HHS Public Access

Author manuscript

*Anal Chem*. Author manuscript; available in PMC 2021 May 09.

Published in final edited form as:

*Anal Chem*. 2020 July 21; 92(14): 9830–9837. doi:10.1021/acs.analchem.0c01328.

# Imidazolium Compounds as Internal Exchange Reporters for Hydrogen/Deuterium Exchange by Mass Spectrometry

**Taylor Murphree**, **Clint Vorauer**, **Marie Brzoska**, **Miklos Guttman***

Department of Medicinal Chemistry; University of Washington, Seattle, WA 98195

## Abstract

Hydrogen deuterium exchange mass spectrometry (HDX-MS) is a powerful tool for protein structure analysis that is well suited for biotherapeutic development and characterization. Because HDX is strongly dependent on solution conditions; even small variations in temperature or pH can have a pronounced effect on the exchange kinetics that can manifest in significant run-to-run variability and compromise reproducibility. Recent attention has been given to the development of internal exchange reporters (IERs), which directly monitor changes to exchange reaction conditions. However, the currently available small peptide IERs are only capable of sampling a very narrow temporal window and are understood to exhibit complex solution dependence in their HDX kinetics. Here we demonstrate the use of imidazolium carbon acids as superior IERs for HDX-MS. These compounds exhibit predictable exchange behavior under a wide variety of reaction conditions, are highly stable, and can be readily modified to exchange over a broad temporal window. The use of these compounds as IERs for solution based HDX-MS could considerably extend the utility of the technique by allowing for more robust empirical exchange correction thereby improving reproducibility.

## INTRODUCTION

Hydrogen-deuterium exchange mass spectrometry (HDX-MS) is a widely utilized technique for the rapid assessment of protein structure and local dynamics. By observing deuterium uptake of back-bone amides via mass spectrometry it is possible to gain information about the local structural stability throughout a protein, map protein-protein and protein-ligand interactions, and monitor allosteric effects[1]. Recent advancements have made HDX-MS amenable to the analysis of large molecule biopharmaceuticals and the FDA now recommends HDX-MS as a tool for establishing “equivalence” between existing biologics and emerging biosimilars[2]. Despite the many advantages of HDX-MS for biophysical evaluation, poor reproducibility has limited its widespread use. The most notable issues pertaining to poor reproducibility are inconsistent sample handling and variation in the exchange reaction conditions[3].

The development of automated HDX-MS systems has made significant progress towards minimizing variation in sample handling, but even with these systems variation in deuterium uptake can vary by as much as ± 5.4% for a given peptide observed in different

*Correspondence: mguttman@uw.edu.

laboratories[4]. This persistent variability is a consequence of the strong solution dependence on backbone amide exchange. Even a small change in the reaction conditions (i.e., temperature, deuterium content, or pH) can have a pronounced effect on the observed exchange[5]. Furthermore, solution conditions can drastically affect both the protein conformational dynamics and the rate of amide intrinsic exchange ($k_{ch}$, the exchange rate of an unstructured amide) differentially. This means that without some means of accounting for altered reaction conditions, any difference in deuterium incorporation observed over two separate experiments could be due to variation in protein dynamics, amide intrinsic exchange, or both[6]. As a result, correcting for variation in reaction conditions has been a focal point among groups trying to improve the reproducibility of HDX-MS[5,6].

Another challenging aspect of studying proteins by HDX-MS is that the exchange of backbone amides can occur over a massive time window, from $10^{-2}$ s to $10^{10}$ s under standard conditions[7]. Performing HDX experiments over this time scale is impractical, not only from a logistical standpoint, but also because many proteins may not be stable enough in solution beyond $10^4$ s. Incomplete coverage of this large time window can significantly limit the ability of HDX-MS to monitor protein dynamics and discern structural perturbations. To overcome this limitation, techniques have been developed to artificially expand the time window for HDX-MS experiments, most often accomplished through the preparation of multiple HDX reaction buffers at different pH[8,9]. These approaches attempt to modify HDX reaction conditions to accelerate or decelerate $k_{ch}$ while minimizing the impact on the conformational dynamics of the protein being studied. This approach, although informative, can provide misleading results if measures are not taken to accurately monitor HDX reaction conditions.

Small peptide internal exchange reporters (IERs) have been developed to monitor HDX reaction conditions. These compounds lack secondary structure, and feature a relatively slow exchanging c-terminal amide. When added to the reaction mixture with the analyte protein, these small peptides undergo exchange under the same conditions as the analyte. Thus, variation in the deuterium uptake by the small peptide IER from run to run can be used to correct for variation in the deuterium uptake by the analyte protein[5,6]. Although these compounds can improve reproducibility, they have limited benefit. For instance, peptide IERs, such as YPI, become fully deuterated within a few minutes at neutral pH and 25°C, making it ineffective for monitoring exchange at longer time points. Additionally, it was recently reported that C-terminal amides exhibit complex pH dependent exchange behavior within physiologically relevant systems[10]. The limitations of existing small peptides IERs encouraged us to explore alternative chemistries capable of extending the utility of IERs in HDX-MS. In this work we demonstrate the capability of imidazolium-based compounds to act as superior IERs for HDX-MS.

## RESULTS AND DISCUSSION

### Exploring different chemistries to find a suitable exchange standard

The currently available small peptide IERs (i.e. the tripeptide: YPI and the tetrapeptide: PPPI) rely on the observed exchange at the C-terminal amide to correct for variation in HDX reaction conditions[5,6]. To better understand the exchange kinetics of the C-terminal amide,

the HDX of YPI was probed under a variety of conditions by time resolved NMR. The spectra show the loss of the isoleucine amide resonance for YPI, over time, as a result of H-D exchange with the buffered NMR solvent ($D_2O$) (Fig. S1). The HDX kinetics were monitored at different pD values, and in all cases the signal decay could be fit to a single exponential to reveal the exchange rate (Fig. 1A). The change in the log rate versus pD for YPI shows a linear relationship, but interestingly, the slope of the fit for these data is only 0.195 (Fig. 1B). This slope deviates significantly from the notion that the intrinsic exchange of backbone amides exhibits a first order relationship with the concentration of hydroxide in solution above the $pH_{min}$ (i.e. the slope of the linear fit for the log rate versus pH above the $pH_{min}$ is expected to be near 1)[11]. The shallow slope suggests, that within the observed pH range, HDX at this site cannot be adequately described by a simple base-catalyzed mechanism. These findings are consistent with c-terminal amides transitioning to an acid-catalyzed mechanism at higher pH compared to other amides[10]. This behavior complicates empirical exchange correction of data collected at different pH values. pH correction below pH 7 by peptide based reporters would likely benefit from C-terminal capping, as this would reduce the contribution of acid-catalysis to the observed exchange. However, the fast exchange rate for unprotected amides will still render peptide based IERs suitable only for measuring very fast time scales (<10 s).

## Identifying a suitable IER with linear pH dependence

Amide proton exchange can acid, base, or water-catalyzed, but above pH 5 amide exchange is predominantly base-catalyzed and the intrinsic exchange rate correlates linearly with $[OH^-]$[12]. We sought to identify alternative chemistries that could exhibit “backbone amide-like” pH dependent HDX behavior across this relevant pH range (5 – 8) and will cover time points relevant to solution HDX-MS studies (i.e. $10^1$-$10^4$ s at neutral pH and 25°C). From extensive screening of various compounds, it was determined that imidazolium containing compounds such as 1,3-dimethylimidazolium showed great promise as IERs for HDX-MS studies. HDX at the C-2 position has been thoroughly investigated and is understood to proceed through base-catalyzed hydrogen transfer followed by deuteration of the anionic C-2 imidazol-2-yl carbene (Fig. 1D)[13]. A similar chemistry has been used to probe the accessibility of histidine through observing the HDX of the C-2 present in the imidazole sidechain[14]. However, imidazole by itself is not viable as an IER, because the HDX rate is strongly dependent on the protonation state of the amines[15].

To test the potential of imidazolium derivatives to act as IERs for HDX-MS the pH dependent exchange behavior of 1,3-dimethylimidazolium was probed by time resolved NMR (Fig. 1C). The comparison revealed that the slope of the change in the log rate as a function of pH is nearly 1 (m=1.05) across a range from pH 6–8 (Fig. 1B), and therefore the exchange at the C-2 proton is first order with respect to hydroxide concentration. Unfortunately, the overall exchange rate was too slow to be useful as a reporter within the typical time window used for HDX-MS experiments ($t_{1/2}$ ~ 30 hours at pD 7.02 and 25°C). Fortunately, conjugated azolium ions are notable for being highly efficient in the transmission of substituent effects to groups bonded to the C-2 positon[16]. This observation suggested that it would be possible to accelerate HDX at the C-2 through the addition of electron withdrawing substituents to the ring system.

A series of C-5 substituted 1,3-dimethylbenzimidazolium derivatives were synthesized and the pH dependence of exchange was measured by NMR (Fig. 2). We focused mainly on three variants: “TM68”, “TM65”, “TM85” (Fig. 2A–C). TM85 contains a highly electron withdrawing nitro group and exchanges relatively quickly with a rate of $1.59 \times 10^{-2}$ $s^{-1}$ ($t_{1/2}$ = 41 s) at pD 7.16. TM65 contains a less electron withdrawing ester group and exchanges around 10-fold slower than TM85 with a rate of $2.38 \times 10^{-3}$ $s^{-1}$ ($t_{1/2}$ = 4.8 min) at pD 7.23. The slowest compound TM68 contains a weakly electron donating C-5 methyl group. The addition of this group results in a nearly 100-fold reduction in the rate of exchange compared to TM85, with a rate of $1.25 \times 10^{-4}$ $s^{-1}$ ($t_{1/2}$ = 1.5 hrs) at pD 7.19. Given the large variation in the exchange rates for these three compounds, ample coverage of the typical HDX-MS time window observed can be achieved. For each compound there was a clear linear relationship between the reaction pD and the log of the exchange rate with a slope of 1 (Fig. 2D). Lastly, the HDX of the three benzimidazolium compounds was also probed at low pH (approx. pD 3.0). For TM85, TM68 and TM65 we observed negligible C-2 exchange (Fig. 2A–C) even after a few hours. In contrast, the YPI showed considerable exchange within tens of minutes at the same pH (Fig. 1A). Therefore, the benzimidazolium compounds will undergo far less back-exchange under HDX-MS quench conditions than small peptides.

We also tested a saturated 1,3-dimethylbenimidazolium-5-methyl ester derivative where positions 3a,4,5,6,7 and 7a were hydrogenated (TM91). This compound exhibited extremely slow C-2 exchange with a rate of $8.45 \times 10^{-6}$ $s^{-1}$ ($t_{1/2}$ = 22 hrs) at pD 8.04 (Fig. S3). While this compound may have been suitable for probing long time scales, we found that the ester was rapidly hydrolyzed at both high and low pH. The hydrolyzed product, containing a free carboxylic acid, had altered exchange kinetics and was therefore not considered further. NMR spectra confirmed that this type of hydrolysis was unique to TM91 as none of the other compounds showed any form of degradation under any condition tested even after extensive incubation (Fig. S5).

In the course of this study we observed a very strong correlation between the rate of exchange and the C-2 proton chemical shift measured by NMR (Fig. 2E). This relationship strongly suggests that the change in NMR chemical shift is proportional to the change in the rate of base-catalyzed exchange. As the imidazolium HDX must proceed through base-catalysis, it is reasonable that the electron withdrawing/donating effects directly and predictably alter the relative acidity of the C-2 proton, and therefore the observed rate of HDX. Taken together, this study demonstrates that imidazolium compounds exchange exclusively through a base-catalyzed mechanism under conditions relevant to HDX-MS and the structures can be tuned to achieve exchange over broad time scales.

## Temperature dependence on exchange kinetics

Temperature is another potential source of day to day and lab to lab variability for HDX-MS studies. Although large variations in temperature are unusual within climate-controlled lab spaces, it is understood that the rate of back-bone amide exchange is exponentially related to the temperature of the exchange reaction[11]. Therefore, even a small change in temperature could have an impact on the observed deuterium uptake by a protein. The change in the rate

of amide intrinsic exchange as a function of temperature is a consequence of the temperature dependence of the equilibrium ionization of water and is therefore predictable within physiologically relevant boundaries[17]. To assess how temperature influences HDX of imidazolium compounds, we observed the exchange of TM85, TM68, and TM65 at temperatures from 12 to 40 °C while maintaining constant solution pH, ionic strength, and solvent composition (Fig. 3A). In all cases there was a linear relationship between the natural log of the exchange rate [$\ln(k_{ex})$] and the inverse temperature (1/K). Using the Arrhenius equation the activation energies of TM85, TM68, and TM65 in the PBS buffer were 27.54, 29.44 and 30.84 Kcal/mol, respectively. We note that the methyl ester present in compound TM65 did not undergo any measurable hydrolysis even after an hour at 40°C.

## Dependence of ionic strength on the exchange kinetics

The rate of amide intrinsic exchange is influenced by electrostatic interactions with neighboring groups and with solvated ions. These interactions are apparent from the complex exchange behavior of amides in salt solutions[18,19]. For example, it is understood that the addition of sub molar quantities of neutral salts, such as sodium chloride, to an aqueous buffered solution (i.e phosphate, citric acid, ect..) results in a change in the activity of water, which can be observed as a decrease in solution pH[20,21]. To test how ionic strength influences HDX of benzimidazolium compounds the HDX of TM85 was measured as a function of solution ionic strength (*i*) from 0 to 500mM NaCl, representing the limits that could be measured by NMR (Fig. 3B). The small decrease in the observed HDX rate at higher *i* is consistent with the theoretical rate calculated by accounting for the effects of salt on the ionization of the reaction buffer. This behavior is highly desirable for an IER because it suggests that the kinetics of exchange are more strongly dependent on the ionization of the solvent than by interactions with dissolved salts. In other words, the salt dependent exchange behavior of benzimidazolium based IERs is a consequence of the effect of the salt on the ionization of the reaction buffer, not the effect of the salt on the electronic structure of the compound. In contrast, YPI exhibited a more than 2-fold increase in the rate of HDX in the presence of 0.5 M sodium chloride (Fig. S4). This behavior suggests that the local electronic environment surrounding the C-terminal amide is significantly perturbed by the presence of neutral salts.

## Influence of organic solvents on exchange kinetics

HDX-MS studies are often performed with an organic co-solvent to help solubilize hydrophobic peptides or small molecule ligands. The addition of miscible organic solvents to water interferes with the bonded structure of water thereby altering ionization within the solution and solvated structures and impacting exchange processes[22,17]. We sought to examine the effect from two commonly used co-solvents: acetonitrile (ACN) and dimethylsulfoxide (DMSO) on the HDX kinetics of the proposed benzimidazolium based IERs. Having noticed from previous studies that compounds exchange faster in the presence of acetonitrile, we elected to focus on the slowest exchanging of the three compounds (TM68) to observe exchange kinetics in the presence of organic solvents at a broad range of mole fractions. As with the previously discussed studies only a single solution condition was varied within the sample set; in this case, the mole fraction of organic solvent (ACN or DMSO).

For both co-solvents, there was a strong positive correlation between the log of the exchange rate and the mole fraction of organic solvent (Fig. 3C). It is notable that the rate of HDX increases more rapidly in the presence of DMSO than ACN, which is consistent with the difference in the activities of ions in the two solvent systems[23,24]. Therefore, it appears the presence of ACN or DMSO does not significantly impact the mechanism of HDX for benzimidazolium derivatives, and they can still accurately report on exchange conditions. Although the rate of HDX for TM68 increases rapidly as the mole fraction of co-solvent increases, the effect is not a practical concern for LC-MS analysis. Even under chromatographic conditions, where the mole fraction of organic is highest, the combined effects of low pH and low temperature will suppress the exchange of benzimidazolium compounds to a much greater extent. For example, the calculated half-life for compound TM85 under HDX-MS chromatographic conditions (pH 2.5 and 0 °C) is estimated to be in excess of 50 hours. This estimate is corroborated by the lack of any observable back exchange from multiple LC-MS studies performed using compounds TM85, TM68, and TM65 as described in the next section.

## Suitability of imidazolium compounds for HDX-MS studies

We next performed a full protein HDX-MS study to test whether imidazolium compounds would serve as useful exchange reporters. TM65, TM68, and TM85 at micromolar concentrations were spiked into a solution of Equine cytochrome C (CytC) and HDX was measured at various time points from 3 seconds to 20 hours. The tripeptide YPI was also included as an alternative IER. The exchange was conducted at pD 7.80 and 7.30 side-by-side at 22°C in standard PBS. Resulting peptides were analyzed for deuterium content along with TM85, TM68, TM65 and YPI. The imidazolium IERs were easily detected by MS due to their fixed positive charge. The uptake of a single deuterium by each IER was evident across the different HDX time points (Fig. S6). We note that the maximum deuterium uptake for all IERs was between 0.85 and 0.90 Da, consistent with the total deuterium content during the HDX reaction (85% D). Some of the deuterium content may be exaggerated due to intensity distortions of isobaric peaks in FT-MS instrumentation[25]. This indicates that back-exchange for imidazolium based IERs under HDX quench conditions is not significant. In contrast, the YPI only showed a maximum shift of 0.60 +/− 0.03 Da consistent with a high degree of back-exchange. Furthermore, the difference in the exchange of the pepsin derived peptides of CytC between pD 7.80 and pD 7.30 (Fig. 4B,C left panels) is paralleled by the observed exchange for all three imidazolium IERs (Fig. 4A).

To test whether the offset in the exchange kinetics at the two pH conditions could be corrected, we shifted the time-axis for the low pD dataset to account for the difference in the $k_{ch}$ between the high and low pH datasets[8,26] (see methods). The pH-based time shifting results in overlays which are still visibly offset (Fig. 4B, C middle panels). As an alternative approach, we tried utilizing the exchange kinetics of the IERs to time shift the pD 7.30 data. The exchange for each of the IERs was fit to a single exponential function (Fig. 4, insets). The ratio of the exchange rate for each imidazolium compound at the high and low pH conditions was fairly consistent (TM85: 2.3; TM65: 2.2; TM68: 2.5), but notably lower for the YPI (1.3). The average ratio from all three imidazolium compounds (2.3) was applied to time-shift all the peptides in the data set (see methods). The IER-based time adjusted

overlays look remarkably consistent, suggesting that using the IER is an accurate approach for time adjustment (Fig. 4B, C right panel). The improvement in time adjustment can be attributed to several things. First, the benzimidazolium based IERs detect reaction buffer pH directly. This insight overcomes some of the error inherent to calculating exchange reaction pD from the reaction buffer pH*[27]. Second, the proposed IERs are sensitive to changes in solution conditions beyond pH. For instance, if there was a subtle offset in temperature or salt concentration between the two samples the benzimidazolium IERs would be able to detect it whereas the pH probe alone would not. This trend was observed at peptides throughout cytochrome C with one exception (Figure S7). The peptides spanning residues 74–80 (YIPGTKM) show a deviation in the later time points in both the pH-corrected and reporter corrected data. This could be reflective of either a pH induced conformational change to the protein which is now accurately detect by the internal reporter. Alternatively, it is possible that this deviation in the HDX-MS profiles results from a pH-dependent interaction with one of the benzimidazolium IERs.

## Overall utility of imidazolium IERs

The proposed benzimidazolium compounds have several advantages that make them ideal IERs for HDX-MS studies: 1) Exchange exclusively through base-catalysis, rendering their exchange rates highly responsive to pH; 2) Exhibit high solubility in water, making it possible to create very concentrated stocks (> 10 mM); 3) Are highly stable in aqueous solvent, so they can be used for long incubations without the risk of degradation; 4) The combination of the three proposed compounds provides coverage of a wide time scale; ranging from seconds to hours at pH 7 and 25°C; 5) The compounds have a fixed positive charge, making them easily detectable by MS; 6) Will not react with protein side-chains[13]. Additionally, these compounds exhibit negligible back-exchange under HDX quench conditions, making it possible to use their maximum deuterium uptake to directly detect the total deuterium content during the exchange reaction.

Beyond these desirable properties a few limitations were observed. Compounds 85, 68 and 65 have exact masses of 192.08, 161.11, 205.10 Da, respectively, which are outside of the mass window typically observed in HDX-MS studies (300 – 2000 *m/z*). Therefore, in order to detect these compounds the *m/z* window was expanded, which can impact sensitivity for some MS instrumentation[28]. A second limitation is poor retention during the LC-MS step. All three compounds were only effectively trapped on C18 columns at 0°C when loaded with a buffer containing less than 2% ACN. None of the compounds were effectively retained on C4 or C8 columns. We note that TM85, the most polar of the proposed compounds, was poorly retained even by C18 chromatography. For this reason, we had to use a nearly 100 times greater concentration of TM85, compared to TM65 and TM68, to obtain sufficient signal. This is not an effect of diminished ionization efficiency for TM85, as direct infusion of all three compounds produced the same signal intensity. TM65 and TM68 on the other hand were easily detectable at starting concentrations lower than the protein being studied. Even if the protein had a strong affinity for the IERs, which is an inevitable caveat of any internal standard, only a fraction of the protein would be bound thereby minimizing the effects on the observed HDX data. Further optimization of imidazolium-based compounds will likely alleviate the aforementioned caveats.

Additionally, it may be necessary for HDX-MS datasets to include a ‘no standard’ control which samples one replicate without the internal standard side-by-side. This will hopefully be sufficient for revealing any major effects from the internal standard on the protein structure without greatly expanding the time required for data collection. Alternatively, the compounds can be analyzed separately (as an external control) to accurately document the conditions and buffers used for a specific HDX-MS dataset.

Despite the current limitations of the three proposed benzimidazolium based IERs, these compounds are capable of making meaningful exchange corrections under a wide variety of experimental conditions. The immediate benefit of the proposed IERs is making comparative studies more robust. Currently, biocomparability studies by HDX-MS are known to be highly variable and only datasets collected and analyzed in parallel provide rigorous comparative power[29]. More recent studies have revealed that HDX-MS studies carried out under carefully matched conditions on an identical protein sample can still vary considerably[4]. Through the use of the proposed benzimidazolium based IERs we were able to effectively compare HDX-MS data collected for a protein under different reaction conditions (Fig. 4B). Admittedly, we only qualitatively assess the effectiveness of reporter-based time adjustments. The appropriate approach for assessing statistical differences between datasets with offset time points still needs to be established.

We believe that the inclusion of imidazolium based IERs could make it possible to accurately reference HDX-MS data collected on different days or even in different labs to a benchmark condition. Currently, HDX-MS studies include specific information on reaction exchange conditions (pH, temp, buffer etc), but this information is largely self-reported, and is therefore of limited utility for rigorous comparison of disparate data sets. Incorporation of the proposed benzimidazolium based IERs would provide a chemical exchange “stamp” that reports the exact conditions of exchange directly from the data set. This “stamp” would also improve the statistical power of comparative studies as it provides unambiguous distinction between effects on the protein structure and dynamics vs. global offsets to intrinsic exchange rate arising from altered reaction conditions.

## METHODS

### Reagents:

$D_2O$, deuterated ACN, and deuterated DMSO were purchased from Cambridge Isotope Labs (Tewksbury, MA, USA). Methyl iodide 99.9%, potassium carbonate, Methyl 3a, 4,5,5,6,7,7a-hexahydro-1H-1,3-benzodiazole-5carboxylate 98%, and 1-methylimidazole 98% were purchased from sigma Aldrich (St. Louis, MO, USA). 5-mehtylbenzimidazole 98%, and 5-nitrobenzimidazole 98+% were purchased through Alfa Aesar (Haverhill, MA, USA). Benzimidazole 98% was purchased from Acros organics (Geel, Belgium). 1-methylbenzimdazole-5-carboxylic acid was purchased from Maybridge chemicals (Altrincham, UK). Before use in alkylation reactions the solvents were dried via distillation over activated 3A molecular sieves. Chromatography solvents were purchased from Fisher Scientific (Hampton, NH, USA).

### HDX kinetics by NMR

Dried samples were re-suspended in an aqueous reaction buffer containing sodium phosphate (50 mM), 3-(Trimethylsilyl)-1-propanesulfonic acid (DSS) (0.104 mM), and 10% $D_2O$ (final volume 600 μL). This aqueous buffer was adjusted to specific pH using small additions of NaOH or HCl. Prior to NMR analysis, the adjusted samples were dried fully by speedvac. The resulting solid was re-suspended in $D_2O$ (99.96%), mixed vigorously for approximately 15 seconds, transferred into a NMR tube, and rapidly loaded into the NMR. For experiments with organic co-solvents, pH adjusted samples were resuspended in a mixture of $D_2O$ and either deuterated DMSO or ACN at a specific ratio.

All NMR experiments were performed on a 499.73 MHz Agilent DD2 spectrometer equipped with a 5 mm triple-resonance $^{1}H(^{13}C/^{15}N)$, z-axis pulsed-field gradient probe head. Course shim values were briefly manually adjusted prior to beginning arrayed $^{1}H$ NMR experiments. Array HDX-NMR studies consisted of identical experiments featuring Watergate solvent suppression separated by a pre-acquisition delay between experiments to allow access to different time scales. The duration of the delay was chosen to allow for the observation of 3 half-lives over 500 total experiments. The dead-time between resuspension and NMR acquisition for each sample was in the range of 45 to 90 seconds. The preparation and adjustment steps necessary for all the replicates of a particular sample set were carried out in the same day. All experiments were conducted at 298 K except for the temperature dependence studies which were sampled at 313 and 285 K. Actual experimental temperatures were determined using solvent chemical shift values as described by Gottlieb et al[30]. Exact pH* was measured for each sample after collection of the NMR kinetic data. Using an empirically derived equation, the pH* value was used to calculate the sample pD[27] (see supporting methods). For experiments carried out at elevated temperature, pH* measurements were made at the experimental temperature via the use of a recirculating water bath.

NMR data was processed using SpinWorks version 4.2.8. Spectra were referenced to DSS (0 ppm) to ensure consistent reporting of chemical shifts. The disappearance of the C-2 $^{1}H$ peak (or C-terminal NH peak for YPI) was analyzed relative to the intensity of a nearby well-resolved aromatic resonance; this was to account for any changes in the total signal intensity during the experiment. The change in the ratio of integrations as a function of exchange in the experiment time was fitted using an exponential decay function.

For ionic strength comparisons a sample of TM85 was prepared at pH 5.5, separated into three identical aliquots, and dried by speedvac. The aliquots were resuspended in 600 μL of either pure water, 250 mM NaCl, or 500 mM NaCl. Each sample was dried again and resuspended in $D_2O$ for HDX measurements. Prior to each NMR measurement a blank containing deuterated reaction buffer or with sodium chloride (250mM or 500mM) was used to roughly tune, lock, and shim the spectrometer for each ionic strength. Corrections to the reaction buffer pD were made using literature values corresponding the to the activity of phosphate buffered water in the presence of sodium chloride (see supporting information).

### Compounds Stability measurements

To assess the solution stability of the proposed benzimidazolium IERs, samples of purified TM85, TM68 and TM65 were reconstituted in $D_2O$ (99.9%), transferred into NMR tubes and sealed with parafilm. The sealed samples were analyzed by $^1H$ NMR immediately after preparation and again after two months of storage at room temperature on the benchtop.

### HDX-MS

Equine Cytochrome C (Sigma Aldrich) was resuspended in PBS (20 mM sodium phosphate 150 mM NaCl, 2 mM DTT) to a concentration of 0.1 mg/mL and adjusted to either pH 7.39 or 7.94 (corresponding to a calculated pD of 7.3 and 7.8, respectively) with small additions of HCl and NaOH. Internal exchange reporters were added to each solution for a final concentration of 6.5 μM TM65, 6.5 μM TM68, and 20 mM TM85. 10 μL of the protein solution was diluted 10-fold into deuterated buffer (for a final content of 85% $D_2O$) and incubated at 22°C for 3 sec, 15 sec, 1 min, 5 min, 30 min, 4 hrs, or 20 hrs. The deuterated buffer had 0.2 μg/mL of bradykinin and angiotensin II to serve as controls for assessing back-exchange[5]. Exchanged samples were added to an equal volume (100 μL) of ice-cold quench buffer (8 M Urea, 0.2% formic acid) for a final pH of 2.5. Samples were flash frozen in an ethanol-dry ice bath (−60 °C) and subsequently stored at −80 °C until LC-MS analysis. The exact pH* during the deuterium reaction was measured using an identical sample in $D_2O$, without protein and used to calculate the pD as described for the NMR methods.

Frozen samples were thawed on a 5 °C block for 4 minutes prior to injection onto a loading loop. The loaded sample was passed over a custom packed pepsin column (2.1 × 50 mm) kept at 8 °C with a flow of 0.1% trifluoroacetic acid (TFA) at 200 μL/min. Digested peptic fragments were trapped onto a Waters BEH trap column (2.1 × 5 mm, 1.7 μm). Waters CSH traps were also found to be effective, but the HSS T3 stationary phase was not effective at retaining the benzimidazolium compounds. After 5 minutes of loading, digestion, and trapping, peptides were resolved on an analytical column (Waters CSH 1 × 100 mm, 1.7μm, 130Å) using a gradient of 3 % to 40 % solvent B for 9 minutes (A: 0.1 % FA, 0.025 % TFA, 2 % ACN; B) 0.1 % FA in ACN). The LC system was coupled to a Thermo Orbitrap performing full scans over the *m/z* range of 150 – 1500 with a resolution setting of 30,000. During the analytical separation step, a series of 250 μL injections were used to clean the pepsin column: 1) 0.1 % Fos-12 with 0.1 % TFA; 2) 2 M GndHCl in 0.1 % TFA; 3) 10 % acetic acid, 10 % acetonitrile, 5 % IPA[31,32]. After each gradient the trapping column was washed with a series of 250 μL injections: 1) 10 % FA; 2) 30 % trifluoroethanol; 3) 80 % MeOH; 4) 66 % isopropanol, 34 % ACN; 5) 80 % ACN. During the trap washes the analytical column was cleaned with three rapid gradients[33]. These cleaning steps were necessary to ensure that the level of carry-over was below 5 % for each peptide analyzed. Undeuterated samples were used to collect MS/MS spectra using data-dependent acquisition. Peptic peptides were identified by exact mass and tandem mass spectrometry (MS/MS) spectra using Byonic (Protein Metrics). Mass shifts were determined using HD-Examiner V2 (Sierra Analytics) and HX-Express v2[34]. The exchange kinetics for each IER were calculated by fitting the data to a single exponential.

Time shifting of the pD 7.30 data was first achieved by assuming a ten-fold increase in the intrinsic amide exchange rate for a pH increase of 1.0. By this criteria, the time axis of the pD 7.30 data set was shifted down by a factor of $10^{(7.94-7.39)}$ or 3.54. Time-shifting using the exchange offset as observed by the benzimidazolium IERs was accomplished by first calculating the difference in exchange rate between the pD 7.80 and 7.30 data sets and then using the average ratio between the rates (average factor of 2.3) to scale the time points of the pD 7.30 data set. For example, the 1 minute time point was shifted down to 26 seconds.

## CONCLUSIONS

Benzimidazolium based IERs exhibit solution dependent HDX behavior ideal for monitoring changes in pH, temperature, salt concentration, or levels of organic co-solvent, and over a wide range of time scales. The use of the IERs presented here can expand the utility of HDX-MS to allow for the analysis of more diverse proteins under highly varied experimental conditions and pave the way to increasing the reproducibility of solution HDX studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

The authors wish to thank David D. Weis and Michele Scian for insightful discussions. We are grateful to Matt McDonald for assistance and guidance with organic synthesis. This work was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R01GM127579.

## REFERENCES

(1). Jensen PF; Rand KD Hydrogen Exchange. In Hydrogen Exchange Mass Spectrometry of Proteins; John Wiley & Sons, Ltd, 2016; pp 1–17. 10.1002/9781118703748.ch1.

(2). Houde D; Berkowitz SA The Role of Hydrogen Exchange Mass Spectrometry in Assessing the Consistency and Comparability of the Higher-Order Structure of Protein Biopharmaceuticals. In Hydrogen Exchange Mass Spectrometry of Proteins; John Wiley & Sons, Ltd, 2016; pp 225–246. 10.1002/9781118703748.ch13.

(3). Hudgens JW; Huang RY-C; D'Ambro E Method Validation and Standards in Hydrogen Exchange Mass Spectrometry. In Hydrogen Exchange Mass Spectrometry of Proteins; John Wiley & Sons, Ltd, 2016; pp 55–72. 10.1002/9781118703748.ch4.

(4). Hudgens JW; Gallagher ES; Karageorgos I; Anderson KW; Filliben JJ; Huang RY-C; Chen G; Bou-Assaf GM; Espada A; Chalmers MJ; Harguindey E; Zhang H-M; Walters BT; Zhang J; Venable J; Steckler C; Park I; Brock A; Lu X; Pandey R; Chandramohan A; Anand GS; Nirudodhi SN; Sperry JB; Rouse JC; Carroll JA; Rand KD; Leurs U; Weis DD; Al-Naqshabandi MA; Hageman TS; Deredge D; Wintrode PL; Papanastasiou M; Lambris JD; Li S; Urata S Interlaboratory Comparison of Hydrogen–Deuterium Exchange Mass Spectrometry Measurements of the Fab Fragment of NISTmAb. Anal. Chem 2019, 91 (11), 7336–7345. 10.1021/acs.analchem.9b01100. [PubMed: 31045344]

(5). Zhang Z; Zhang A; Xiao G Improved Protein Hydrogen/Deuterium Exchange Mass Spectrometry Platform with Fully Automated Data Processing. Anal. Chem 2012, 84 (11), 4942–4949. 10.1021/ac300535r. [PubMed: 22571272]

(6). Toth RT; Mills BJ; Joshi SB; Esfandiary R; Bishop SM; Middaugh CR; Volkin DB; Weis DD Correction to Empirical Correction for Differences in Chemical Exchange Rates in Hydrogen

Exchange-Mass Spectrometry Measurements. Anal. Chem 2017, 89 (24), 13673–13673. 10.1021/acs.analchem.7b04690. [PubMed: 29210567]

(7). Hamuro Y Determination of Equine Cytochrome c Backbone Amide Hydrogen/Deuterium Exchange Rates by Mass Spectrometry Using a Wider Time Window and Isotope Envelope. J. Am. Soc. Mass Spectrom 2017, 28 (3), 486–497. 10.1007/s13361-016-1571-1. [PubMed: 28108962]

(8). Goswami D; Devarakonda S; Chalmers MJ; Pascal BD; Spiegelman BM; Griffin PR Time Window Expansion for HDX Analysis of an Intrinsically Disordered Protein. J Am Soc Mass Spectrom 2013, 24 (10), 1584–1592. 10.1007/s13361-013-0669-y. [PubMed: 23884631]

(9). Masson GR; Burke JE; Ahn NG; Anand GS; Borchers C; Brier S; Bou-Assaf GM; Engen JR; Englander SW; Faber J; Garlish R; Griffin PR; Gross ML; Guttman M; Hamuro Y; Heck AJR; Houde D; Iacob RE; Jørgensen TJD; Kaltashov IA; Klinman JP; Konermann L; Man P; Mayne L; Pascal BD; Reichmann D; Skehel M; Snijder J; Strutzenberg TS; Underbakke ES; Wagner C; Wales TE; Walters BT; Weis DD; Wilson DJ; Wintrode PL; Zhang Z; Zheng J; Schriemer DC; Rand KD Recommendations for Performing, Interpreting and Reporting Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS) Experiments. Nat Methods 2019, 16 (7), 595–602. 10.1038/s41592-019-0459-y. [PubMed: 31249422]

(10). Zhang J; Goswami D; Zhang Z New Insight into Differences in Intrinsic HDX Rates at Different pH and Temperature. 67th ASMS Conference on Mass Spectrometry and Allied Topics, Atlanta, GA, June 2–6, 2019.

(11). Englander SW; Mayne L; Bai Y; Sosnick TR Hydrogen Exchange: The Modern Legacy of Linderstrøm-Lang. Protein Sci 1997, 6 (5), 1101–1109. [PubMed: 9144782]

(12). Bai Y; Milne JS; Mayne L; Englander SW Primary Structure Effects on Peptide Group Hydrogen Exchange. Proteins: Structure, Function, and Bioinformatics 1993, 17 (1), 75–86. 10.1002/prot.340170110.

(13). Amyes TL; Diver ST; Richard JP; Rivas FM; Toth K Formation and Stability of N-Heterocyclic Carbenes in Water: The Carbon Acid PKa of Imidazolium Cations in Aqueous Solution. J. Am. Chem. Soc 2004, 126 (13), 4366–4374. 10.1021/ja039890j. [PubMed: 15053626]

(14). Hayashi N; Kuyama H; Nakajima C; Kawahara K; Miyagi M; Nishimura O; Matsuo H; Nakazawa T Imidazole C-2 Hydrogen/Deuterium Exchange Reaction at Histidine for Probing Protein Structure and Function with MALDI Mass Spectrometry. Biochemistry 2014, 53 (11), 1818–1826. 10.1021/bi401260f. [PubMed: 24606199]

(15). Bradbury JH; Chapman BE; Pellegrino FA Hydrogen-Deuterium Exchange Kinetics of the C-2 Protons of Imidazole and Histidine Compounds. J. Am. Chem. Soc 1973, 95 (18), 6139–6140. 10.1021/ja00799a063. [PubMed: 4733837]

(16). Attanasi O; Bartoli G; Todesco PE Base-Catalyzed Hydrogen-Deuterium Exchange in Some 5-Substituted Benzothiazoles and in Thiazolo-[4,5-c]Pyridine. Effect of Substituents and Polar Effects of the Aza Group. Tetrahedron 1976, 32 (3), 399–401. 10.1016/0040-4020(76)80054-3.

(17). Englander SW; Kallenbach NR Hydrogen Exchange and Structural Dynamics of Proteins and Nucleic Acids. Quarterly Reviews of Biophysics 1983, 16 (4), 521–655. 10.1017/S0033583500005217. [PubMed: 6204354]

(18). Tüchsen E; Woodward C Mechanism of Surface Peptide Proton Exchange in Bovine Pancreatic Trypsin Inhibitor Salt Effects and O-Protonation. Journal of Molecular Biology 1985, 185 (2), 421–430. 10.1016/0022-2836(85)90413-9. [PubMed: 2414452]

(19). Christoffersen M; Bolvig S; Tüchsen E Salt Effects on the Amide Hydrogen Exchange of Bovine Pancreatic Trypsin Inhibitor. Biochemistry 1996, 35 (7), 2309–2315. 10.1021/bi951711q. [PubMed: 8652571]

(20). Voinescu AE; Bauduin P; Pinna MC; Touraud D; Ninham BW; Kunz W Similarity of Salt Influences on the PH of Buffers, Polyelectrolytes, and Proteins. J. Phys. Chem. B 2006, 110 (17), 8870–8876. 10.1021/jp0600209. [PubMed: 16640447]

(21). Critchfield FE; Johnson JB Effect of Neutral Salts on PH of Acid Solutions. Anal. Chem 1959, 31 (4), 570–572. 10.1021/ac50164a034.

(22). Yang J-Z; Xu W-G Medium Effect of an Organic Solvent on the Activity Coefficients of HCl Consistent with Pitzer's Electrolyte Solution Theory. J Solution Chem 2005, 34 (1), 71–76. 10.1007/s10953-005-2073-0.

(23). Archer MD; Gasser RPH Electrolyte Solutions in Dimethyl Sulphoxide. Part 2.—Caesium Iodide. Trans. Faraday Soc 1966, 62 (0), 3451–3458. 10.1039/TF9666203451.

(24). Porras SP; Sarmini K; Fanali S; Kenndler E Medium Effect (Transfer Activity Coefficient) of Methanol and Acetonitrile on β-Cyclodextrin/Benzoate Complexation in Capillary Zone Electrophoresis. Anal. Chem 2003, 75 (7), 1645–1651. 10.1021/ac026407z. [PubMed: 12705597]

(25). Burns KM; Rey M; Baker CAH; Schriemer DC Platform Dependencies in Bottom-up Hydrogen/Deuterium Exchange Mass Spectrometry. Mol. Cell Proteomics 2013, 12 (2), 539–548. 10.1074/mcp.M112.023770. [PubMed: 23197788]

(26). Hamuro Y Determination of Equine Cytochrome c Backbone Amide Hydrogen/Deuterium Exchange Rates by Mass Spectrometry Using a Wider Time Window and Isotope Envelope. J. Am. Soc. Mass Spectrom 2017, 28 (3), 486–497. 10.1007/s13361-016-1571-1. [PubMed: 28108962]

(27). Krężel A; Bal W A Formula for Correlating PKa Values Determined in D2O and H2O. Journal of Inorganic Biochemistry 2004, 98 (1), 161–166. 10.1016/j.jinorgbio.2003.10.001. [PubMed: 14659645]

(28). Schwartz JC; Senko MW; Syka JEP A Two-Dimensional Quadrupole Ion Trap Mass Spectrometer. J. Am. Soc. Mass Spectrom 2002, 13 (6), 659–669. 10.1016/S1044-0305(02)00384-7. [PubMed: 12056566]

(29). Houde D; Berkowitz SA; Engen JR The Utility of Hydrogen/Deuterium Exchange Mass Spectrometry in Biopharmaceutical Comparability Studies. J Pharm Sci 2011, 100 (6), 2071–2086. 10.1002/jps.22432. [PubMed: 21491437]

(30). Gottlieb HE; Kotlyar V; Nudelman A NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities. J. Org. Chem 1997, 62 (21), 7512–7515. 10.1021/jo971176v. [PubMed: 11671879]

(31). Majumdar R; Manikwar P; Hickey JM; Arora J; Middaugh CR; Volkin DB; Weis DD Minimizing Carry-over in an Online Pepsin Digestion System Used for the H/D Exchange Mass Spectrometric Analysis of an IgG1 Monoclonal Antibody. J. Am. Soc. Mass Spectrom 2012, 23 (12), 2140–2148. 10.1007/s13361-012-0485-9. [PubMed: 22993047]

(32). Hamuro Y; Coales SJ Optimization of Feasibility Stage for Hydrogen/Deuterium Exchange Mass Spectrometry. J. Am. Soc. Mass Spectrom 2018, 29 (3), 623–629. 10.1007/s13361-017-1860-3. [PubMed: 29299838]

(33). Fang J; Rand KD; Beuning PJ; Engen JR False EX1 Signatures Caused by Sample Carryover during HX MS Analyses. Int J Mass Spectrom 2011, 302 (1–3), 19–25. 10.1016/j.ijms.2010.06.039. [PubMed: 21643454]

(34). Guttman M; Weis DD; Engen JR; Lee KK Analysis of Overlapped and Noisy Hydrogen/Deuterium Exchange Mass Spectra. J. Am. Soc. Mass Spectrom 2013, 24 (12), 1906–1912. 10.1007/s13361-013-0727-5. [PubMed: 24018862]

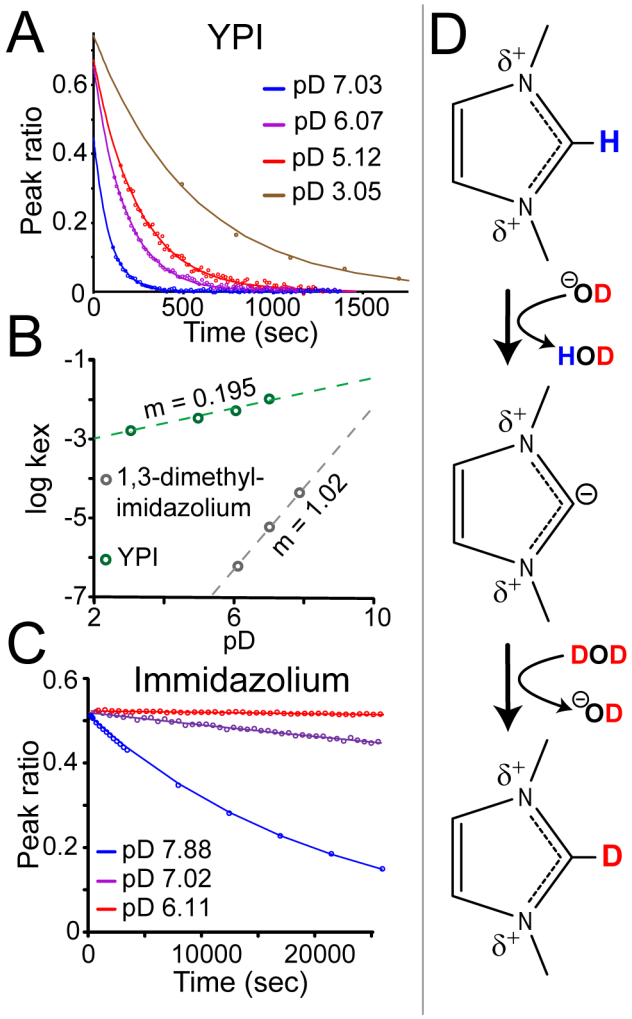


**Figure 1:**

**A)** HDX rates for the c-terminal isoleucine in peptide YPI measured by NMR at various pD. Solid lines represent fits of exponential decay functions. **B)** Plot of the log of the exchange rate, log($k_{ex}$), as a function of pD for both YPI (green) and 1,3-dimethylimidazolium (gray). The slopes (m) of the lines are shown next the linear fit. **C)** pD dependence of HDX at the C-2 proton for 1,3-dimethylimidazolium measured by NMR at various pD. **D)** General mechanism for HDX at the C-2 proton for 1,3-dimethylimidazolium.

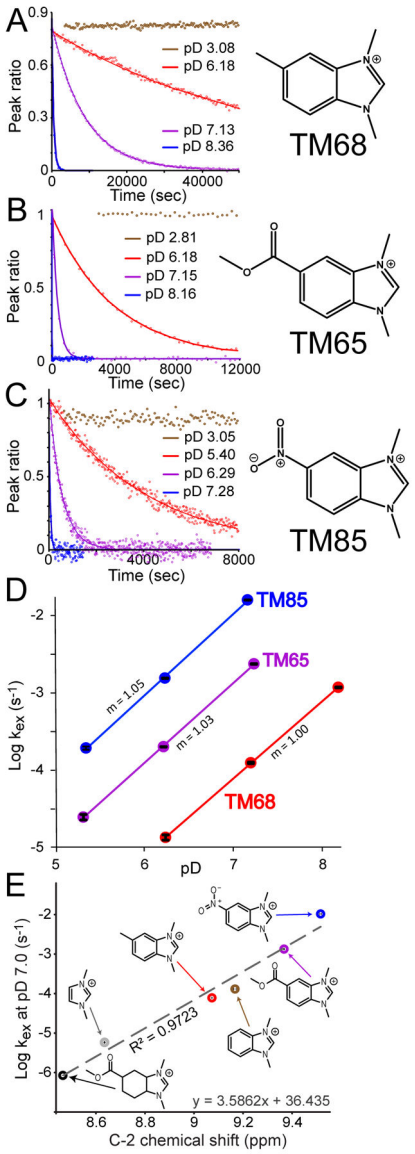


**Figure 2:**

pD dependence of HDX rates for the C-2 proton in TM68 **(A)**, TM65 **(B)**, and TM85 **(C)** measured by NMR. Lines represent exponential decay functions. Chemical structures are shown on the right. **D)** The observed rate of exchange ($k_{ex}$) for compounds TM85 (blue), TM68 (red), and TM65 (purple) as a function of pD. The slope of each line (m) is indicated next to the linear fit. **E)** The rate of exchange for compounds TM85 (blue), TM68 (red), TM65 (purple), TM39 (brown), TM31 (gray), TM91 (Black) at pD 7.0 as a function of C-2 chemical shift (ppm). Error bars show standard deviations from triplicate measurements.

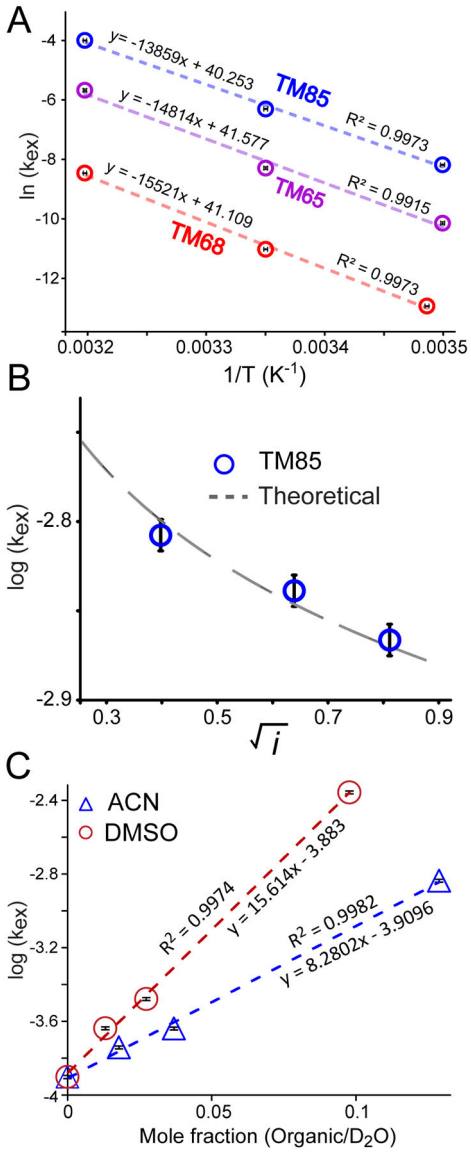


**Figure 3:**

**A)** Arrhenius plots showing the temperature dependence on the HDX rates of compounds TM85 (blue), TM68 (red), and TM65 (purple). The equations for the linear fits and $R^2$ values are shown above each line. **B)** The observed rate of exchange for TM85 as a function of the square root of the solution of ionic strength (0.0 M, $i$=0.158 M, 0.250 M, $i$=0.408 M and 0.50 M, $i$=0.658 M) (blue circles). The dashed line corresponds to the theoretical rate of exchange ($\log_{10}$) for compound TM85 as function of the solution ionic strength. **C)** The observed rate of exchange for TM68 as a function of the fraction of organic solvent in the $D_2O$ reaction buffer with either DMSO (red) or ACN (blue). Equations for the linear fits are shown under each line.

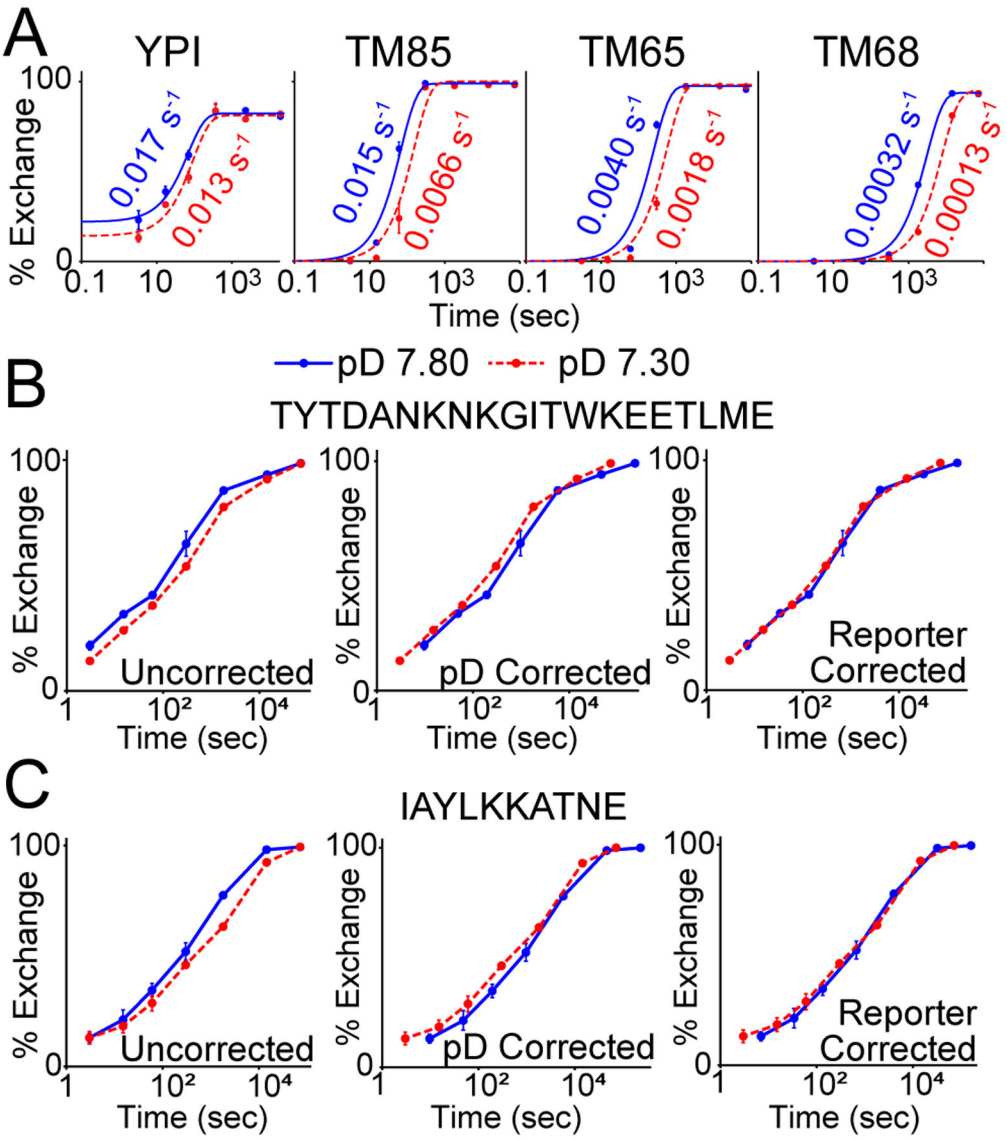


**Figure 4:**

Solution HDX-MS data for Cytochrome C and internal exchange reporters. A) Deuterium uptake plots for YPI, TM85, TM65, and TM68 is shown for various timepoints at pD 7.8 (blue) and 7.3 (red). Solid and dashed lines show a single exponential fit. Example deuterium uptake plots for two peptides of Cytochrome C: TYTDANKNKGITWKEETLME **(B)** and IAYLKKATNE **(C)** are shown. B/C) The plots on the left show the uncorrected deuterium uptake at pD 7.8 (blue) and 7.3 (red). The plots in the center show the deuterium uptake after time-shifting the pD 7.8 data to match the exchange conditions based on the pD difference. The plots on the right show the deuterium uptake after time-shifting the pD 7.8 data based on the difference in rates measured using the exchange rates of the exchange reporters TM85, TH65, TM 68 shown in (A). Additional examples are shown in figure S7 and data for all peptides are provided in the supporting data file.