrose than in mannitol, limiting overall exchange. Though reliable glass transition temperatures (T_g) could not be obtained here, higher T_g s have been reported in dry sucrose powders (~60–75°C)^{42,43} than in dry mannitol powders (~10–20°C)⁴⁴, consistent with greater restriction of protein dynamics in sucrose. Pepsin digestion was used to probe the local effects of sucrose and mannitol on deuterium uptake.

Pepsin-Digested Peptides—Samples were subjected to pepsin digestion after exposure to D₂O for 48 h, a time within the slow deuterium uptake phase. Digestion yielded 38 peptides suitable for further analysis (Fig. 4) in that they showed no overlap of isotope clusters with other features, were identifiable by MS/MS analysis and were present in sufficient abundance. In the peptic fragments, deuterium incorporation varies with peptide sequence/location, extent of hydration, and matrix type (Fig. 5; see also Fig. S2). As in the intact protein, deuterium uptake for peptic fragments from mannitol samples is greater than or equal to that in sucrose samples. The difference in deuterium uptake is greatest at intermediate hydration ($a_w = 0.43$) and smallest at low and high hydration ($a_w = 0.11, 1.0$). We define the “relative protection” (RP) as the difference in deuterium uptake in the mannitol and sucrose samples (i.e., $RP = \% \text{ exchange in mannitol} - \% \text{ exchange in sucrose}$). RP curves for selected peptides (Fig. 6) show that RP varies with hydration, with no significant difference at $a_w = 0.11$, reaches a maximum at $a_w = 0.43$ and is negligible in solution. The magnitude of the RP maximum at $a_w = 0.43$ is peptide-specific. These results indicate that protection from exchange is not related to matrix water content alone, but varies with additive and location in the protein sequence.

Derived Peptides—From the 38 original peptides, deuterium uptake for 16 smaller peptides was obtained by subtraction (Fig. S3, Table S2A). Uptake values for 11 single amino acids were obtained similarly (Fig. S4, Table S2B). Ribbon diagrams summarize the effects of hydration on Mb HDX in mannitol and sucrose samples (Fig. 7). The diagrams were constructed using the shortest amino acid sequences available, providing at least peptide-level resolution for all segments.

Results for the pepsin-digested and derived peptides show the effects of a_w on the local pattern of exchange, and should be contrasted with previous HDX studies for Mb in solution at 22 °C⁴⁵. The pattern of HDX at 5 °C reported here is broadly consistent with the earlier report, even in the driest solids, showing the greatest protection from exchange in the helices (e.g., V10, L32–F33, E105, A110–I112, L125–R139, Fig. 7) and the least protection in loops and near the C-terminus (e.g., D20–I21, S58–D60, G150–G153, Fig. 7). This is consistent with retention of Mb secondary structure in the solids. Interestingly, the results for solids show several distinct differences from HDX patterns in solution. For example, at $a_w = 0.23$, the regions most protected from exchange include M55–A57 in helix D and K63–L69 near the N-terminal end of helix E, in addition to the regions that are most protected in solution (Fig. 7). That M55–A57 and K63–L69 are not highly protected in solution at 5 °C suggests that the sucrose and mannitol matrices confer additional protection in these regions. As a_w decreases, only two of the regions that are least protected in solution also show minimal protection in solids: D20–I21 and the C-terminus. Limited protection in these regions may involve the inhomogeneous distribution of D₂O in the solid and/or protein-matrix interactions, as well as local protein structure and dynamics.

DISCUSSION

In neutral solution, HDX is base catalyzed. The reaction involves the initial abstraction of the amide proton, typically by [−]OD, followed by the formation of a new N-D bond to D₂O with regeneration of [−]OD⁴⁶. The intrinsic rate of exchange in unstructured peptides, k_{int} , is affected by temperature, amino acid sequence, pH, and ionic strength³⁹. However, far greater effects are exerted by local protein structure. It is generally accepted that exchangeable amide hydrogens are either solvent exposed or located in domains in which “opening events” allow for transient disruption of intra-chain hydrogen bonds. Opening events can involve local fluctuations or sub-domain unfolding, as well as global unfolding of the molecule as a whole⁴⁷. Protected amide residues extensively involved in intramolecular hydrogen bonds and those in more rigid structural domains exchange slowly, if at all. The

observed rate of exchange in solution, k_{obs} , reflects both transient opening events ($k_o/k_c = K_{op}$) and the intrinsic chemical rate of exchange (k_{int}), as described by the Linderstrom-Lang scheme⁴⁸

In this conception of solution HDX, the protein exists in a network of hydrogen bonds, predominantly comprised of intra-chain hydrogen bonds and hydrogen bonds between exposed amino acid residues and water. Opening events (k_o , k_c) involve the transient breaking of intra-chain hydrogen bonds, allowing bonds with water to form. In the exchange reaction (k_{int}), hydrogen bonds are involved in the ^-OD catalyzed abstraction of the exchanging proton and in the donation of deuterium from D_2O . Both opening and chemical exchange occur in the dynamic extended network of hydrogen bonds that characterize liquid water.

HDX in amorphous solids differs from solution HDX in several respects. First, and most obviously, the concentration of water (or D_2O) in amorphous solids is less than in than aqueous solutions and varies with RH (i.e., a_w). Opening events and the exchange reaction show dependences on a_w , a phenomenon not observed in solution. Second, hydrogen bonds form between the protein and components of the solid other than water. These interactions partially couple the protein to the solid matrix and restrict protein dynamics. As a result, some dynamic modes available in solution are not attainable and the rates of opening events and exchange may be altered. Third, because amorphous solids are spatially and dynamically heterogeneous, the local environment for exchange is likely to vary within the solid matrix, producing protein subpopulations that undergo opening and exchange events at different rates. For example, the local concentrations of water and buffer salts may vary spatially within the matrix, influencing the effective local pH. Finally, because solid-state HDX involves sorption of D_2O from the vapor phase and its subsequent diffusion in the solid, these rate processes may influence the observed rate of exchange, though this is not the case in the studies reported here. None of these differences necessarily invalidates the Linderstrom-Lang scheme, which is non-committal about the nature of an opening event and the environment in which it occurs.

For exchange to occur at a particular amide proton in a solid sample, it must at least have access to a source of deuterium. Since D_2O is the only source of deuterium in these studies, exchange is evidence that the site has been exposed to D_2O or species derived from it. Exposure to deuterium may occur through the local interaction of the amide group with sorbed D_2O or related species, or through conduction of deuterium through hydrogen bond networks in the solid. In this sense, then, the propensity for exchange HDX in solids is a measure of the local hydration of the amide group. This differs from solution HDX, in which D_2O is present in excess and does not limit exchange. However, the exposure of a particular amide group to D_2O in a solid sample is a necessary, but not sufficient, condition for exchange. As in solution, intramolecular hydrogen bonds must also be broken (i.e., in an opening event) for exchange to occur. Since hydrogen bonds link the protein to components of the solid other than water, an opening event may also require that these bonds be broken.

Accordingly, we propose that solid HDX data can be interpreted as a measure of protein conformation and dynamics in amorphous solids, according to the Linderstrom-Lang scheme, with the following provisos: (i) the rate (k_o , k_c , k_{int}) and extent of ssHDX may depend on a_w and composition as well as protein conformation and dynamics, (ii) the rate and extent of HDX may vary spatially within the solid matrix, reflecting heterogeneities in solid composition and protein dynamics and (iii) other kinetic processes, such as D_2O vapor

sorption/diffusion and structural relaxation may influence solid HDX when they occur on the timescale of the experiment.

Our results support this proposal. FTIR results reported by us elsewhere^{21, 23, 24} demonstrate that solids that show protection from exchange by HDX also show retention of structure by FTIR, and, conversely, that solids that show loss of secondary structure by FTIR (e.g., including guanidine hydrochloride) also show reduced protection from exchange. The results presented here also show that exchange in Mb solids varies with time, a_w , composition and position in the protein sequence (Figs. 5, 7). The dependence on a_w reflects changes in the hydration of exchangeable amide groups, as well as changes in protein conformation and dynamics induced by the changing water content of the solid. The results also show that HDX depends on solid composition. That the extent of exchange is generally greater in mannitol samples than in sucrose samples (Figs. 3, 5, 7), despite similar or lower bulk water content in mannitol (Fig. 1), implies that a_w and solid composition are not strict covariates. That is, composition has an effect on solid HDX apart from its effect on bulk water content. The dependence of HDX on position in the protein sequence and the difference in the two solids (Fig. 5) suggest that HDX in solids is not merely solution HDX at a slower rate. Rather, this is consistent with an effect of the solid matrix on exchange through hydrogen bonds to the protein and/or the restriction of opening events.

CONCLUSIONS

The studies reported here address the effects of hydration on Mb HDX in amorphous solids containing mannitol or sucrose. The extent of exchange varied with matrix type, a_w and position in the protein backbone. Since HDX requires the exposure of an amide group to D₂O or species derived from it, the extent of exchange in solids is a measure of the hydration of exchangeable amide groups, as well as protein conformation and dynamics. HDX with pepsin digestion allows these effects to be mapped with peptide-level resolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support was provided through NIH RO1 GM085293 (PI: E. Topp), through a Pharmaceutical Research and Manufacturers of America Foundation postdoctoral fellowship (to A. Sophocleous) and from the College of Pharmacy at Purdue University. The authors are grateful to Dr. David D. Weis of the Department of Chemistry at The University of Kansas for helpful discussions.

SUPPORTING INFORMATION AVAILABLE Figures and tables designated “S” within the manuscript are presented in on-line supplemental information. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

REFERENCES

1. Ediger MD. Spatially heterogeneous dynamics in supercooled liquids. *Annu. Rev. Phys. Chem.* 2000; 51:99–128. [PubMed: 11031277]
2. Rupley JA, Careri G. Protein hydration and function. *Adv. Protein Chem.* 1991; 41:37–172. [PubMed: 2069077]
3. Careri G. Cooperative charge fluctuations by migrating protons in globular proteins. *Prog. Biophys. Mol. Bio.* 1998; 70:223–249. [PubMed: 9830313]
4. Hill JJ, Shalaeve EY, Zograf G. Thermodynamic and dynamic factors involved in the stability of native protein structure in amorphous solids in relation to levels of hydration. *J. Pharm. Sci.* 2005; 94(8):1636–1667. [PubMed: 15965985]

5. Young RD, Fenimore PW. Coupling of protein and environment fluctuations. *Biochim. Biophys. Acta.* 2011; 1814:916–921. [PubMed: 21621015]
6. Frauenfelder H, Chen G, Berendzen J, Fenimore PW, Jansson H, McMahon BH, Stroer IR, Swenson J, Young RD. A unified model of protein dynamics. *P. Natl. Acad. Sci. USA.* 2009; 106(13):5129–5134.
7. Doster W, Cusack S, Petry W. Dynamical transition of myoglobin revealed by inelastic neutron scattering. *Nature.* 1989; 337:754–756. [PubMed: 2918910]
8. Magazu S, Migliardo F, Benedetto A. Puzzle of protein dynamical transition. *J. Phys. Chem. B.* 2011; 115(24):7736–7743. [PubMed: 21612196]
9. Doster W, Settles M. Protein-water displacement distributions. *Biochim. Biophys. Acta.* 2005; 1749(2):173–186. [PubMed: 15893505]
10. Cornicchi E, Marconi M, Onori G, Paciaroni A. Controlling the protein dynamical transition with sugar-based bioprotectant matrices: A neutron scattering study. *Biophys. J.* 2006; 91(1):289–297. [PubMed: 16617083]
11. Schiro G, Sclafini M, Caronna C, Natali F, Plazenet M, Cupane A. Dynamics of myoglobin in confinement: An elastic and quasi-elastic neutron scattering study. *Chem. Phys.* 2008; 345(2–3): 259–266.
12. Khodadadi S, Pawlus S, Sokolov AP. Influence of hydration on protein dynamics: Combining dielectric and neutron scattering spectroscopy data. *J. Phys. Chem. B.* 2008; 112(45):14273–14280. [PubMed: 18942780]
13. Schiro G, Cupane A, Vitrano E, Bruni F. Dielectric relaxations in confined hydrated myoglobin. *J. Phys. Chem. B.* 2009; 113(28):9606–9613. [PubMed: 19537774]
14. Dong AC, Prestrelski SJ, Allison SD, Carpenter JF. Infrared spectroscopic studies of lyophilization-induced and temperature-induced protein aggregation. *J. Pharm. Sci.* 1995; 84:415–424. [PubMed: 7629730]
15. Luthra S, Kalonia DS, Pikal MJ. Effect of hydration on the secondary structure of lyophilized proteins as measured by Fourier transform infrared (FTIR) spectroscopy. *J. Pharm. Sci.* 2007; 95(4):781–789.
16. Dong JP, Hubel A, Bischof JD, Aksan A. Freezing-induced phase separation and spatial microheterogeneity in protein solutions. *J. Phys. Chem. B.* 2009; 113(30):10081–10087. [PubMed: 19580250]
17. Krushelnitsky A, Zinkevich T, Mukhametshina N, Tarasova N, Gogolev Y, Gnezdilov O, Fedotov V, Belton P, Reichert D. C-13 and N-15 NMR study of the hydration response of T4 lysozyme and alpha B-crystallin internal dynamics. *J. Phys. Chem. B.* 2009; 113(29):10022–10034. [PubMed: 19603846]
18. Reif B, Xue Y, Agarwal V, Pavlova MS, Hologne M, Diehl A, Ryabov YE, Skrynnikov NR. Protein side-chain dynamics observed by solution- and solid-state NMR: Comparative analysis of methyl H-2 relaxation data. *J. Am. Chem. Soc.* 2006; 128(38):12354–12355. [PubMed: 16984151]
19. Yoshioka S, Miyazaki T, Aso Y. Degradation rate of lyophilized insulin, exhibiting an apparent Arrhenius behavior around glass transition temperature regardless of significant contribution of molecular mobility. *J. Pharm. Sci.* 2006; 95(12):2684–2691. [PubMed: 16892208]
20. Yoshioka S, Miyazaki T, Aso Y, Kawanishi T. Significance of local mobility in aggregation of beta-galactosidase lyophilized with trehalose, sucrose or stachyose. *Pharm. Res.* 2007; 24:1660–1667. [PubMed: 17404806]
21. Li Y, Williams TD, Schowen RL, Topp EM. Characterizing protein structure in amorphous solids using hydrogen/deuterium exchange with mass spectrometry. *Anal. Biochem.* 2007; 366(1):18–28. [PubMed: 17490599]
22. Sinha S, Li YS, Williams TD, Topp EM. Trehalose and calcium exert site-specific effects on calmodulin conformation in amorphous solids. *Biotechnol. Bioeng.* 2007; 97(6):1650–1653. [PubMed: 17286268]
23. Li Y, Williams TD, Topp EM. Effects of excipients on protein conformation in lyophilized solids by hydrogen/deuterium exchange with mass spectrometry. *Pharm. Res.* 2008; 25(2):259–267. [PubMed: 17597380]

24. Sinha S, Li YS, Williams TD, Topp EM. Protein conformation in amorphous solids by FTIR and by hydrogen/deuterium exchange with mass spectrometry. *Biophys. J.* 2008; 95(12):5951–5961. [PubMed: 18835903]
25. Kaltashov IA, Bobst CE, Abzalimov RR. H/D exchange and mass spectrometry in the studies of protein conformation and dynamics: Is there a need for a top-down approach? *Anal. Chem.* 2009; 81(19):7892–7899. [PubMed: 19694441]
26. Engen JR. Analysis of protein conformation and dynamics by hydrogen/deuterium exchange MS. *Anal. Chem.* 2009; 81(19):7870–7875. [PubMed: 19788312]
27. Tsutsui Y, Wintrode PL. Hydrogen/deuterium, exchange-mass spectrometry: A powerful tool for probing protein structure, dynamics and interactions. *Curr. Med. Chem.* 2007; 14(22):2344–2358. [PubMed: 17896983]
28. Schinkel JE, Downer NW, Rupley JA. Hydrogen-exchange of lysozyme powders - hydration dependence of internal motions. *Biochemistry-US.* 1985; 24(2):352–366.
29. Hutcheon GA, Parker MD, Moore BD. Measuring enzyme motility in organic media using novel H-D exchange methodology. *Biotechnol. Bioeng.* 2000; 70(3):262–269. [PubMed: 10992230]
30. Desai UR, Klibanov AM. Assessing the structural integrity of a lyophilized protein in organic solvents. *J. Am. Chem. Soc.* 1995; 117(14):3940–3945.
31. Sophocleous A, Topp EM. Localized hydration in lyophilized myoglobin by hydrogen-deuterium exchange mass spectrometry. 2. Exchange kinetics. *Mol. Pharmaceut.* 2012 submitted.
32. Wang LT, Pan H, Smith DL. Hydrogen exchange-mass spectrometry: Optimization of digestion conditions. *Mol. Cell. Proteomics.* 2002; 1(2):132–138. [PubMed: 12096131]
33. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: Some practical advice. *Pharm. Res.* 1997; 14(8):969–975. [PubMed: 9279875]
34. Meyer JD, Nayar R, Manning MC. Impact of bulking agents on the stability of a lyophilized monoclonal antibody. *Eur. J. Pharm. Sci.* 2009; 38(1):29–38. [PubMed: 19467324]
35. Greenspan L. Humidity fixed points of binary saturated aqueous solutions. *J. Res. NBS A Phys. Ch.* 1977; 81(1):89–96.
36. Hnojewy WS, Reyerson LH. Further studies on the sorption of H₂O and D₂O vapors by lysozyme and the deuterium-hydrogen exchange effect. *J. Phys. Chem.* 1961; 65(10):1694–1698.
37. Keppel T, Jacques M, Young R, Ratzlaff K, Weis D. An efficient and inexpensive refrigerated LC system for H/D exchange mass spectrometry. *J. Am. Soc. Mass Spectr.* 2011; 22(8):1472–1476.
38. Weis DD, Engen JR, Kass II. Semi-automated data processing of hydrogen exchange mass spectra using HX-express. *J. Am. Soc. Mass Spectr.* 2006; 17(12):1700–1703.
39. Bai YW, Milne JS, Mayne L, Englander SW. Primary structure effects on peptide group hydrogen exchange. *Proteins.* 1993; 17(1):75–86. [PubMed: 8234246]
40. Hunter NE, Frampton CS, Craig DQM, Belton PS. The use of dynamic vapour sorption methods for the characterisation of water uptake in amorphous trehalose. *Carbohydr. Res.* 2010; 345(13):1938–1944.
41. Dong AC, Kendrick B, Kreilgard L, Matsuura J, Manning MC, Carpenter JF. Spectroscopic study of secondary structure and thermal denaturation of recombinant human factor XIII in aqueous solution. *Arch. Biochem. Biophys.* 1997; 347(2):213–220. [PubMed: 9367527]
42. Tebooy M, Deruiter RA, Demeere ALJ. Evaluation of the physical stability of freeze-dried sucrose-containing formulations by differential scanning calorimetry. *Pharm. Res.* 1992; 9(1):109–114. [PubMed: 1589394]
43. Shamblyn SL, Huang EY, Zografi G. The effects of co-lyophilized polymeric additives on the glass transition temperature and crystallization of amorphous sucrose. *J. Therm. Anal.* 1996; 47:1567–1579.
44. Yu L, Mishra DS, Rigsbee DR. Determination of the glass properties of D-mannitol using sorbitol as an impurity. *J. Pharm. Sci.* 1998; 87(6):774–777. [PubMed: 9607958]
45. Pan J, Han J, Borchers CH, Konermann L. Hydrogen/deuterium exchange mass spectrometry with top-down electron capture dissociation for characterizing structural transitions of a 17 kDa protein. *J. Am. Chem. Soc.* 2009; 131:12801–12808. [PubMed: 19670873]

46. Hvidt A, Nielsen SO. Hydrogen exchange in proteins. *Adv. Protein Chem.* 1966; 21:287–386. [PubMed: 5333290]
47. Maity H, Lim WK, Rumbley JN, Englander SW. Protein hydrogen exchange mechanism: Local fluctuations. *Protein Sci.* 2003; 12(1):153–160. [PubMed: 12493838]
48. Berger A, Linderstrom-Lang K. Deuterium exchange of poly-DL-alanine in aqueous solution. *Arch. Biochem. Biophys.* 1957; 69:106–118. [PubMed: 13445185]
49. Maurus R, Overall CM, Bogumil R, Luo Y, Mauk AG, Smith M, Brayer GD. A myoglobin variant with a polar substitution in a conserved hydrophobic cluster in the heme binding pocket. *BBA Protein Struct. M.* 1997; 1341(1):1–13.

Figure 1. Water vapor sorption isotherms for Mb in lyophilized solids containing mannitol (1:1 w/w; closed symbols) or sucrose (1:1 w/w; open symbols) at 5°C.

Figure 2. Second-derivative FTIR spectra of Mb in lyophilized samples containing mannitol (1:1 w/w; solid line) or sucrose (1:1 w/w; dashed line).

Figure 3.

Kinetics of deuterium uptake at 5°C for intact Mb in lyophilized solids containing 1:1 w/w (a) mannitol or (b) sucrose and exposed to 11% (closed diamond), 23% (open diamond), 33% (closed triangle), 43% (open triangle) or 75% RH (closed square) and compared to a fully hydrated, solution control (open square) ($n = 3 \pm \text{SE}$). Dashed lines represent fits of data in solids to a biexponential model; see text for details.

Figure 4.

Mb pepsin digestion map. Original peptides are shown in black with the two N-terminal amino acids in dark grey to indicate that these do not contribute to measured deuterium uptake. Derived peptides are shown in light grey. Secondary structure is shown above the amino acid numbers, with cylinders representing α -helices.

Figure 5.

Deuterium uptake for peptic peptides (P1–P38) from Mb in lyophilized solids containing 1:1 w/w (a) mannitol and (b) sucrose at $a_w = 0.11$ (blue), 0.23 (red), 0.33 (green), 0.43 (purple) and 0.75 (pink), and compared to a fully hydrated, solution control (orange) ($n = 3$). The data is replotted for individual peptides with SE in Fig. S2.

Figure 6.

The protection of the sucrose matrix, relative to mannitol, calculated as the difference in the percent exchange (mannitol – sucrose; Fig. 5) for selected peptic peptides: (long-dashed line) P16, (dotted line) P26, (short-dashed line) P37, and (solid line) average of P1–P24; ($n = 3 \pm \text{SE}$). See also Fig. 7B.

Figure 7.

(A) High-resolution ribbon diagram of hydration effect on deuterium uptake for Mb in mannitol (top) and sucrose (bottom). From left to right: $a_w = 0.11, 0.23, 0.33, 0.43, 0.75, D_2O$ solution. The deuterium uptake for each region was determined by selecting the shortest fragment corresponding to that particular region. Images were generated using PyMOL (PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC) from the RCSB PDB (www.pdb.org) crystal structure of PDB ID 1WLA⁴⁹. The deuterium uptake for each region was determined by selecting the shortest fragment corresponding to that particular region. See Table S3 for fragment assignments. (B) High-resolution diagrams of hydration effect on deuterium uptake for Mb in mannitol (solid line) and sucrose (dashed), shown as a function of amino acid number. Figures (a–f) correspond to $a_w = 0.11, 0.23, 0.33, 0.43, 0.75, D_2O$ solution, respectively. The difference in protection from exchange afforded by the two additives (i.e., sucrose vs. mannitol) can be estimated from the difference between the two plots.