Supporting Information

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
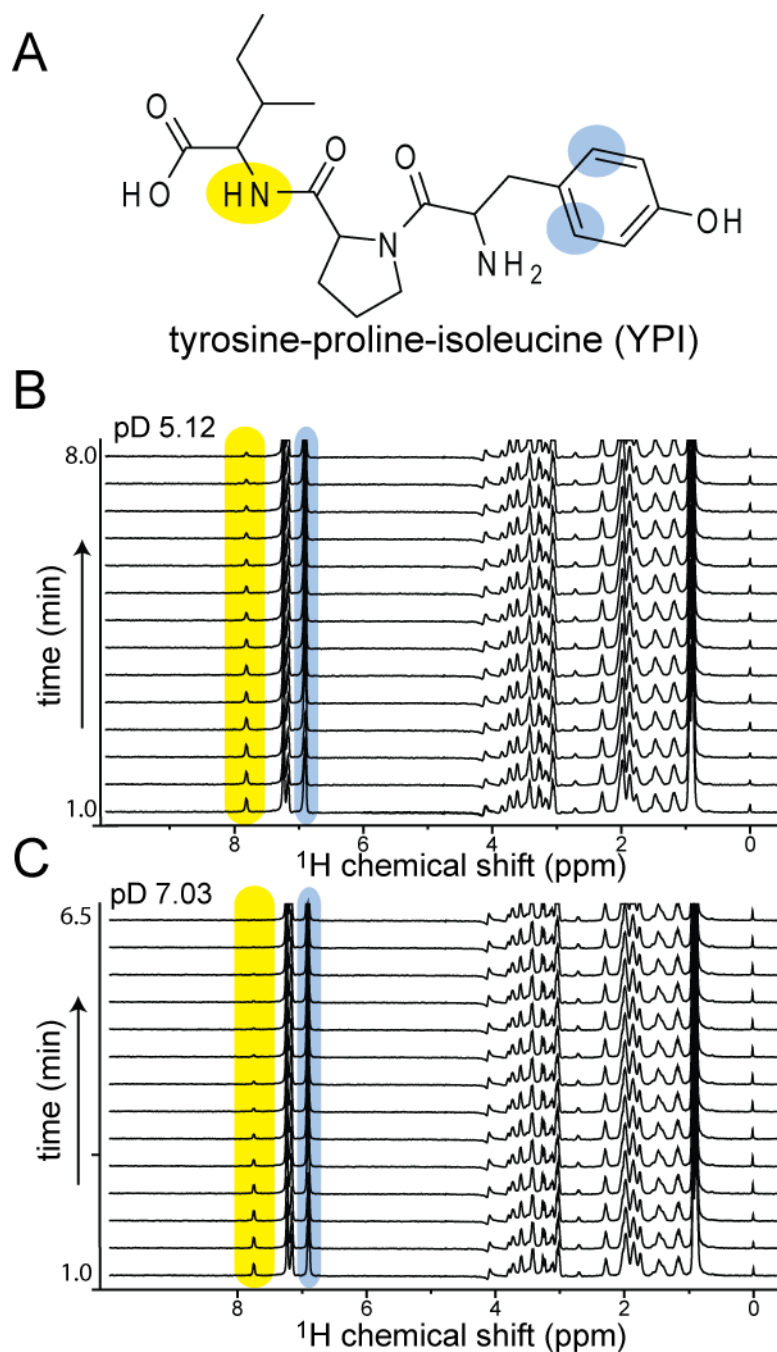
*Correspondence: mguttman@uw.edu

Contents:

Figure S1: Structure and NMR spectra for YPI HDX kinetic measurements. **A)** The chemical structure of YPI with the C-terminal amide that can be measured by HDX is highlighted in yellow. Stacked ^{1}H NMR spectra measuring the kinetics of HDX at the C-terminal amide are shown for YPI at pD 5.12 **(B)** and pD 7.03 **(C)**. The disappearance of the amide peak (yellow) relative to the aromatic peak (light blue), which does not exchange was used to measure the rate of exchange.
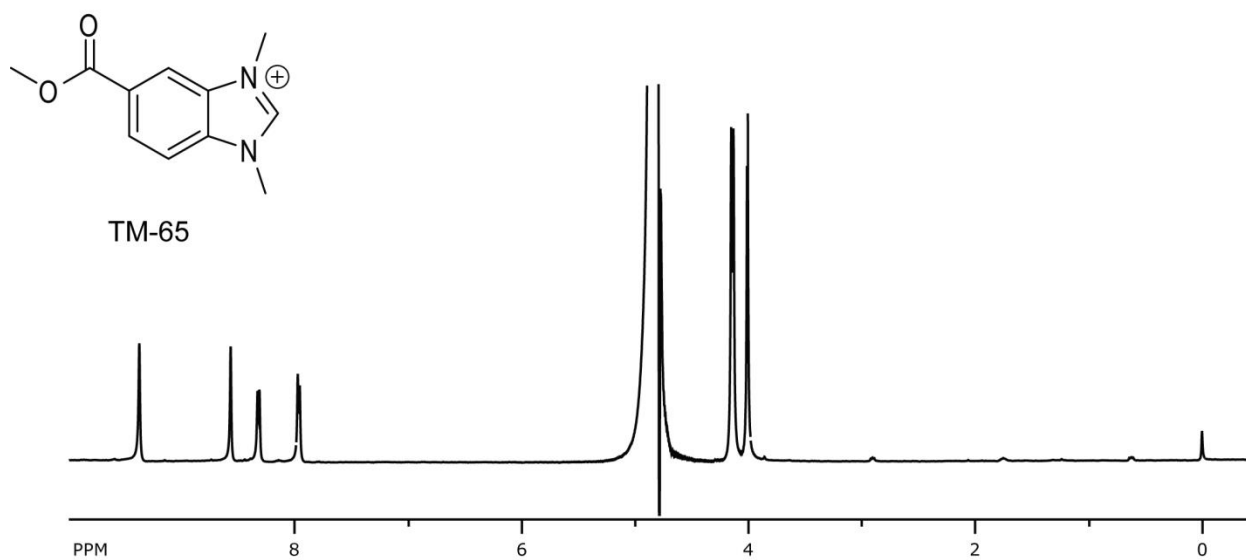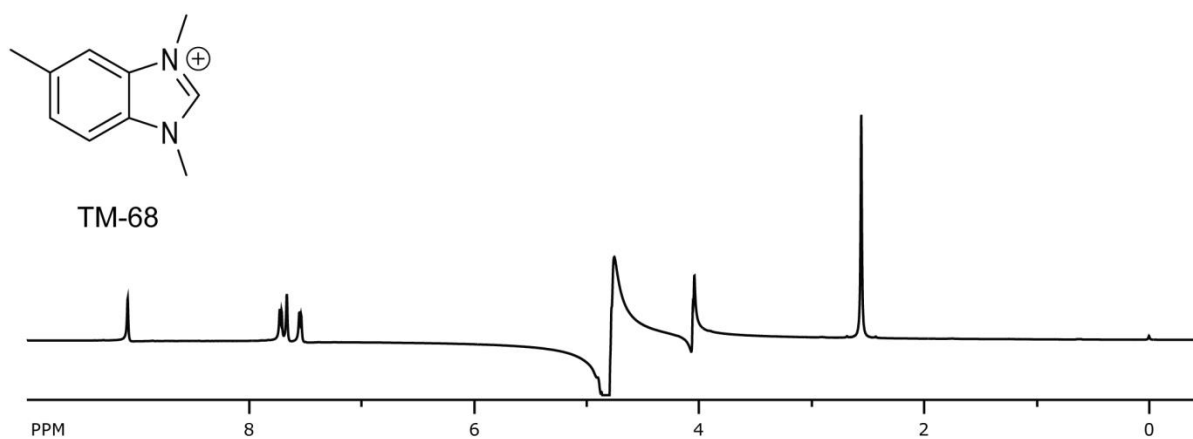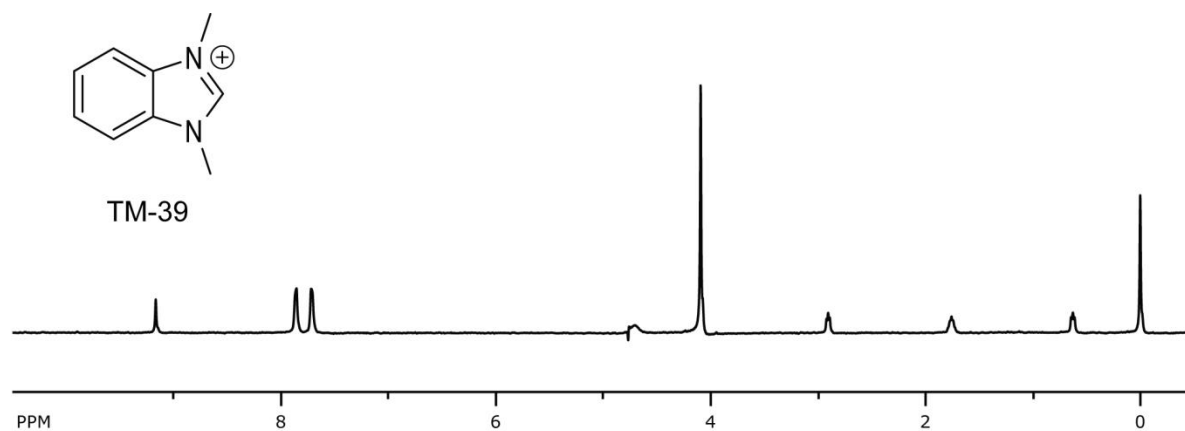
Figure S2: ^{1}H NMR spectra of all compounds examined.
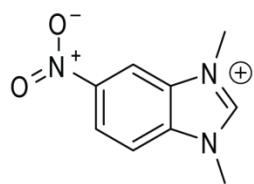
TM-85
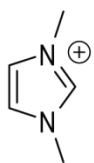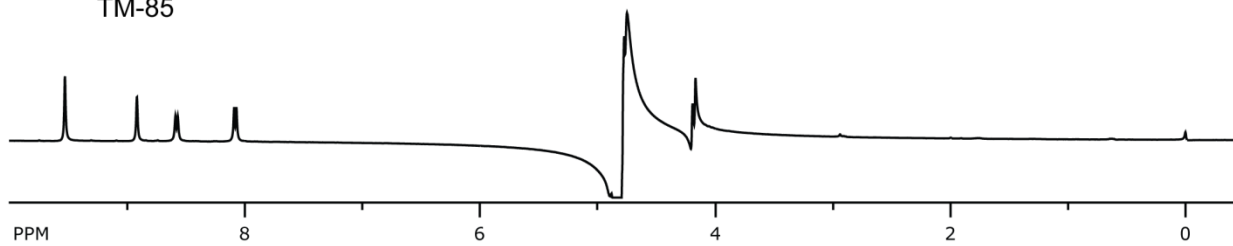
TM-31
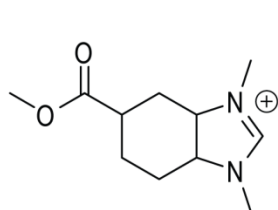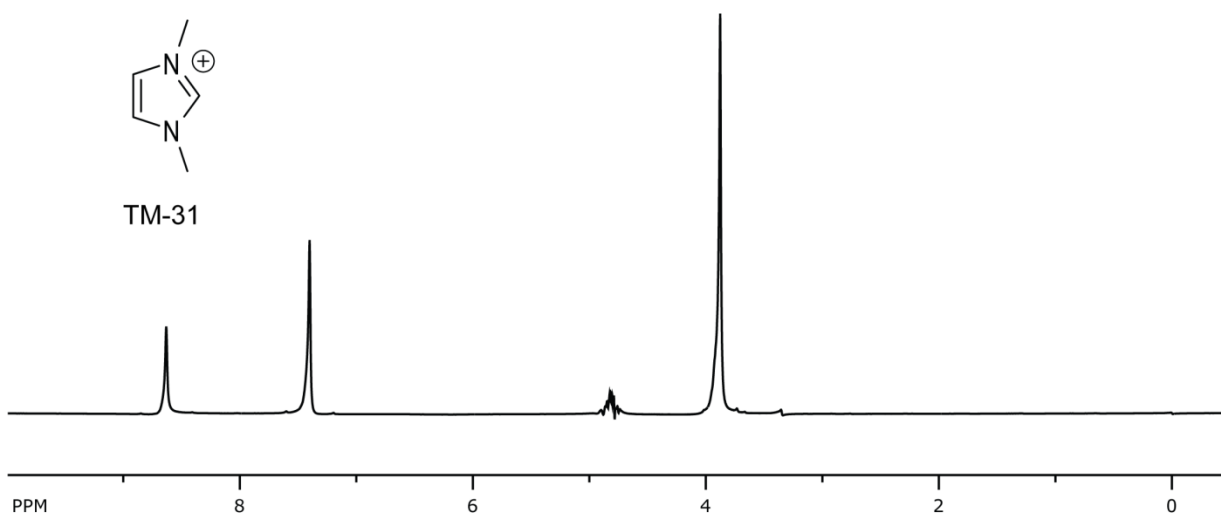
TM-91
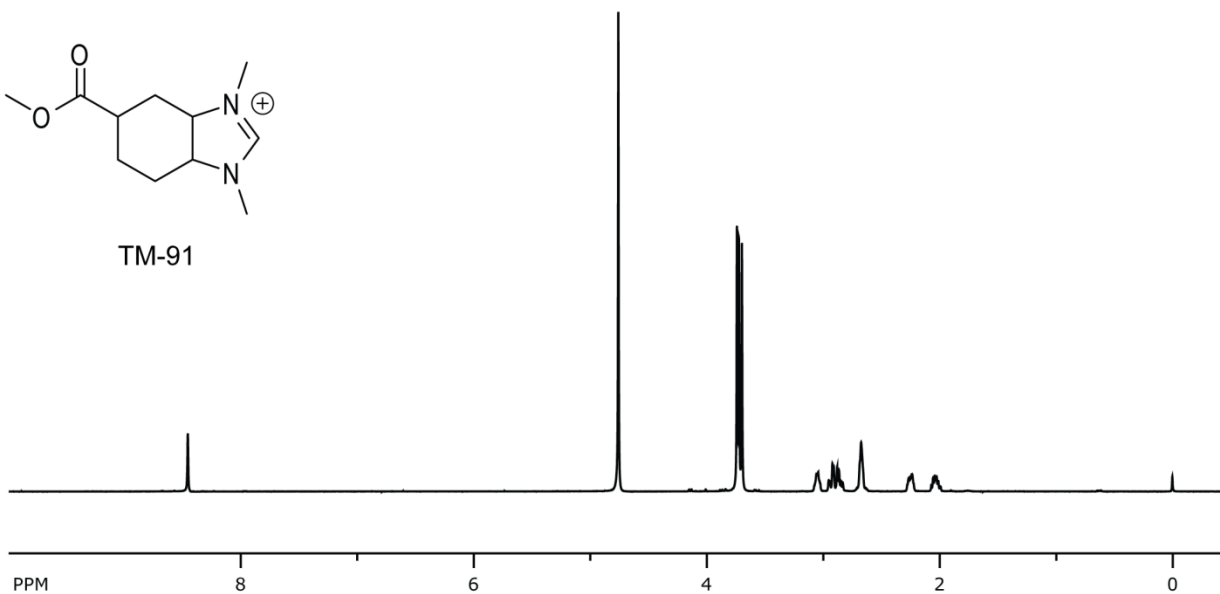
Figure S2 (cont.): ^{1}H NMR spectra of all compounds examined.
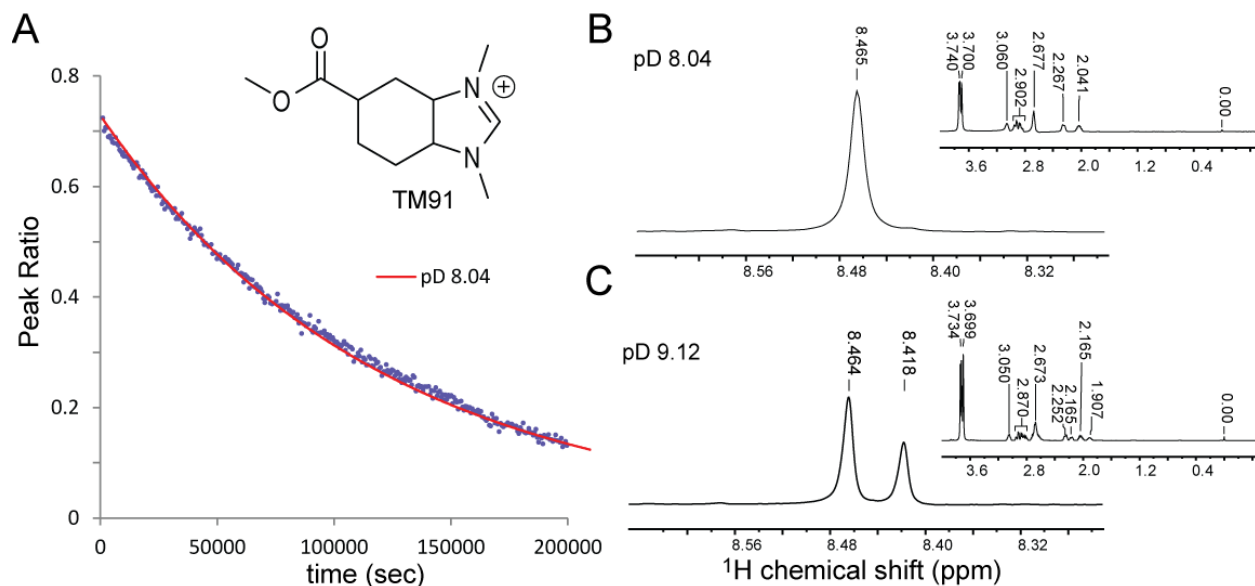
Figure S3: **A)** HDX at C-2 for compound TM91 at pD 8.04 as observed by ^{1}H NMR. The red line shows the fit to a single exponential decay (k_{ex}= 8.45x10-6 s^{-1}, $t_{1/2}$ =22 h at pH 8.04). ^{1}H NMR spectra of TM91 in 10% D_2O at pD 8.04 **(B)** and pD 9.12 **(C)**. The insert shows the upfield portion of the spectrum.
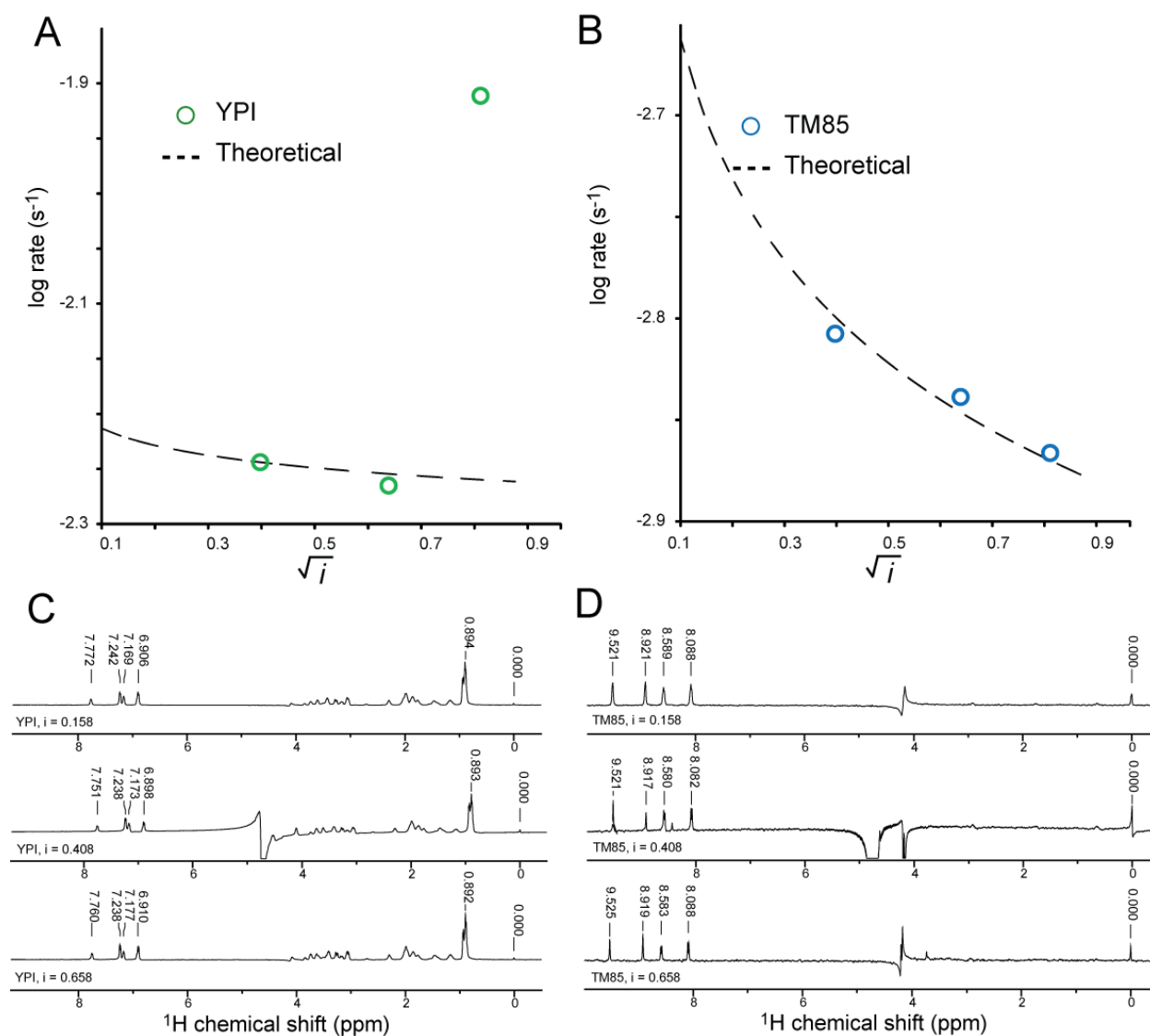
Figure S4) Dependence of C-terminal HDX for YPI **(A)** and the C-2 proton of TM85 **(B)** on the solution ionic strength as observed by ^{1}H NMR. ^{1}H NMR spectra are shown for YPI **(C)** and TM85 **(D)** at pD 6.07 and 6.24 respectively; calculated ionic strengths for NMR samples are reported (i is given in units of mol/L).
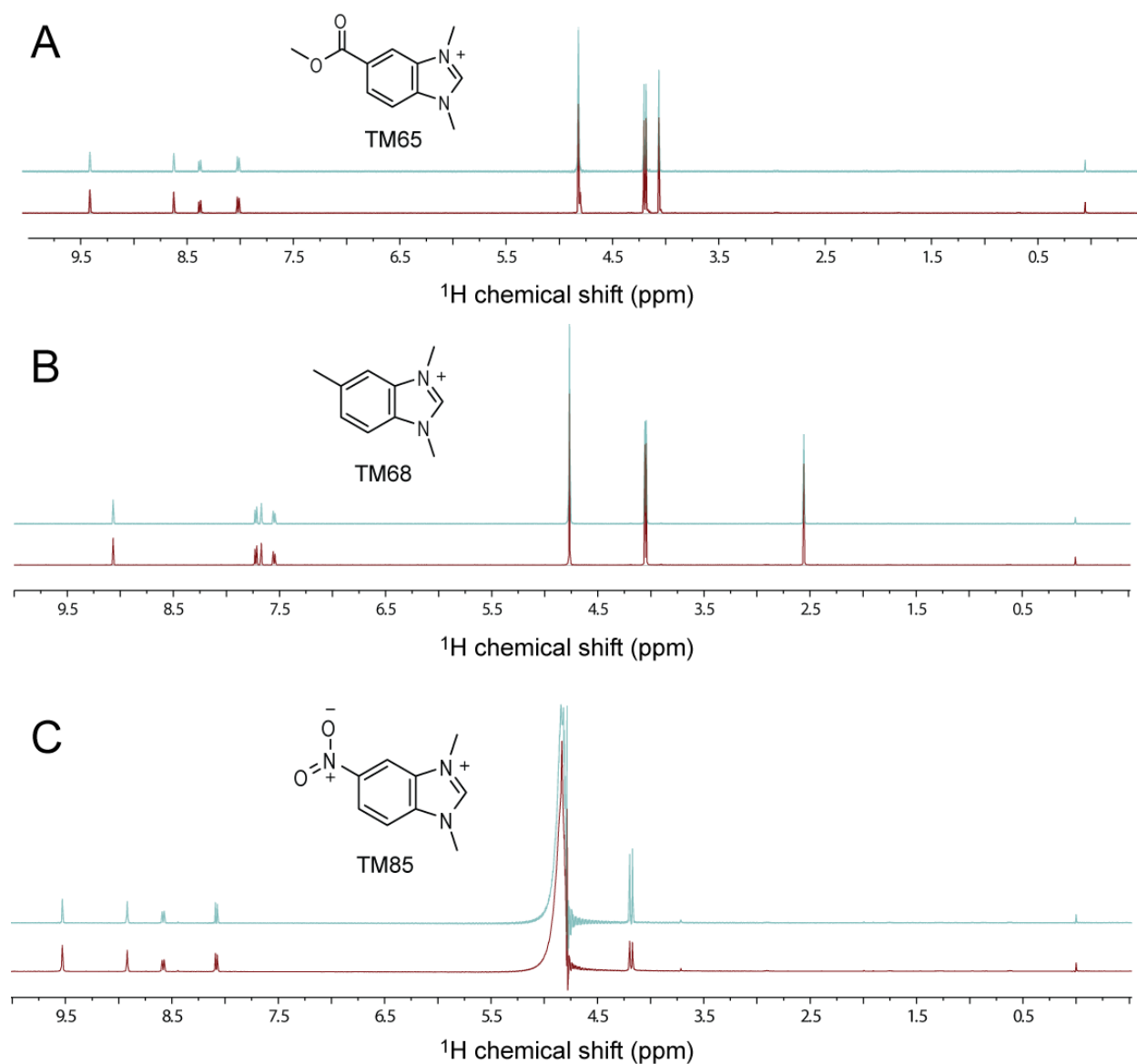
Figure S5: Stability of reporter compounds TM65 **(A)**, TM68 **(B)**, and TM85 **(C)** in 10% D_2O at pH 7.0 by ^1H NMR. Spectra of each compounds are shown immediately after resuspension (brown) and after two months of storage in the buffered aqueous solution at room temperature (light blue).
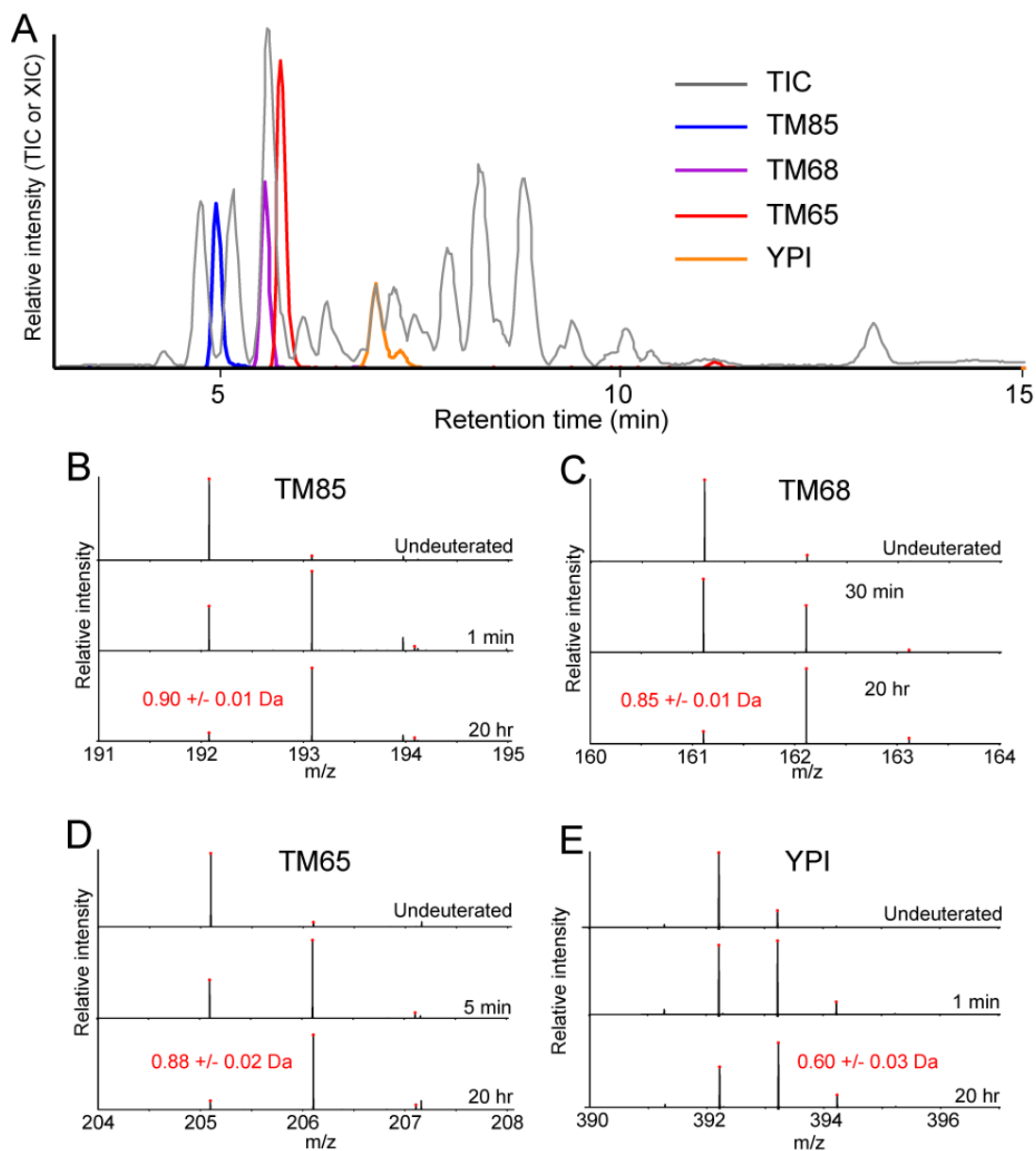
Figure S6: HDX by LC-MS with IERs. **A)** The LC chromatogram of an undeuterated pepsin digest of Cytochrome C (TIC shown in the gray trace). Extracted ion chromatograms (XIC) for the IERs are shown: TM85 (blue), TM68 (purple), TM65 (red), and YPI (orange). **B-E)** Mass spectra for each IER in the undeuterated state (top), or at two deuterium time points (middle and lower panel) with deuterium exchange times indicated on the right side. Numbers indicate the total mass shift at the longest (20 hour) time point (average +/- standard deviation from triplicate measurements).
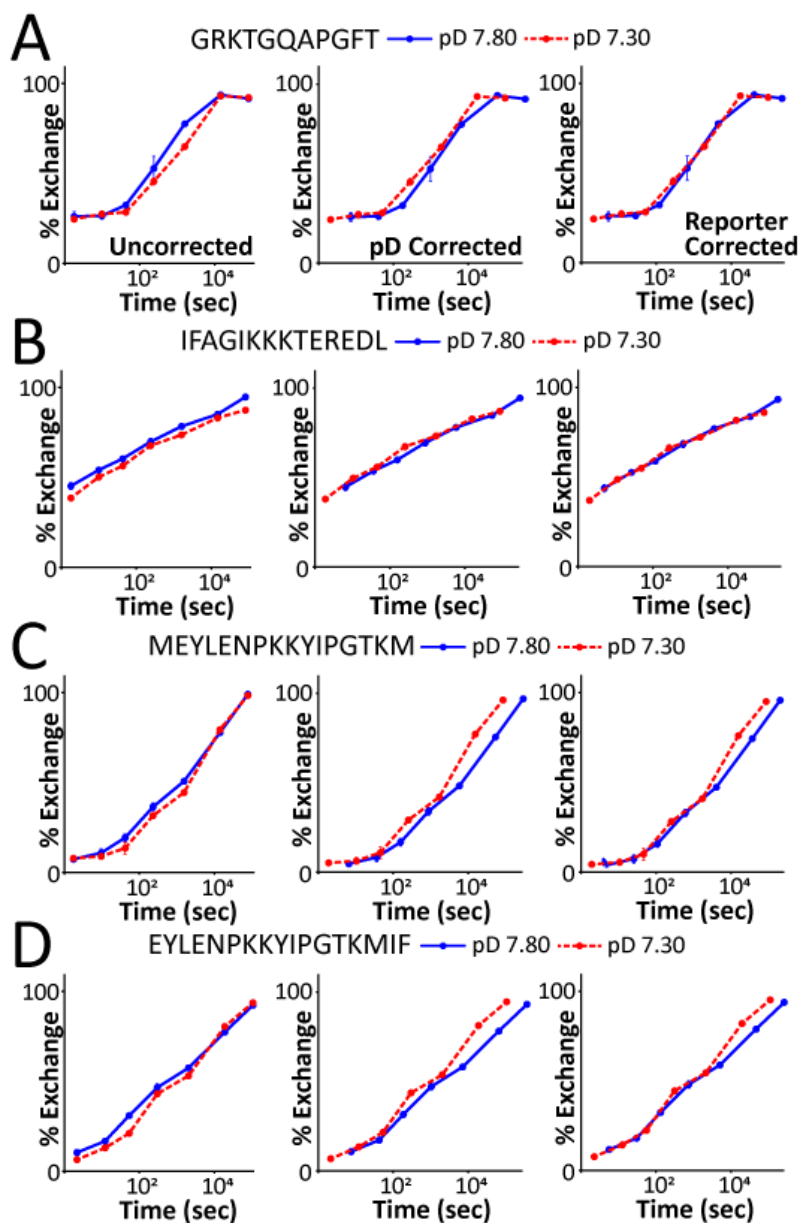
Figure S7: HDX-MS of additional Cytochrome C peptides. For additional examples of HDX-MS profiles of peptides at pD 7.3 (red) and pD 7.8 (blue) as described in Figure 4. While panels A and B show additional examples where the reporter-corrected data align well, panels C and D show two overlapping peptides at a specific region of Cytochrome C that show deviations at later time points. The offset could be due to a change in the protein dynamics at the higher pH leading to more protection. Alternatively, the standards may be interacting with the protein at this region in a pH-dependent manner to offset the kinetics.

SYNTHESIS and COMPOUND CHARACTERIZATION

Alkylation of imidazole derivatives: To an oven dried round bottom flask containing a magnetic stir bar benzimidazole (0.84mmol, 1.0 eq) and potassium carbonate (1.26mmol, 1.5 eq) were added. The vessel was fitted with a rubber septum and flushed with Argon *(g).* Approximately 30ml of dried acetonitrile was added via cannula to the vessel via argon pressure. Next the mixture was set to stir and methyl iodide (4,23mmol, 5.0 eq) was added dropwise to the stirring solution. The vessel was then fitted with a liquid cooled reflux condenser and heated to reflux. The mixture was allowed to stir at this temperature for approximately 12 hours or until all starting material appeared to be consumed by TLC. Excess solvent was removed under reduced pressure. The residue was resuspended in mobile phase (9:1 H_2O:ACN w/ 0.1% TFA) and filtered using a syringe driven filter (0.22µM). The effluent was purified via reverse phase HPLC (gradient elution: 15-95% ACN in H_2O with 0.1% TFA), to yield 1,3-dimethlybenzimidazolium (TM39) as a yellowish solid, 89%.

1,3-dimethlybenzimidazolium (TM39) purified by HPLC (gradient elution: 15-95% ACN in H_2O with 0.1% TFA) to yield yellowish solid 89%, 73% yield; ^{1}H NMR (499.73 MHz, D_2O, ppm): δ 9.17 (S, 1H),7.79 (D, 4H) 4.09 (S, 6H); MS (ESI) calcd for $C_9H_{11}N_2$: 147.09, found: 147.1

1,3-dimethylimidazolium (TM31) isolated via filtration to yield white solid 97%, 90% yield; ^{1}H NMR (499.73 MHz, D_2O, ppm): δ 8.64 (S, 1H), 7.41 (S, 2H), 3.884 (S, 6H)

1,3-dimethylbenzimidazolium-5-methyl ester (TM65) purified via HPLC (isocratic elution: 10% ACN in H_2O with 0.1% TFA) to yield white solid 98%, 40% yield; ^{1}H NMR (499.73 MHz, D_2O, ppm): δ 9.36 (S, 1H), 8.57 (S, 1H), 8.325 (D, 1H), 7.96 (D, 1H), 4.14 (6H, SS), 4.01 (S, 3H); MS (ESI) calcd for $C_9H_{13}N_2O_2$: 205.10, found: 205.10

1,3,5-triimethylbenzimdazolium (TM68) purified via HPLC (gradient elution: 15-95% ACN in H_2O with 0.1% TFA) to yield white solid 95%, 68% yield; ^{1}H NMR (499.73 MHz, D_2O, ppm): δ 9.07ppm (S, 1H), 7.67 (S, 1H), 7.55 (D, 1H), 7.72 (D, 1H), 4.05 (S, 6H), 2.56 (S, 3H); MS (ESI) calcd for $C_{10}H_{13}N_2$: 161.11, found: 161.11

5-nitro-1,3-dimethlybenzimidazolium (TM85) purified via HPLC (isocratic elution: 20% ACN in H$_2$O with 0.1% TFA) to yield yellow solid 98%, 65% yield; ^{1}H NMR (499.73 MHz, D$_2$O, ppm): δ 9.51 (S, 1H), 8.92 (S, 1H), 8.58 (D, 1H), 8.08 (D, 1H), 4.19 (S, 3H), 4.17 (S, 3H); MS (ESI) calcd for C$_9$H$_{10}$N$_3$O$_2$: 192.08, found: 192.08

3a,4,5,6,7,7a-hexahydro-1,3-dimethylbenzimidazolium-5-methyl ester (TM91) purified via HPLC (gradient elution: 10-95% ACN in H$_2$O) to yield white solid 98%, 85% yield; ^{1}H NMR (499.73 MHz, D$_2$O, ppm): δ 8.46 (S, 1H), 3.74-3.70 (9H, SSS), 3.08-3.02 (m, 1H) 2.95-2.83 (m, 3H), 2.69-2.66 (m, 3H), 2.28-2.22 (m, 1H), 2.07-1.99 (m, 1H); MS (ESI) calcd for C$_9$H$_{19}$N$_2$O$_2$: 211.14, found: 211.1

Solid phase peptide synthesis: in a glass fritted 2 port solid state synthesis vessel 300mg of chlorotrityl resin preloaded with Fmoc-L-isoleucine (approx. 0.1mmol) was allowed to swell in 5ml of dried DMF under an N$_2$ atmosphere with gentle agitation for 30 minutes. Next the residual DMF was pushed through the frit using N$_2$ gas pressure. The beads were then resuspended in approximately 5ml of 20% piperidine in Dry DMF. The beads were agitated under a nitrogen atmosphere for 5 minutes, before the solvent was removed via gas pressure. This step was repeated until cleavage of the Fmoc group was complete by ninhydrin assay (Kaiser test). Following successful FMOC cleavage, the solvent was pushed out via gas pressure and the beads were washed with several 5ml portions of dry DMF. While washing the beads, the coupling reaction mixture containing Fmoc-proline (0.5mmol) was set to stir in dry DMF with HATU (0.51mmol) and triethylamine (1.0mmol) in a separate vessel. The coupling reaction mixture was added to the gas dried beads and agitated under a nitrogen atmosphere for approximately 8 hours. Washing, cleavage and couplings steps were repeated for the addition of Fmoc-O-tert-butyl-L-tyrosine with no changes made to the reaction stoichiometry. After completing the coupling of tyrosine, simultaneous deprotection of the tyrosine O-tertbutyl and Fmoc protecting groups and cleavage was achieved by agitating the bead bound peptide in 5ml of 95% trifluoroacetic acid (TFA) for approximately 1 hour at room temperature. The cleaved crude tri-peptide, YPI, was

recovered via vacuum filtration. The effluent was concentrated under reduced pressure and purified via HPLC (gradient elution 2ml/min 10-95% ACN in H_2O with 0.1% TFA). The resulting white solid was characterized via mass spectrometry and proton NMR.

tyrosine-proline-isoleucine (YPI) purified via HPLC (gradient elution: 5-95% ACN in H_2O with 0.1% TFA) to yield white solid 98%, 35% yield (calculated overall); ^{1}H NMR (499.73 MHz, D_2O, ppm): δ 7.81 (m, 0.5H), 7.24-7.17 (m, 2.5), 6.907 (m, 2H), 4.10-3.04 (m, 6H), 2.29 (m,1H), 1.99-1.77 (m, 6H), 1.47 (m, 1H) 1.28 (m, 1H), 0.95-0.88 (m, 8H); MS (ESI) calcd for $C_{19}H_{27}N_3O_5$: 391.21, found 392.21

CALCULATIONS

The activation energy values (E_a) for compounds TM85, TM68 and TM65 were calculated using a modified Arrhenius equation[1] (Eq. 1). Where R is the ideal gas constant, $\ln(k_{ex})$ is the natural log of the observed rate of exchange, T is the temperature in kelvin and $\ln(A)$ is the pre-exponential factor associated with each of the reactions.

Eq. 1)

$$\ln(k_{ex}) = {}^{-E_a}\!/_R \cdot {}^1\!/_T + \ln A$$

The pD values for each exchange reaction were calculated using an empirical relationship[2] described by equations Eq. 2, Eq. 3. In Eq. 2 pH* is the pH of the deuterium rich reaction buffer as measured via glass electrode. In Eq. 3 pH is value calculated using Eq. 2 using the observed pH* measurement.

Eq. 2)

$$pH = 0.929 pH^* + 0.41$$

Eq. 3)

$$pD = pH \cdot 1.076$$

Adjusted pD values used to predict the salt effected rate of HDX for TM85 and YPI were calculated using equation Eq. 4. The adjusted pD values were calculated using empirically determined values for the activity of phosphate buffered water in the presence of sodium chloride via equations described by Voinescu and collegues[3,4].

Eq. 4)

$$pH\_adj = -\log\_10 \left(\left(kw \mathbin{/} \left([OH^\wedge -\,] \cdot \left((\gamma OH \cdot \gamma H\_3\,O) \mathbin{/} (\gamma H\_3\,O) \right) \right) \right) \right)$$

The $\log_{10}$ of the salt effected rate of HDX for TM85 was calculated using equation Eq. 5.

Eq. 5)

$$k_{ex} = \left(1.055 \cdot pH_{adj} \right) - 9.3705$$

CONSIDERATIONS FOR ERROR REPORTING

Variation in calculated exchange rates at a single reaction pD: NMR kinetics experiments were repeated in triplicate for compounds TM85, TM68 and TM65 at three reaction pD values. The standard deviation between the calculated rates at a single reaction pD was used to generate the vertical error bars in the main text figure 2, D. Where NMR kinetics experiments were carried out at different reaction temperatures or in the presence of salt or organic solvents and vertical error bars are shown, the standard deviation corresponding to the calculated rate at the reaction pD was used.

Variation in reaction pD: NMR kinetics experiments were repeated in triplicate for compounds TM85, TM68 and TM65 at three reaction pD values. To determine the standard deviation of reaction pD each set of three samples was measured using the same probe in succession.

Variation in NMR temperature: Actual NMR experimental temperatures were determined using the solvent chemical shift as described by Gottlieb et al[5]. Horizontal error bars used to describe variance in the NMR experimental temperature (main text Fig. 3, A) correspond to values reported in the aforementioned manuscript.

REFERENCES

(1)   Jensen, P. F.; Rand, K. D. Hydrogen Exchange. In *Hydrogen Exchange Mass Spectrometry of Proteins*; John Wiley & Sons, Ltd, 2016; pp 1–17. https://doi.org/10.1002/9781118703748.ch1.

(2)   Kręzel, A.; Bal, W. A Formula for Correlating PKa Values Determined in D2O and H2O. *Journal of Inorganic Biochemistry* **2004**, *98* (1), 161–166. https://doi.org/10.1016/j.jinorgbio.2003.10.001.

(3)   Voinescu, A. E.; Bauduin, P.; Pinna, M. C.; Touraud, D.; Ninham, B. W.; Kunz, W. Similarity of Salt Influences on the PH of Buffers, Polyelectrolytes, and Proteins. *J. Phys. Chem. B* **2006**, *110* (17), 8870–8876. https://doi.org/10.1021/jp0600209.

(4)   Harned, H. S.; Owen, B. B. The Physical Chemistry of Electrolytic Solutions, Reinhold Pub. *Corp., New York* **1958**, *354*.

(5)   Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities. *J. Org. Chem.* **1997**, *62* (21), 7512–7515. https://doi.org/10.1021/jo971176v.