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Empirical Correction for Differences in Chemical Exchange Rates in Hydrogen Exchange-Mass Spectrometry Measurements

Ronald T. Toth, IV,[†] Brittney J. Mills,[†,∥] Sangeeta B. Joshi,[†] Reza Esfandiary,[‡] Steven M. Bishop,[‡] C. Russell Middaugh,[†] David B. Volkin,[*,†] and David D. Weis[*,§]

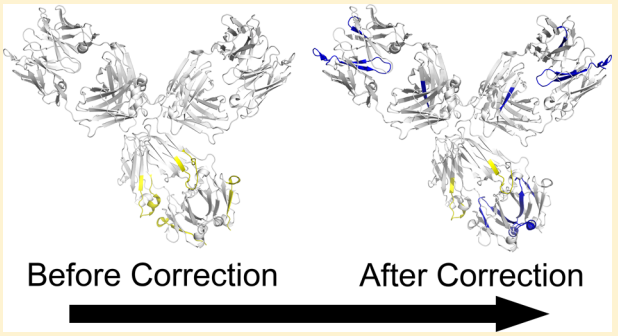
[†]Department of Pharmaceutical Chemistry, Macromolecule and Vaccine Stabilization Center, University of Kansas, 2030 Becker Drive, Lawrence, Kansas 66049, United States

[‡]Department of Formulation Sciences, MedImmune LLC, Gaithersburg, Maryland 20878, United States

[§]Departments of Chemistry and Pharmaceutical Chemistry, University of Kansas 1251 Wescoe Hall Drive, Lawrence, Kansas 66045, United States

Supporting Information

ABSTRACT: A barrier to the use of hydrogen exchange-mass spectrometry (HX-MS) in many contexts, especially analytical characterization of various protein therapeutic candidates, is that differences in temperature, pH, ionic strength, buffering agent, or other additives can alter chemical exchange rates, making HX data gathered under differing solution conditions difficult to compare. Here, we present data demonstrating that HX chemical exchange rates can be substantially altered not only by the well-established variables of temperature and pH but also by additives including arginine, guanidine, methionine, and thiocyanate. To compensate for these additive effects, we have developed an empirical method to correct the hydrogen-exchange data for these differences. First, differences in chemical exchange rates are measured by use of an unstructured reporter peptide, YPI. An empirical chemical exchange correction factor, determined by use of the HX data from the reporter peptide, is then applied to the HX measurements obtained from a protein of interest under different solution conditions. We demonstrate that the correction is experimentally sound through simulation and in a proof-of-concept experiment using unstructured peptides under slow-exchange conditions (pD 4.5 at ambient temperature). To illustrate its utility, we applied the correction to HX-MS excipient screening data collected for a pharmaceutically relevant IgG4 mAb being characterized to determine the effects of different formulations on backbone dynamics.



Hydrogen exchange (HX) provides medium- to high-resolution information about higher-order structure and dynamics of proteins. When a protein of interest, prepared in an H_2O buffer, is diluted into a D_2O buffer, labile protium atoms will exchange with deuterium atoms from the solvent; the consequent increase in mass can be measured by mass spectrometry (MS). Because the rate of HX in a folded protein depends on the flexibility of the protein backbone, HX-MS provides a mechanism to measure conformational dynamics of both the whole protein and peptide segments.[1,2] If the protein is perturbed, HX-MS data can be used to map the effects of perturbations to specific segments of the protein by comparison to an unperturbed reference state.

The propensity of differing solution conditions to alter chemical exchange rates (sometimes also referred to as intrinsic exchange) in HX experiments is a potential barrier to the use of HX-MS in many applications. This barrier is present in any scenario where HX is compared between protein samples in solutions with different compositions, such as during excipient screening experiments in formulation development. More generally, this barrier has been noted in studies from other laboratories under any circumstances where there is a difference in the composition of the solutions used for labeling.[3−5] The dependence of chemical exchange on solution pH is well understood[6] and can be readily predicted and corrected.[7] Such corrections have allowed for the comparison of HX at different pH values.[8−11] On the other hand, the dependence of chemical exchange on other differences in solution composition at the same pH is not well understood, although methods have been developed and implemented to correct for these less predictable effects.[4,5]

To better understand how differing solution conditions can have an effect on HX of a protein molecule, it must be understood that a number of factors can alter the HX rate. To undergo exchange, the amide hydrogen must be released from protective hydrogen bonds. This release occurs through protein dynamic opening and closing events, often referred to as

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"breathing" motions. Once released, well-defined chemical exchange processes cause the exchange. Within the Linderstrøm−Lang mechanism, the overall HX rate is a function of three processes, represented by three rate constants: dynamic opening events (k_{op}) and closing events (k_{cl}) and the rate at which the reaction itself occurs once the residues are available for exchange (which is known as the chemical exchange rate, k_{ch}). The observed rate, k_{HX}, is described by eq 1:

$$k_{HX} = \frac{k_{op}k_{ch}}{k_{op} + k_{cl} + k_{ch}} \quad (1)$$

Under the EX2 limit, k_{cl} is much larger than k_{ch}, such that eq 1 can be reduced to eq 2:

$$k_{HX} = \frac{k_{op}}{k_{cl}}k_{ch} \quad (2)$$

We note here that exchange under the EX1 limit does not depend on the rate of chemical exchange. Exchange under this limit is unusual at physiological pH and exhibits a characteristic bimodal isotopic distribution in the isotope cluster.[12] Thus, the observed HX rate is a combination of the chemical rate and protein dynamics. When a difference is observed in the HX rate between two conditions, the difference might be due to either a change in protein dynamics, a change in the chemical rate, or both. Conversely, when no difference in HX rate is observed between conditions, the apparent similarity might arise because there was no change in protein dynamics or it could be that a change in dynamics was compensated for by a change in chemical exchange. Consequently, to draw conclusions regarding protein dynamics based on HX compared between solutions, it is vital to rule out or correct for differences in chemical exchange.

Changes in the rate of chemical exchange between conditions can occur as a result of a number of effects. Changes in pH affect HX rates by altering the concentrations of hydronium and hydroxide ions. Temperature affects HX rates both by altering water autoprotolysis and also by increasing the rate of the rate-limiting step. Additionally, neighboring side chains influence the rate of chemical exchange through well-understood effects.[13,14] Differences in ionic strength can also affect chemical exchange, presumably by screening the polarity of adjacent side chains.[13] Beyond these predictable effects, the addition of solutes and cosolvents can also alter chemical exchange. Unlike pH and temperature effects, though, the extent of the effects of such additives on HX rate of peptides and proteins are not as predictable.

Results from previous studies in our laboratory[15,16] have demonstrated that HX-MS can be used to evaluate the effects of solutes on protein backbone flexibility, which in turn can be correlated with the effects of additives on the conformational stability and aggregation propensity of the protein. Particularly in a pharmaceutical context, these additives can be used as stabilizing excipients in a drug product formulation. HX-MS thus has the potential to be a useful tool for the analysis of excipients' effects on therapeutic proteins and as a potential predictor of excipient effects on long-term storage stability.[15] Changes in chemical exchange rates between solution conditions is a barrier to such work. In fact, most researchers take great pains to maintain identical labeling conditions between protein states in order to rule out altered chemical exchange. To remove this barrier, here we present a method to correct HX-MS measurements carried out under conditions of altered chemical exchange. In this method, differences in chemical exchange rates are monitored with an unstructured reporter peptide in different solutions, either mixed with the protein of interest as an internal standard or in separate measurements. An empirical chemical exchange correction factor, determined by use of the HX data from the reporter peptide in different solutions, is then applied to the HX measured in peptides derived from the protein of interest in the same solutions. To illustrate the application, we apply the correction procedure to an HX-MS excipient screening study on an IgG4 monoclonal antibody. In this particular application, the goal is to maximize the number of additives screened by minimizing the amount of data acquired for each additive. In this application, each additive is screened at a single HX time. The method can, however, be applied more generally to any HX-MS data where the conditions being compared have different chemical exchange characteristics.

EXPERIMENTAL SECTION

Materials. An IgG4 (mAb-D) was provided by MedImmune (Gaithersburg, MD) at a concentration of 40 mg·mL^{-1}. To quantify the protein, triplicate samples were prepared by diluting the stock mAb-D solution 1:50 into the same buffer. The absorbance at 280 nm was averaged over triplicate analyses and an extinction coefficient of 1.68 mL·mg^{-1}·cm^{-1} was used to calculate the protein concentration. Absorption was measured with an Agilent 8453 UV−visible spectrophotometer (Palo Alto, CA). The reporter peptide YPI was purchased from AnaSpec (Fremont, CA). The FKPGI reporter peptide was purchased from GenScript (Piscataway, NJ).

Trehalose dihydrate was purchased from Pfanstiehl (Waukegan, IL). Arginine monohydrochloride, deuterium oxide (99+ %D), methionine, D-mannitol, porcine pepsin, sodium sulfate, polysorbate 20, and liquid chromatography (LC)-grade acetic acid and phosphoric acid were purchased from Sigma−Aldrich (St. Louis, MO). Premium-grade tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) and LC-MS-grade formic acid (+99%) were purchased from Thermo Scientific (Rockford, IL). Sodium phosphate dibasic (anhydrous), citric acid (anhydrous), and sodium thiocyanate were purchased from Acros Organics (Fair Lawn, NJ). Sodium chloride, guanidine hydrochloride (GdnHCl) , LC-MS-grade water, acetonitrile, and 2-propanol were purchased from Fisher Scientific (Fair Lawn, NJ).

Sample Preparation. Stock solutions of mAb-D were dialyzed into 5 mM citrate−phosphate (CP) buffer at pH 6.5 or 7.4, with or without 150 mM NaCl. Stock solutions of each of eight additives, chosen such that both commonly reported stabilizing and destabilizing additives were represented, were also prepared in 5 mM CP buffer at pH 6.5 or 7.4, with or without 150 mM NaCl, at a higher concentration than desired in the final sample. The mAb-D sample was then diluted with the corresponding CP buffer and the appropriate additive stock solution to achieve a protein concentration of 5 mg·mL^{-1} and the desired additive concentration (0.3 M arginine, 0.3 M guanidine, 0.3 M sodium thiocyanate, 0.3 M sodium sulfate, 0.2 M methionine, 0.4 M trehalose, 0.8 M mannitol, or 0.05% polysorbate 20). After addition of the additive and before addition of mAb-D, the pH of the buffer was adjusted to be within 0.02 pH unit of the desired pH. Control samples of mAb-D were prepared with only CP buffer at the appropriate pH, with or without 150 mM NaCl.

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For the HX studies, additives containing exchangeable hydrogens (arginine, guanidine, mannitol, methionine, and trehalose) were fully deuterated before use. Each additive was prepared in D_2O at a concentration slightly higher than the final concentration (to account for dilution effects) and incubated for 30 min. The additive solution was vacuum-dried at 30 °C for 48 h in an Eppendorf Vacufuge (Hamburg, Germany). Two additional cycles of dissolution in D_2O followed by evaporation were performed. The resulting solid was dissolved in the appropriate volume of CP buffer prepared with D_2O. The pD of all labeling solutions was adjusted with deuterium chloride or deuterium oxide. To account for the offset associated with measuring pD with a pH meter, solutions were adjusted to a pH 0.4 unit lower than the desired value.[17]

Hydrogen Exchange-Mass Spectrometry. HX-MS experiments were performed on a quadrupole time-of-flight (QTOF) mass analyzer (Agilent 6530, Santa Clara, CA) with a three-pump LC system (Agilent 1260, Santa Clara, CA). Sample preparation was performed by an H/DX PAL robot (LEAP Technologies, Carrboro, NC).

For YPI studies, 3 μL of YPI was incubated with 21 μL of deuterated additive (a 1:8 dilution into D_2O) at 25 °C. Labeling was performed with each of 27 different labeling buffers: 5 mM CP buffer at either pD 6.5 with 150 mM NaCl, pD 7.4 with 150 mM NaCl, or pD 7.4 without salt, with and without addition of each of the eight additives. Incubation at each labeling time was performed in triplicate. After labeling for the designated time, the exchange was quenched by a 1:1 dilution into quench buffer (4 M GdnHCl, 0.2 M phosphate, pH 2.5) at 1 °C for 60 s. Twenty-five μL of the quenched YPI was injected into the sample loop of the refrigerated compartment of the H/DX PAL (maintained at 0 °C), containing a reversed-phase trap (Poroshell 120 EC-C8, 2.1 × 5 mm, 2.7 μm particle diameter, Agilent, Santa Clara CA). The sample was desalted for 1 min at a flow rate of 200 μL min^{-1} with 0.1% formic acid, eluted with a 4 min gradient from 1% to 100% B, with a mobile phase A of 0.1% formic acid and a mobile phase B of 90% acetonitrile in 0.1% formic acid. The trap was re-equilibrated with 1% A for 2 min before the next injection.

For studies using predigested mAb-D peptides, mAb-D was diluted to 5 mg·mL^{-1} in quench buffer containing 500 mM tris(2-carboxyethyl)phosphine (TCEP), 4 M guanidinium hydrochloride, 0.2 M phosphate, pH 2.5 and then passed over an immobilized pepsin column (50 mm × 2.1 mm, pepsin was immobilized and packed as described previously[15]) at 200 μL min^{-1} at 25 °C. The collected peptides were vacuum-dried at 30 °C for 1 h and reconstituted in 5 mM CP buffer at either pH 6.5 or 7.4. The samples were then labeled as described above, except that the labeling buffer used was 5 mM CP buffer at pD 4.5, with and without addition of 6 M urea. After labeling, the HX reaction was quenched using a 1:1 dilution into quench buffer at 1 °C for 60 s. Quenched mAb-D peptide (25 μL) was injected into the sample loop of a refrigerated compartment maintained at 1 °C, containing, in addition to the reversed-phase trap, a reversed-phase column (Zorbax 300SB-C18 2.1 × 50 mm, 1.8 μm particle diameter, Agilent, Santa Clara, CA). An 18 min gradient from 1% to 95% B was used.

In formulation screening studies of mAb-D, mAb-D samples, prepared at 5 mg·mL^{-1} in excipient-containing CP buffers, were subjected to HX as described, with the addition of a pepsin column to the refrigerated compartment. Labeling was performed in each of 27 different labeling buffers, 5 mM CP buffer at either pD 6.5 with 150 mM NaCl, pD 7.4 with 150 mM, or pD 7.4 without additional NaCl, with and without addition of each of the eight additives.

Collision-induced dissociation and MS/MS analysis were used to identify the mAb-D peptic peptides. From a set of 216 peptides covering 100% of the light chain and 87% of the heavy chain, a subset of 40 peptides was chosen for analysis such that a similar number of peptides covered each domain of mAb-D. The HX-MS data were processed with HDExaminer software (Sierra Analytics, Modesto, CA). The results are expressed in terms of mass differences for each peptide obtained by subtracting the mass of each peptide after labeling in a baseline buffer (CP control, condition b) from that of the peptide labeled in the presence of an additive (condition a):

$$\Delta m_{ab}(t) = \Delta m_a(t) - \Delta m_b(t) \quad (3)$$

where Δm_{ab} is the mass difference and Δm_a and Δm_b are the measured peptide mass increases under conditions a and b, respectively. A positive value indicates that the peptide became more deuterated under condition a relative to b; a negative value indicates the opposite. For mass difference plots, a significance criterion at 98% confidence was established by propagation of random error with the 98th percentile of the standard deviation from triplicate measurements, as described previously.[18]

Simulations of Hydrogen Exchange Kinetics. H → D chemical exchange kinetics were simulated by use of the sequence-dependent rate constants described by Englander and co-workers.[13] The experimental parameters were 298 K with the polyalanine model under the high ionic strength limit. Total HX of the peptide was simulated by summing the single exponentials for each measurable amide rate constant $k_{ch,i}$ as a function of time (t), $\sum_{i=3}^{n}(1 - e^{-k_{ch,i}t})$. In this context, of the n residues in a peptide, the ones with measurable exchange were taken to be the nonproline residues beginning at the third residue ($i = 3$). Calculations were carried out with a modified version of an Excel spreadsheet provided by the Englander laboratory at the University of Pennsylvania (http://hx2.med.upenn.edu/download.html).

Cubic Spline Interpolation. Cubic spline interpolation was performed with 300 points between minimum and maximum t values with the "natural" boundary condition, by use of Origin (OriginLab, Northampton, MA). Inversion of the spline function (i.e., determining t given Δm) was carried out numerically by use of Solver in Excel (Microsoft, Redmond, WA) with the spline coefficients.

Homology Model. A homology model of mAb-D was developed from PDB 5DK3, the crystal structure of pembrolizumab, a full-length IgG4 antibody.[19] To assign protection/deprotection effects observed in individual peptides, statistically significant differential HX was mapped onto all residues in the peptide, including prolines and rapidly back-exchanging N-terminal amides. In cases where there were conflicts between significant and insignificant effects in partially overlapping peptides, the strongest effect was applied along the entire length of the overlap. There were no cases of conflicts between significant effects of opposite sign.

RESULTS

Additives Alter Chemical Hydrogen Exchange. To determine the effect of differences in experimental solution conditions on the chemical exchange rate, the reporter peptide

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YPI was used. YPI cannot form secondary structures because it is only three residues long and contains a proline.[20] Hence, the single amide hydrogen is not expected to be protected by intramolecular hydrogen bonds. This amide undergoes slow exchange because of both the steric influence of the isoleucine side chain and its proximity to the C-terminal carboxylate. These factors make YPI useful for directly measuring the chemical exchange rate on the seconds-to-minutes time scale, similar to a PPPI reporter peptide described previously.[5]

For these studies, we investigated the exchange of YPI at both pD 6.5 and 7.4 in the presence of a variety of additives to determine if its chemical exchange rate is affected by solution conditions. As shown in Figure 1, HX of YPI is slower at pD 6.5 (Figure 1A) than at pD 7.4 (Figure 1B,C), as expected on the basis of the well-known effect of pH on chemical exchange. Additionally, panels B and C show that the addition of salt to otherwise identical buffer conditions increases the HX rate, as expected on the basis of previous work.[13] The differences in HX between the solution conditions at pD 7.4 are smaller in the presence of NaCl (Figure 1B) than in the absence of NaCl (Figure 1C) because the added NaCl mitigates differences in the ionic strengths of the solutions. The observed differences in HX illustrate how chemical exchange can be strongly influenced by these additives. At the final labeling time, measured HX of YPI in all solution conditions at both pD values converge at the same deuteration level, confirming that all of the solutions have the same D:H isotope ratio. Thus, chemical exchange exhibited by the unstructured YPI peptide is altered under certain solution conditions. Subsequently, we will show how these measurements with YPI can be used to correct for altered chemical exchange rates.

Correcting for Differences in Chemical Hydrogen Exchange. In this section, we advance an approach that can compensate for the effects of altered chemical exchange on the observed rate of HX so that the effects of differing solution conditions on protein dynamics can be directly compared. Because HX measured at the peptide level is a multiexponential process, we have found that compensating for altered chemical exchange based on rate constants and curve-fitting is unreliable (results not shown). Thus, we have selected an empirical approach based on time-scale adjustment as used previously in correction for pH.[6,7] However, unlike pH corrections, which are predictable on the basis of hydrogen-exchange theory, the effects of additives are more difficult to predict. By measuring HX with a suitable unstructured reference, such as YPI, in each of the solution conditions, we can estimate the magnitude of the effect of differences in solution composition on the chemical exchange process. On the basis of this measurement, a mathematical correction to the observed differences in HX in different solutions can be applied to compensate for the altered chemical exchange.

From the hypothesis that the change in solution conditions will have nearly equal effects on the kinetics of HX at each residue, we can derive a chemical exchange correction factor, χ_{ab} (see Supporting Information), defined in eq 4:

$$\chi_{ab} \equiv \frac{k_a}{k_b} \tag{4}$$

where k denotes a rate constant for chemical exchange under a condition of interest, a, and a baseline or reference condition b. From this relationship follows a method to identify an HX time under baseline condition b, $t_{eq,b}$, that will have equivalent chemical exchange as HX time t_a under condition a:

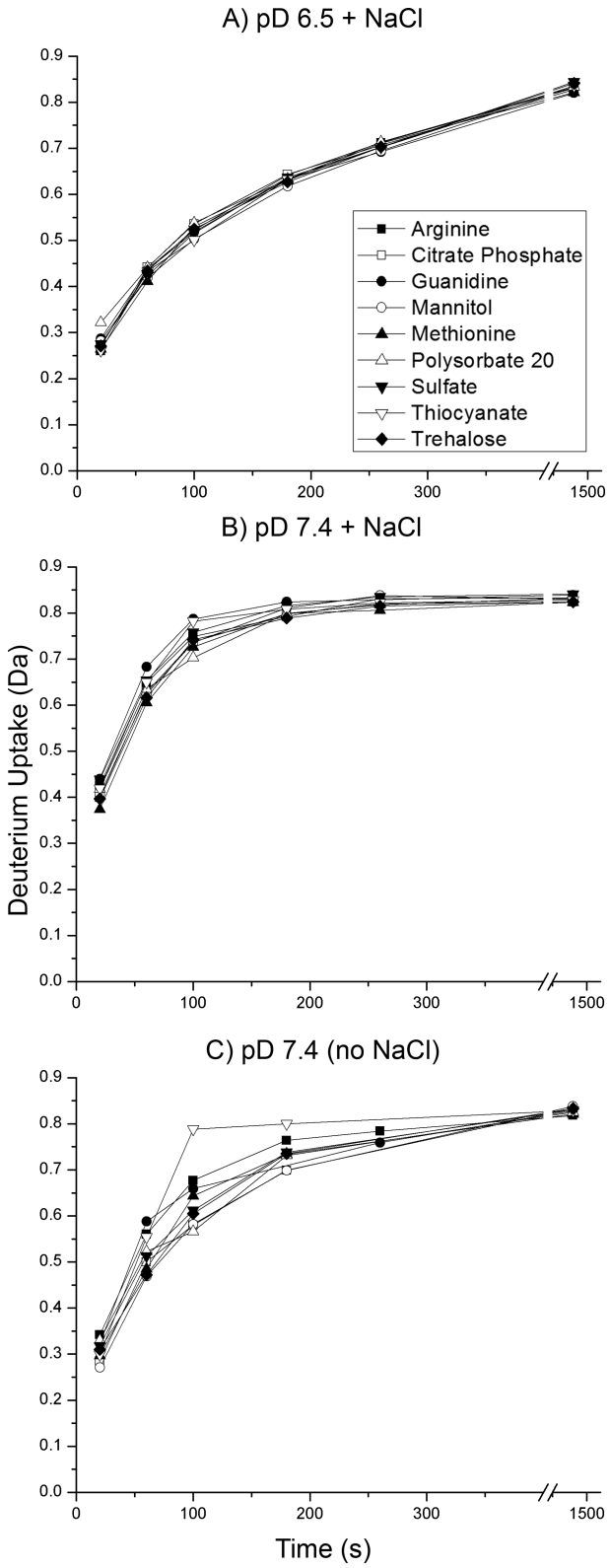

Figure 1. HX rates are substantially altered by additives, so a correction is needed. Deuterium uptake curves are shown for YPI in citrate−phosphate (CP) buffer containing the additives listed in the legend under the following conditions: (A) pD 6.5 with 150 mM NaCl, (B) pD 7.4 with 150 mM NaCl, and (C) pD 7.4 without additional salt.

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$$t_{eq,b} = \frac{t_a}{\chi_{ab}} \quad (5)$$

as detailed in Supporting Information. In the absence of any changes in protection factor (i.e., $PF_{a,i} = PF_{b,i}$), such as in an unstructured reporter peptide, the difference in HX between the conditions, obtained at these two *different* HX times, can be attributed completely to differences in chemical exchange. Thus, a value for χ_{ab} may be determined by identifying times of equal exchange in conditions a and b for amides that are unprotected.

The relationship in eq 5 also establishes a mechanism by which an HX time for baseline condition b can be identified from measurements of χ_{ab} to compensate for differences in chemical HX for the protein of interest. The difference between the HX measurements

$$\Delta m_{ab} = \Delta m_a(t_a) - \Delta m_b(t_{eq,b}) \quad (6)$$

can then be attributed to differences in protection of the protein of interest between the conditions. After establishing the empirical correction method in this section, we will demonstrate the validity of the hypothesis underlying eq 4 in the following section. This method is analogous to the time-shifting methods explored previously[7] but without knowledge of the magnitude of time shifting prior to experimentation.

The correction consists of two distinct steps, illustrated in Figures 2 and 3 and explained in greater detail in the following text. In the first step, the chemical exchange correction factor, χ_{ab}, is measured from the HX kinetics of an unstructured reporter peptide under two conditions. In the second step, the correction, based on χ_{ab}, is applied to the protein HX data by adjusting the measured HX in one of the conditions. The adjustment is obtained by interpolation with a shifted time defined by eq 4. This shift in HX time adjusts the measured HX for differences arising from chemical exchange differences between the two conditions. Finally, we illustrate this correction in the context of a protein formulation study where the goal is to identify changes in HX induced by various additives relative to a baseline condition by using a minimal set of HX measurements across many different additives. For the sake of efficiency, HX-MS by the protein in the baseline condition is measured at several different HX times, while the effect of each additive is measured only at one carefully selected HX time. However, the method is easily extended to any two-state HX-MS experiment with an arbitrary number of HX measurement times; we will expand on this point in the Discussion.

The value of χ_{ab} is determined from reporter peptide hydrogen-exchange kinetics in each solution condition. Here, we illustrate the process by applying it to a pH change, where the required correction is large and known a priori. A pD 7.00 buffer is solution condition a, and a pD 7.25 buffer is solution condition b, both at 25 °C. From standard reference data for chemical exchange in model peptides,[13] the rate constants for HX by the single amide hydrogen in YPI are $k_a = 4.6 \times 10^{-3}$ s^{-1} and $k_b = 8.2 \times 10^{-3}$ s^{-1}, yielding an anticipated χ_{ab} value of 0.56 (i.e., $10^{\Delta pH} = 10^{-0.25} = k_a/k_b = 0.56$). For changes other than pH, the χ value must be determined empirically, as illustrated in Figure 2. To obtain this empirical correction, HX is first measured at one HX time, t_a, under condition a (○ in Figure 2). Second, HX is measured under baseline condition b at several HX times (● in Figure 2) such that the measured HX values bracket the value measured for condition a. We estimate

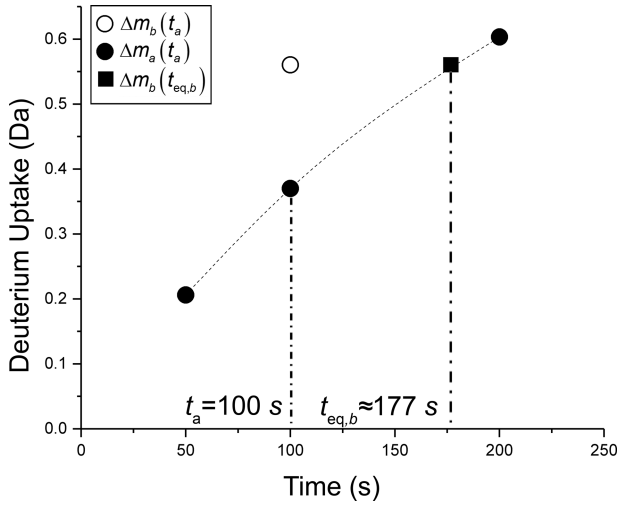

Figure 2. Establishment of a chemical exchange correction factor by use of the reporting peptide YPI. Data shown are from a simulation of HX by the YPI peptide at pD 7.00 buffer (condition b, the baseline condition, ●) and pD 7.25 buffer (condition a, ○). The dashed line is a cubic spline interpolation of the baseline condition, a. (■) HX value used for inverse interpolation to determine $t_{eq,b}$. The ratio of t_a to $t_{eq,b}$ is used to establish χ_{ab} (here, χ = 100 s/177 s = 056) to correct for differences in chemical exchange between the conditions as shown in Figure 3.

that bracketing times of $t_a/2$ and $2t_a$ will be suitable for most cases. Next, the functional form of the baseline peptide exchange under condition b is estimated with a cubic spline[21] function, f (dotted line in Figure 2):

$$\Delta m_b(t) \approx f(t) \quad (7)$$

The time to reach equivalent exchange under condition b, $t_{eq,b}$, is then estimated by inverting the cubic spline function given the HX under condition a, $\Delta m_a(t_a)$, as shown in Figure 2 (■):

$$t_{eq,b} = f^{-1}[\Delta m_a(t_a)] \quad (8)$$

In other words, this is the HX time under condition b that produces the same extent of exchange as found at HX time t_a under condition a. In this case, $t_{eq,b}$ was determined to be 177 s from eq 8, inversion of the spline function. The ratio of t_a to $t_{eq,b}$ then becomes the correction factor, χ_{ab}. Here, 100 s/177 s = 0.56, matching the expected value exactly. While in this case the ratio of the empirically determined HX times is identical to the ratio of calculated rate constants, the interpolation method could introduce error into χ due to errors introduced by estimating HX kinetics through interpolation. The use of more measurements can, of course, increase the accuracy of the spline interpolation. In this work, we chose to use the reporter peptide as an external standard, measuring HX by the peptide separately from our antibody to avoid any potential interactions between peptide and antibody. The reporter peptide can also be incorporated as an internal standard if there is no concern about interactions between the protein(s) of interest and the reporter.

Once the correction factor, χ_{ab}, has been determined, it can be used to correct HX-MS data acquired on proteins of interest under the two conditions. Figure 3 illustrates how the χ value is used to correct the HX-MS data from peptides, in this case simulated HX of unstructured Leu-enkephalin, YGGFL, also at pD 7 and pD 7.25. To correct for altered chemical exchange,

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HX is first measured for each peptic peptide at one HX time under the condition of interest, condition a, $\Delta m_a(t_a)$, and a minimum of three bracketing times under the baseline condition, b, as shown in Figure 3A. It is important to note that these HX labeling times do *not* need to be the same ones that were used to determine χ_{ab}. A cubic spline function is again found to fit the condition b data. The YPI-derived value of χ_{ab} is now used to determine $t_{eq,b}$ by rearranging eq 4 as follows:

$$t_{eq,b} = \frac{t_a}{\chi_{ab}} \tag{9}$$

Next, the cubic spline interpolation (eq 7) is used to determine the extent of HX in baseline condition b at $t_{eq,b}$: that is, $\Delta m_b(t_{eq,b}) \approx f(t_{eq,b})$ (see Figure 3B, ■). This value, $\Delta m_b(t_{eq,b})$, becomes the corrected deuteration of the peptide under condition b. The magnitude of the difference in HX between the conditions that is attributable to altered protection is then

$$\Delta m_{ab} \equiv \Delta m_a(t_a) - \Delta m_b(t_{eq,b}) \tag{10}$$

This is the difference shown between ○ and ■ in Figure 3C. As a guide to the eye, it is useful to translate this corrected value back to t_a for easier comparison with $\Delta m_a(t_a)$ (see Figure 3C, ▲). It can also be instructive to examine the magnitude of the required correction, that is, the difference between HX measured under condition b at the HX time for condition a and at the time corrected by χ_{ab}, $\Delta m_b(t_a) - \Delta m_b(t_{eq,b})$. In this example, we simulated a peptide with no protection. Therefore, the corrected differential HX should be zero since the differences in HX arise only due to effects on the rate of chemical exchange. The slight differential HX apparent in Figure 3C (○ vs ▲) is an error introduced by estimating HX kinetics with a cubic spline function.

Testing the Hypothesis of Uniform Effects on Chemical Exchange. The derivation of eq 4, as detailed in Supporting Information, is based on the hypothesis that the effects on chemical exchange are, at least to a first approximation, uniform effects; that is, χ_{ab} is uniform for all residues. While convenient if true, this assumption must be tested. Here we advance a method to test the reliability of the approximation and present the results of that test. If the hypothesis is valid in a system with experimental data in which it is known that the protection factor is identical between conditions (i.e., $PF_a = PF_b$), then differential HX will be near zero following the correction because the only changes in HX are due to an altered rate of chemical exchange. Here we eliminate protection by using unstructured peptides obtained by predigestion of mAb-D with pepsin such that $PF_a = PF_b = 1$. We selected peptides less than 10 residues in length, short enough to minimize the formation of secondary structure.[22,23] However, since the amides in unstructured peptides exchange more quickly than amides in structured proteins, it was necessary to lower the pD to 4.5 to bring HX into a range measurable with standard HX methods (i.e., seconds to hours). Additionally, low pD requires the use of a reporting peptide that exchanges more quickly than YPI. Thus, for this part of the work, the peptide FKPGI was used as a reporting peptide. In addition to being too short to form two helical turns, FKPGI contains a helix-breaking proline and other residues with poor helical propensity.[20] The peptide also contains one slowly exchanging and one rapidly exchanging amide hydrogen, G and I, respectively,[13] making it useful for measuring exchange over a wide range of time scales and solution conditions.

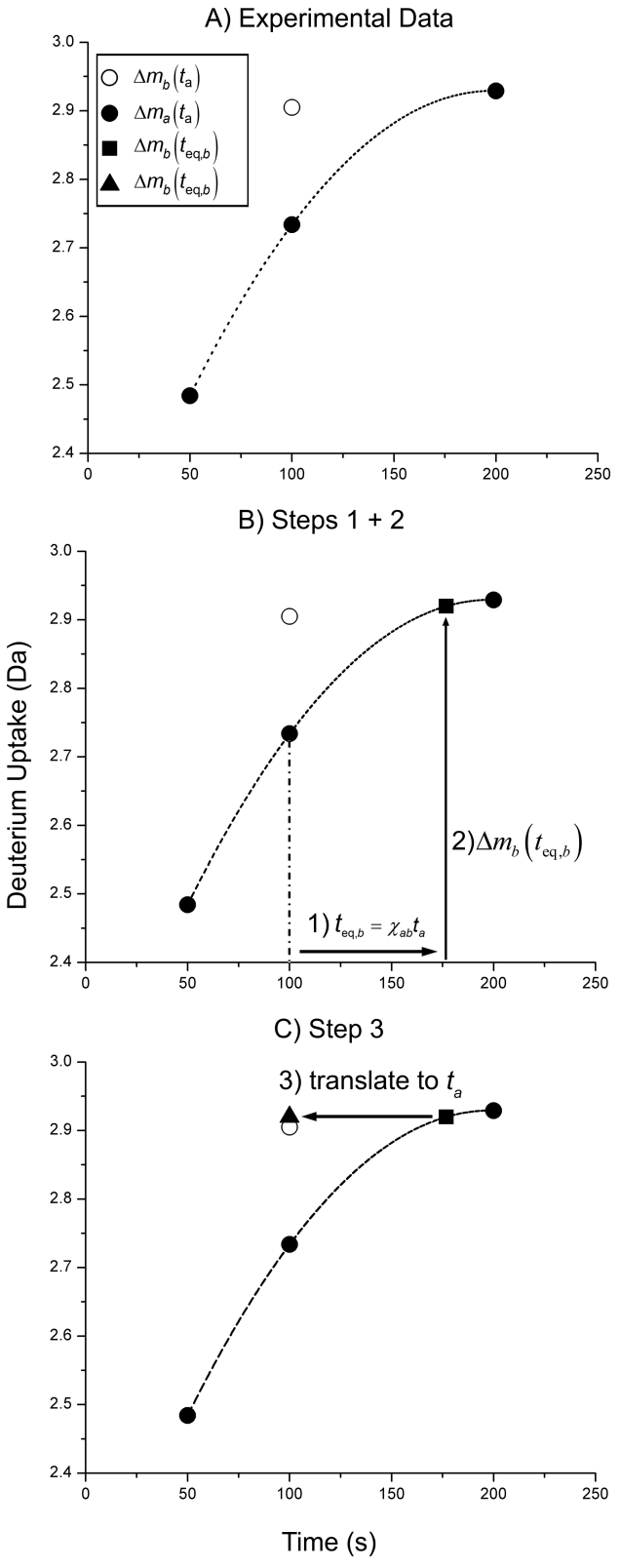

Figure 3. Outline of the correction procedure for peptic peptides. (A) Data from a simulation of HX by the three slowly back-exchanging amides of YGGFL peptide at pD 7.00 (condition b, ●) and at pD 7.25 (condition a, ○). The dashed line is a cubic spline interpolation of the baseline condition, b. (B) ■ represents a point calculated by use of the established chemical exchange correction factor (χ_{ab}, determined to be 0.56 in Figure 2). (C) χ_{ab} is used to calculate ▲, a corrected HX value $\Delta m_b(t_{eq,b})$, translated to t_a as a guide to the eye.

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The procedure for correction uses a determination of χ with FKPGI, followed by application of the correction to the peptides. Figure 4 shows the measured HX of FKPGI reporter

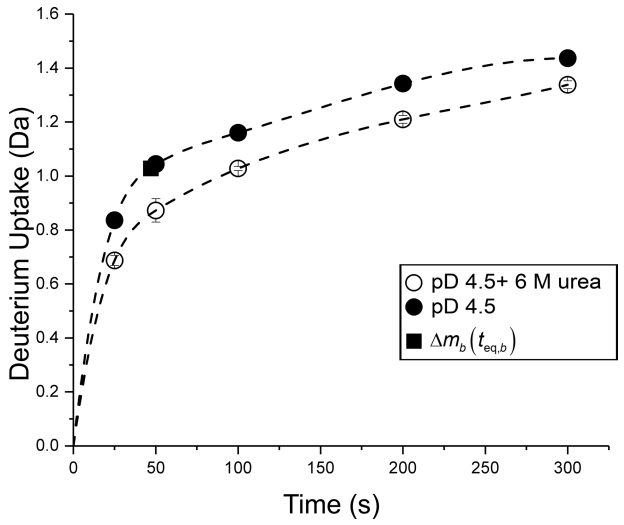

Figure 4. Chemical HX rates are substantially different at pD 4.5 in the presence of 6 M urea. In this measurement, pD 4.5 CP buffer is condition b, and pD 4.5 CP buffer with 6 M urea is condition a. A t of 100 s with bracketing HX times of 25, 50, and 200 s was used to estimate $t_{eq,b}$ of 47 s (■), as described previously, yielding χ = 2.12. ■ represents the point on the pD 4.5 interpolated curve that reaches the same level of exchange as 100 s of HX time in pD 4.5 + 6 M urea. The dashed lines are cubic spline interpolations. χ = 2.12 was used to correct deuterium uptake of peptides in Figure 5. Error bars, where large enough to be visible, are sample standard deviations from triplicate measurements.

peptide in CP buffer at pD 4.5 (baseline condition b, ●) and in the same buffer with the addition of 6 M urea (condition a, ○). As the change in condition between buffer alone and buffer with urea was too extreme for a bracketing HX time of $t/2$ to be sufficient, an additional HX time of $t/4$ was used. Figure 4 demonstrates that HX rates are substantially slower in the presence of urea. Presumably, urea reduces the apparent acidity of the amide hydrogens, as noted previously.[3] Under these conditions, the chemical exchange correction factor χ_{ab} was determined to be 2.12 by the approach described in the previous section as shown in Figure 4: t_a of 100 s was used, and $t_{eq,b}$ of 47 s was determined from 100 s/2.12 = 47 s (■). To test the uniformity of the correction, we applied it to peptides other than the reporter itself. HX was measured for unprotected peptides at 100 s of labeling in 6 M urea (condition b) and at 25, 50, 100, and 200 s of labeling in the absence of urea (condition a). The χ value of 2.12 was used to correct for differences in chemical exchange rates as described in the previous section. Figure 5 shows the differential HX before (left side) and after correction (right side). The large nonzero values before correction arise because of the strong effect of urea on chemical exchange.[3] After application of the correction, the differential HX is near zero, within the experimental error limits. These results confirm the hypothesis that the effect of the difference in conditions is approximately uniform for all residues in the peptides tested. These results suggest that, at least in these cases, any specific interactions between certain residues and solutes, if present, are insignificant compared to the global effects on chemical exchange.

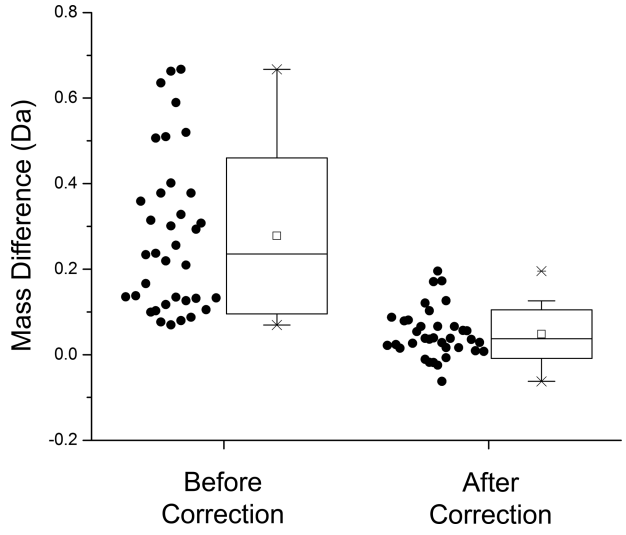

Figure 5. Effect of 6 M urea was approximately uniform for unstructured peptides. Differential HX between unprotected peptides undergoing HX under differing solution conditions are shown before and after correction by the method described in the text. The box and data plots represent the difference in hydrogen exchange between predigested mAb-D peptides labeled for 100 s at 25 °C at pD 4.5 in CP buffer with and without 6 M urea. (□) mean; (×) 1st and 99th percentiles. Before correction, substantial differences exist in differential HX between conditions, that is, the mass differences are not zero. The correction procedure using the chemical exchange correction factor χ_{ab} = 2.12 (see Figure 4) brings differential HX to near zero, demonstrating that chemical exchange effects can be approximated as uniform.

Application of Correction to a Monoclonal Antibody Formulation Screening Study. In this section, we demonstrate the application of the chemical exchange correction method in a formulation screening study on mAb-D, an IgG4 monoclonal antibody. HX was measured for mAb-D in 5 mM CP buffer alone or including each one of eight additives, at pD 6.5, at pD 7.4, and at pD 7.4 in the presence of 150 mM NaCl. After the reaction was quenched at different HX times, peptic peptides were generated and analyzed by LC−MS to determine deuterium uptake for a subset of 40 peptides covering each domain of mAb-D with a similar number of peptides. A chemical exchange correction factor was established as described previously for each different solution condition by use of the YPI data shown in Figure 1 at t = 100 s, with 5 mM CP buffer at the corresponding pD serving as condition a in all cases. The χ values are shown in Table 1, and the resulting magnitudes of correction $[\Delta m_b(t_a) - \Delta m_a(t_{eq,b})]$ for each of the 40 peptides are shown in aggregate in Figure 6. The correction values range from 0.05 Da (for 0.8 M mannitol), to −0.6 Da (for 0.3 M sodium thiocyanate). Thus, both positive and negative corrections were required, and the magnitude of correction depended strongly on the solution condition. Additives such as trehalose, polysorbate 20, and mannitol had only a minimal effect on chemical HX rates (see Figure 1). Consequently, in the presence of these additives, χ was near unity and thus only a negligible correction was required (as shown in Figure 6). HX studies could be conducted for such excipients without correction for differences in HX rates due to the additive. On the other hand, arginine, guanidine, and thiocyanate substantially increased the chemical HX rates (see Figure 1) and consequently have χ values much less than unity

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Table 1. Chemical Exchange Correction Factor Values Determined from Hydrogen Exchange of YPI

5 mM CP buffer + additive	pD 6.5 + NaCl[a]	pD 7.4 + NaCl[b]	pD 7.4[b]
none	1.00	1.00	1.00
arginine (0.3 M)	1.00	0.90	0.69
guanidine (0.3 M)	1.00	0.77	0.69
mannitol (0.8 M)	1.11	0.98	1.05
methionine (0.2 M)	1.05	1.10	0.88
polysorbate 20 (0.05%)	0.97	1.01	0.92
sulfate (0.3 M)	1.05	0.85	0.87
thiocyanate (0.3 M)	1.08	0.86	0.6
trehalose (0.4 M)	1.05	1.02	0.94

[a]Chemical exchange correction factors were determined with 5 mM CP buffer as the baseline condition, at 100 s of HX labeling time, by use of data shown in Figure 1. [b]Chemical exchange correction factors were determined with 5 mM CP buffer as the baseline condition, at 60 s of HX labeling time, by use of data shown in Figure 1.

and require substantial negative corrections (Figure 6). In addition to direct effects on chemical exchange, such additives also have stronger effects on chemical exchange here because their addition has a larger effect on the ionic strength of the solution, previously shown to alter HX rates.[13] The addition of 150 mM NaCl seems to mitigate some of the ionic strength effects caused by the additives, as is evident by χ values becoming closer to unity in the presence of 150 mM NaCl.

To further illustrate the application of exchange correction to a mAb formulation study, Figure 7 shows differential HX plots before and after correction for mAb-D formulated with the eight different additives in 5 mM CP buffer at pD 7.4 without additional sodium chloride. The baseline state (i.e., state b) is mAb-D in 5 mM CP buffer at pD 7.4 without additional sodium chloride. The pH 7.4 with no added salt condition was selected because it had the greatest variability in chemical exchange rates for the YPI reporter (see Figure 1 and Table 1). In general, there were fewer statistically significant differences in HX after the chemical exchange correction was applied. The post-correction results reveal the extent to which these

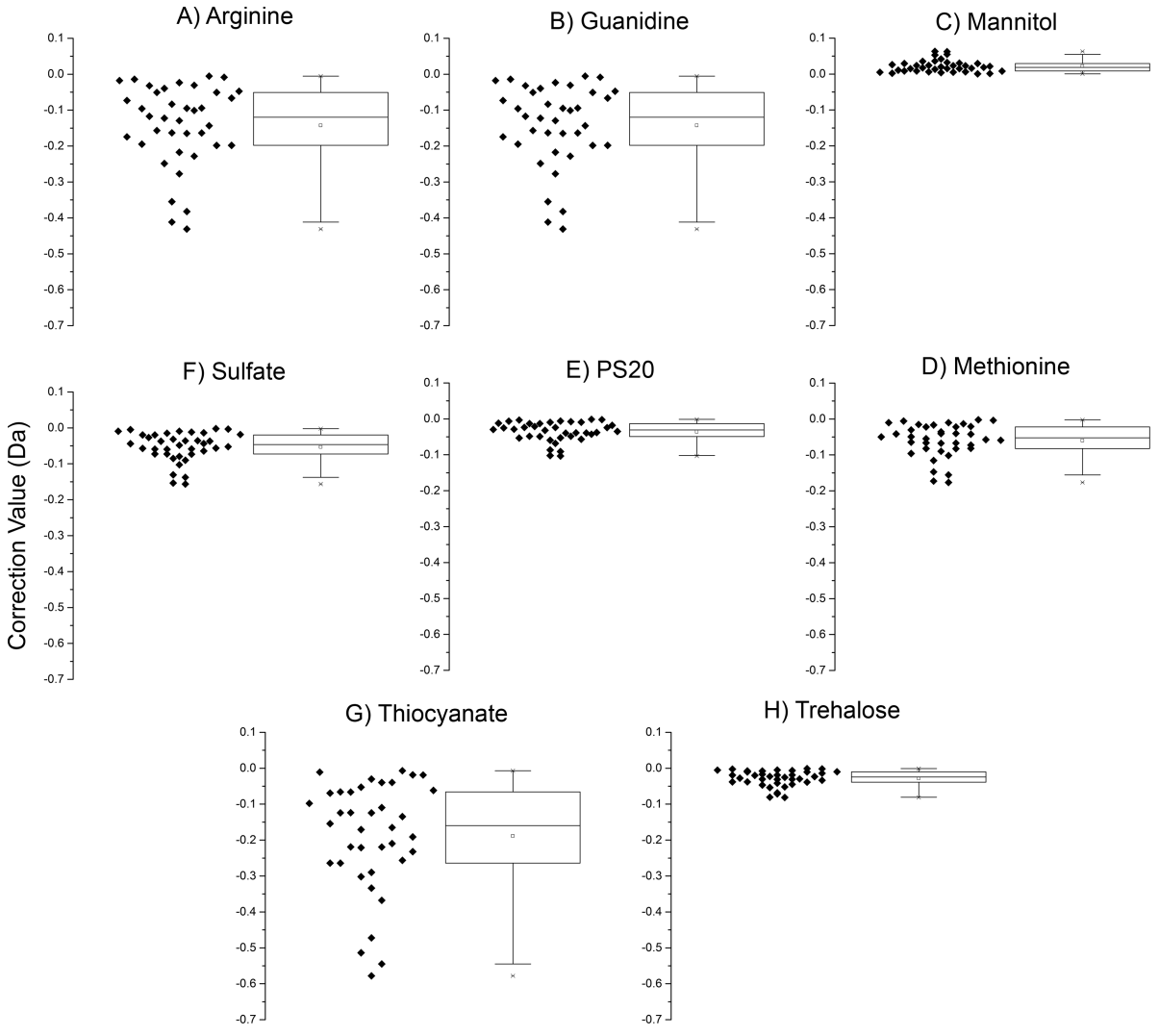

Figure 6. Substantial corrections were required to adjust for altered chemical exchange for mAb-D peptides in some formulations. The plots display the magnitudes of correction for the subset of mAb-D peptides examined at pD 7.4 without additional NaCl by use of the chemical exchange correction factors established in Table 1. The values represent the portion of differential deuterium uptake that can be attributed to the difference in chemical exchange rate between CP buffer alone and CP buffer containing (A) arginine, (B) guanidine, (C) mannitol, (D) methionine, (E) polysorbate 20, (F) sulfate, (G) thiocyanate, or (H) trehalose.

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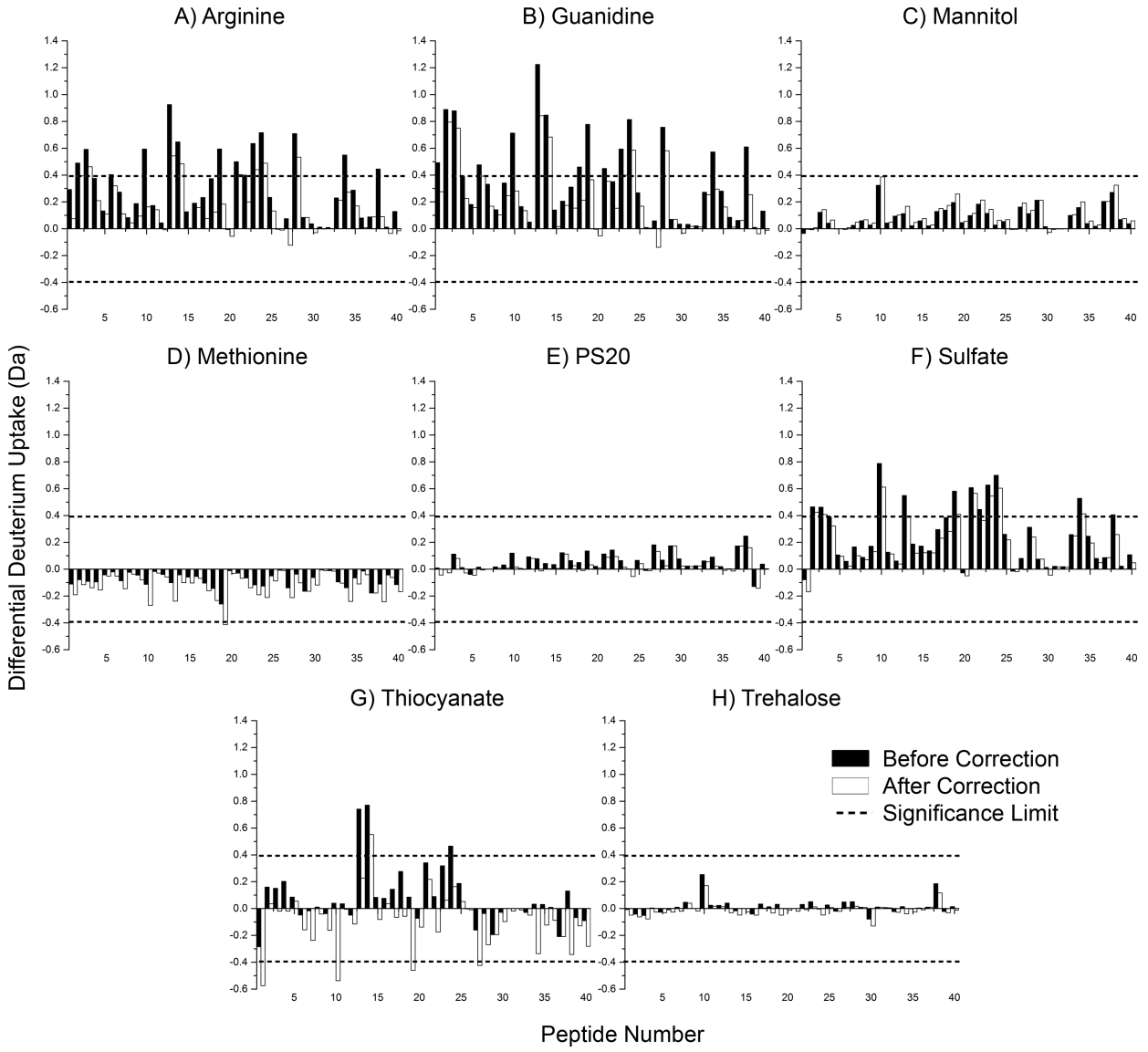

Figure 7. Corrections can alter differential HX analysis of some mAb-D peptides in some formulations. HX was quenched after 125 s of exchange. Difference plots exhibit the differential exchange by mAb-D formulated at pD 7.4 in CP buffer (no NaCl) containing (A) 0.3 M arginine, (B) 0.3 M guanidine, (C) 0.8 M mannitol, (D) 0.2 M methionine, (E) 0.05% polysorbate 20, (F) 0.3 M sodium sulfate, (G) 0.3 M thiocyanate, and (H) 0.4 M trehalose (condition a) vs 5 mM CP buffer (no NaCl) without additional additives (condition b). Data are shown before and after correction for differences in chemical exchange rates according to the chemical exchange correction factors in Table 1. Forty peptides covering all domains of mAb-D, numbered sequentially from the N terminus of the heavy chain to the C terminus of the light chain, are shown. The dashed lines represent the significance limit at 98% confidence, as defined in the text.

formulations altered the backbone flexibility of mAb-D relative to the reference condition. Arginine, guanidine, and sulfate induced moderate increases in HX in specific regions of mAb-D. Thiocyanate induced both increases and decreases in HX. The remainder of the additives had only negligible effects on the HX of mAb-D. The correlation of these effects with the physical stability of mAb-D is the subject of a separate study.[26]

For further illustration, the HX differences for mAb-D in the thiocyanate formulation are shown, mapped onto a homology model in Figure 8 both before and after correction. With the uncorrected HX data, it appears that multiple regions had significantly increased HX rates (see Figure 8A). Additionally, several regions appear to be unaffected. However, based on the corrected HX data, these regions actually became significantly protected. In other words, some of the thiocyanate effects would have been attributed to changes in protection, when in reality the majority of the effects were due to differences in chemical exchange rates between conditions. The converse is also true: conditions where the differential HX was slight become significant after correction, because the change in HX rates are compensated for by the change in protection. The most notable increases in backbone dynamics caused by thiocyanate are localized to the C_H2 aggregation hotspot region that we have previously identified in IgG1 mAbs.[15,24] In addition, this work reveals several regions of IgG4 mAbs that became protected in the V_H, C_H1, C_H3, and V_L domains in the presence of thiocyanate. However, the limited set of 40 peptides analyzed here does not comprehensively cover the entire antibody sequence.

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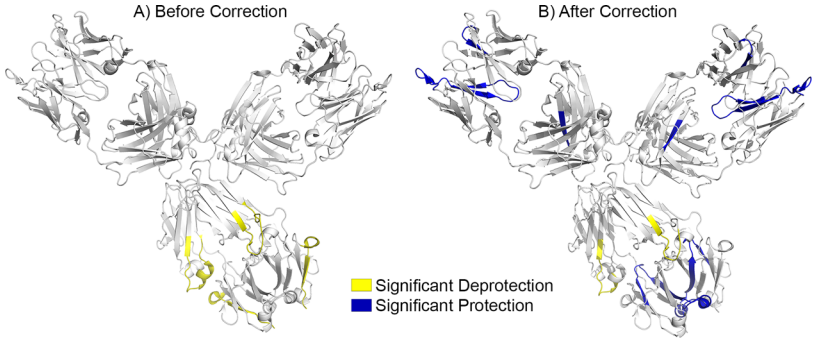

Figure 8. Homology model of mAb-D showing effects of thiocyanate on the local flexibility of mAb-D as measured by HX-MS at pD 7.4. Apparent differences are shown (A) before correction for chemical exchange effects of the additive and (B) after correction. Regions shown in yellow exhibited significant increases in hydrogen exchange, and regions in blue exhibited significant decreases in hydrogen exchange. The mAb homology model is based on PDB 5DK3.[19]

DISCUSSION

Effects of Additives on Chemical Exchange. It is interesting to note that there seem to be pH-dependent effects. For example, comparing the values in Table 1 for the cases where additional NaCl was added, it seems that guanidine, sulfate, and thiocyanate have little effect on chemical exchange at pD 6.5 but a strong effect at pD 7.4. This is puzzling since it seems reasonable to expect that the effects of these additives on chemical HX would be uniform over this narrow pH range. All of these solutions are far from ideal behavior because of high concentrations of additives and high ionic strength. It is possible that the one-unit pH change has some unexpected effect on secondary solution properties such as ion pairing or water autoprotolysis. In addition, the pH electrode responds to proton activity, not concentration, while the rate of chemical exchange depends on hydroxide ion concentration. Alternatively, these discrepancies may have arisen due to differences in the subtleties of the mixed buffer system or its preparation such as pH adjustment. While understanding the general properties of additives on chemical exchange kinetics is out of the scope of the present work, these unexpected results underscore the need for methods to detect altered chemical HX and correct for it.

Generalizability of the Reporter Peptide Approach. We have presented this method for chemical exchange correction within the context of a formulation study, where the objective is to assay a wide range of conditions with a minimal number of HX measurements. However, the reporter peptide chemical exchange correction method can be extended to more traditional HX experiments in which many HX labeling times are used. The interpolation method illustrated in Figure 3 can readily be extended to consecutive groups of three HX labeling times such that each HX measurement, except either the first or last HX measurement, could be adjusted by interpolation between its bracketing measurements. This extension will be the subject of a subsequent publication.

Use of Reporter Peptide in Longitudinal Hydrogen Exchange. A persistent problem in large-scale HX-MS measurements is that HX kinetics are sensitive to subtle environmental changes such as temperature drift or minor variations in pH of labeling conditions between labeling buffer lots. This is particularly problematic when HX-MS experiments span weeks to months. It is generally recognized that when HX-MS data are acquired months apart or at different facilities,[25] they cannot be easily compared without tight controls. Our work here suggests a potential solution to these problems. An unstructured reporter peptide, such as YPI, can be incorporated in all HX-MS experiments. Examination of the HX kinetics of the reporter peptide could be used to detect the presence of drift in the chemical exchange rate over time. The reporter could be incorporated internally or externally. An external reporter would be useful for tracking interday variability, for example, while an internal reporter could track run-to-run variability. If chemical exchange drift is detected, the interpolation method described here could be applied to adjust HX-MS measurements for compatibility with other data sets of interest. Such a correction, using a PPPI reporter, was suggested in earlier work,[5] but that correction requires the use of a complex model of the HX process. Our method, in contrast, uses a much simpler empirical correction. This extension will also be the subject of a subsequent publication.

CONCLUSIONS

A barrier to the use of HX-MS in situations where changes to solution conditions are required has been that differences in solution composition can alter the chemical exchange rates. We have developed and experimentally validated an empirical strategy to help overcome discrepancies in HX measurements that are caused by these differences in chemical exchange rates. The method involves measuring differences in HX rates with a reporter peptide and then applying a correction, based on those measured differences in HX rates, for peptides derived from the protein of interest. This correction allows for direct comparisons between measurements of a protein prepared in differing solution conditions; for example, this technique can be used as a structurally resolved formulation screening technique with protein therapeutic candidates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01396.

Additional text and equations giving a mathematical derivation and justification of the chemical exchange correction method (PDF)

AUTHOR INFORMATION

Corresponding Authors

*(D.B.V.) E-mail volkin@ku.edu.
*(D.D.W.) E-mail dweis@ku.edu.

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ORCID

David D. Weis: 0000-0003-3032-1211

Present Address

[‖](B.J.M.) AbbVie, 1 N. Waukegan Rd., North Chicago, IL 60064.

Notes

The authors declare no competing financial interest.

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