# Metal Ion-Specific Screening of Charge Effects in Protein Amide H/D Exchange Follows the Hofmeister Series

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***Supporting Information***

**Materials and Methods:**

**Preparation of Lys-acetyl charge ladders for Mb:** Approximately 100 mg of Mb (isolated from horse heart, Sigma-Aldrich®, St. Louis, MO, USA) was dissolved in 150 mL of 100 mM HEPBS buffer (N-(2-Hydroxyethyl)piperazine-N'-(4-butanesulfonic acid)), pH 9.0. The resulting solution was divided into six fractions and increasing aliquots of 0.5 M acetic anhydride (prepared by dilution of pure acetic anhydride into 1,4-dioxane) were added to each of the six fractions. After acetylation, proteins were transferred to 100 mM citrate buffer (pH 7.4) using a centrifugal filtration device (5 kDa cutoff, Corning® Spin-X® UF, Corning, NY, USA). For this transfer, proteins were diluted ~ 20 fold into citrate buffer and concentrated. This concentration-dilution was repeated ~ 5 times and the final pH of the solution was measured to be pH 7.4. The six aliquots of progressively acetylated protein (Figure 1) were combined to obtain a 600 µM Mb charge ladder in a 100 mM citrate buffer, which was stored at -80 °C in 50 µL aliquots.

**Adjustment of pH of Mb solutions upon the addition of Hofmeister cations:** In order to counteract the drift of pH upon the addition of cations to $D_2O$ solutions, 50 mM Tris-DCl was incorporated into all of the $D_2O$/Hofmeister solutions made, allowing for more accurate readings from the pH electrode. The pD of each deuterated salt solution was verified to be at pD 7.8 before H/D exchange. The values of pD reported in this paper for salt solutions are values of $pH_{read}$ (reported by the pH meter) with a correction of 0.4 units added to the measured value (e.g., a solution with $pH_{read}$ = 7.4 is referred to as pD = 7.8).

**Hydrogen/Deuterium exchange measured by LC-ESI-MS:** In order to initiate H/D exchange, two 50 µL samples of the Mb charge ladder were removed from the freezer, thawed and centrifugally concentrated at 3500 × g for ~ 4 minutes using a centrifugal filtration device (5 kDa cutoff). The final concentration of Mb was 1.2 mM using UV-Vis spectroscopy ($\lambda_{max}$ = 280 nm, ε = 13,940 $M^{-1}.cm^{-1}$). A 45 µL aliquot of this Mb-Ac solution was added to 405 µL of 50 mM Tris-DCl (pH 7.4) in 99.9 % $D_2O$ (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). At the desired time point of the H/D exchange reaction (i.e., 5, 10, 15, 25, 35, 45, 60, 70, and 80 min) three aliquots (10 µL each) were flash frozen in liquid nitrogen (to quench H/D exchange) and stored at -80 °C for < 6 hours until mass spectrometric analysis.

Protein solutions that were frozen at various time points were removed from the freezer and instantly thawed, followed by the addition of 190 µL of ice-chilled 0.3 % formic acid to the 10 µL protein solution (i.e., a 1:20 dilution). The samples were then immediately injected into the LC-ESI-MS. We used a Waters Synapt ESI-HDMS (Waters Inc., Manchester, UK) to measure H/D exchange. The ESI-MS capillary voltage was 3.5 kV and the source temperature was 120 °C. At the end of the H/D exchange experiment, we also generated perdeuterated charge ladders (to quantify back-exchange) by heating 40 µL of Mb-Ac/$D_2O$ solutions in a Peltier device (Mastercycler® pro, Eppendorf, Hauppauge, NY, USA) at 70 °C for 10 minutes. Each sample was then equilibrated to room temperature (23 °C), from which a 20 µL aliquot of the denatured sample was injected into the ESI-MS after adding 380 µL (i.e., a 1:20 dilution) of ice-chilled 0.3 % formic acid solution.

The front of the mass spectrometer was equipped with a Rheodyne® valve that was submerged in ice, with a front syringe injection port. A 1 cm protein desalting column (Michrom BioResources, Inc., Auburn, CA, USA) was placed between the valve and the MS to trap the Mb-Ac and to permit the desalting of protein solutions prior to MS. After trapping the Mb-Ac, the column was rapidly washed with 1.5 mL of ice-chilled 0.3 % formic acid solution (in less than 10 seconds) in order to remove any salt or buffer residuals.

The Mb protein charge ladder was eluted from the HPLC column with the following mixture: 59.85 % acetonitrile, 39.85 %, $H_2O$, and 0.30 % formic acid. The flow rate of the HPLC system was 400 µL/min. The resulting mass spectra were deconvoluted using the MaxEnt 1 feature within MassLynx$^{TM}$ (Waters Corporation, Milford, MA, USA). The entire process of thawing solutions, desalting, and MS analysis required approximately 5 min and could be reproduced at a variability of ± 1 min.

**Results and Discussion:**

**Myoglobin retains its heme group upon acetylation.** The thermogram of unacetylated and acetylated Mb (Figure 2E) exhibited a $T_m$ = 80 °C for Mb-Ac(0) and $T_m$ = 68 °C for Mb-Ac(~16), which confirmed that Mb was in its holo state (the $T_m$ of unacetylated apo-Mb = 63 °C[1]). The 12 °C reduction in $T_m$ of holo-Mb that occurred upon acetylation does not suggest that acetylation dissociated the heme group, as these magnitudes of destabilization have been observed for other non-heme proteins.[2] Moreover, Mb retained its heme group upon acetylation as measured by the peak at $\lambda_{max}$ = 408 nm in UV-Vis spectra for both Mb-Ac(0) and Mb-Ac(~16) (Figure S5).

## References:


(1) Nelson, D. L.; Lehninger, A. L.; Cox, M. M. *Lehninger principles of biochemistry*; W.H. Freeman: New York, **2008**, *5*, 141.

(2) Shaw, B. F.; Arthanari, H.; Narovlyansky, M.; Durazo, A.; Frueh, D. P.; Pollastri, M. P.; Lee, A.; Bilgicer, B.; Gygi, S. P.; Wagner, G.; Whitesides, G. M. *J. Am. Chem. Soc.* **2010**, *132*, 17411-17425.


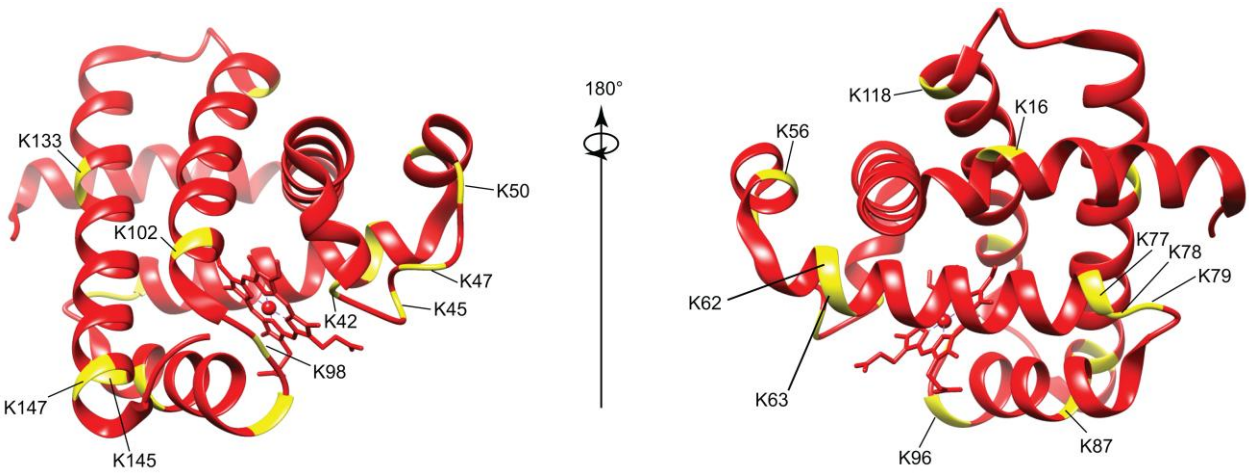


**Figure S1.** Secondary structure cartoon of holo-Mb (PDB ID: 1DWR) with all 19 lysine residues numbered (highlighted in yellow).

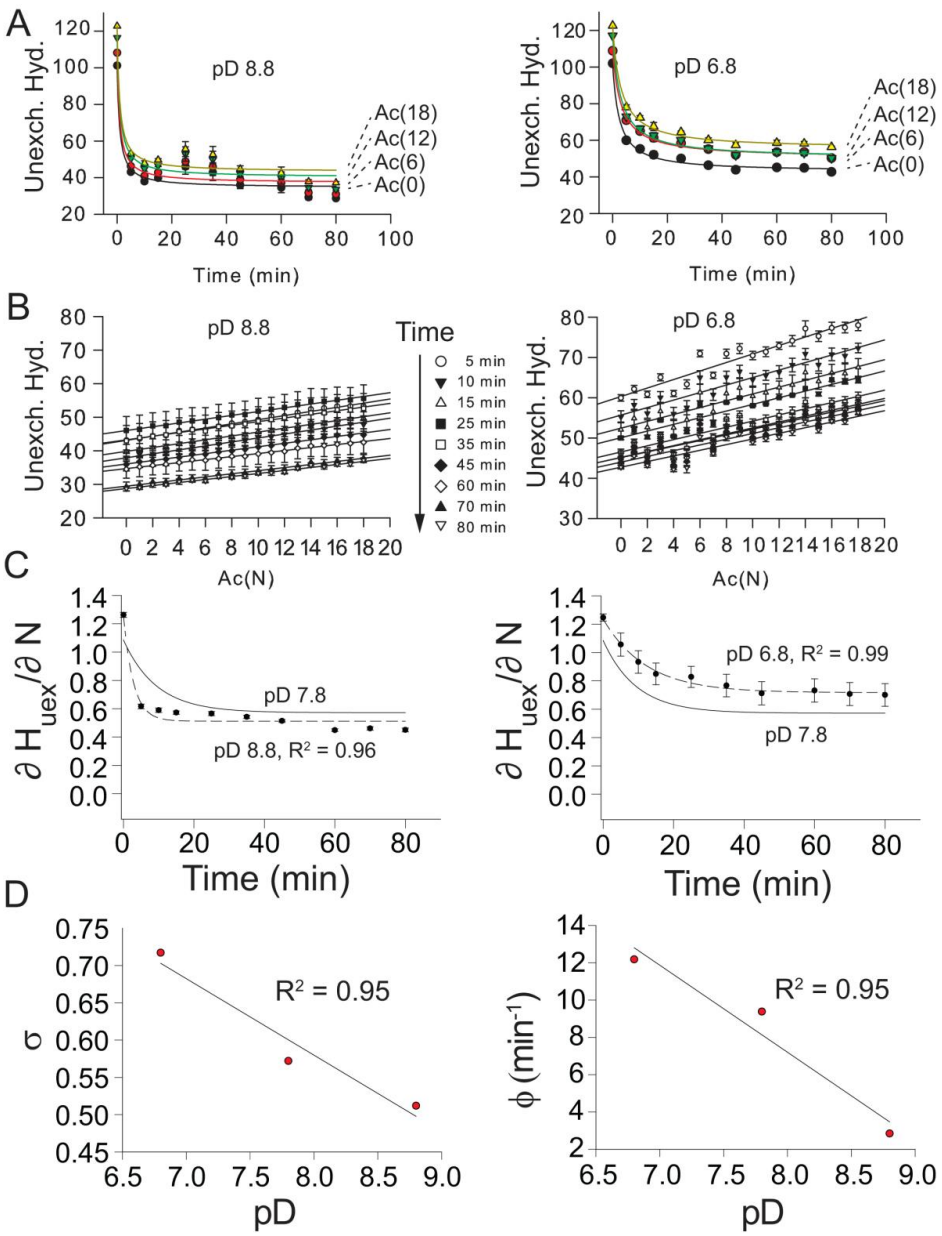


**Figure S2.** Amide H/D exchange of Mb-Lys-Ac(N) charge ladder measured at different pH with mass spectrometry (left column = pD 8.8; right column = pD 6.8). **(A)** Plots of H/D exchange as a function of time (0 M Hofmeister salt); pD = 7.8. Error bars are the standard deviation of three separate measurements. **(B)** Plots of the number of unexchanged hydrogens in each rung of the Mb charge ladder at each time point (5 min to 80 min in $D_2O$) versus the acetylation number, i.e., a plot of $\partial H_{uex}/\partial N$ for all 19 rungs. Error bars are the same standard deviation shown in part (A). **(C)** Plots of $\partial H_{uex}/\partial N$ (the slope of each line in part (A)) as a function of time. Error bars are the standard errors associated with the linear fit of $\partial H_{uex}/\partial N$ points from part (B). The longitudinal plot of $\partial H_{uex}/\partial N$ at pD 7.8 is shown as a solid black line in both panels as reference. **(D)** Plots of "penetration" constant ($\sigma$) and "shielding" decay constant ($\phi$) as a function of pD.

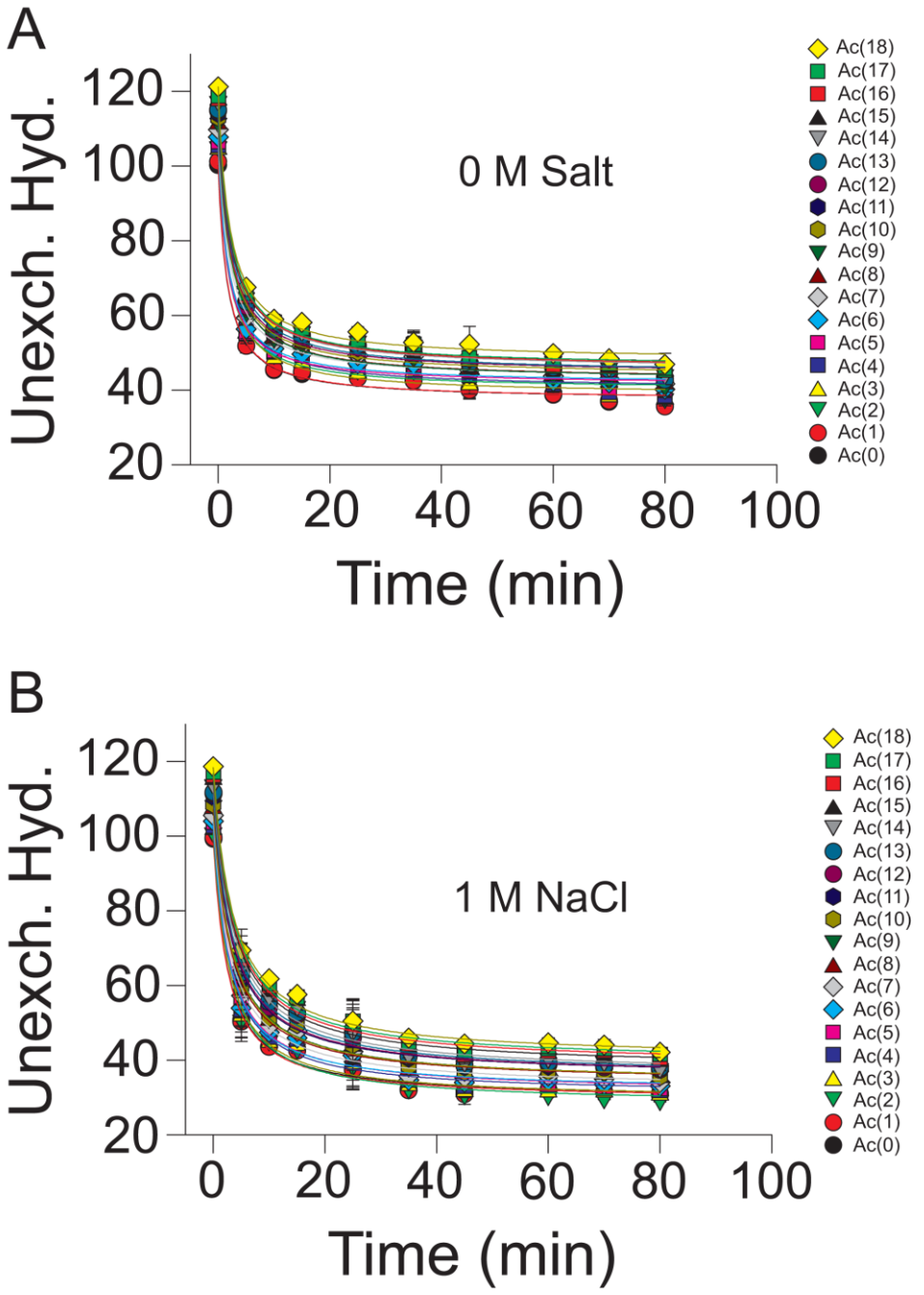


**Figure S3.** Amide H/D exchange for all 19 rungs of the Mb charge ladder in the **(A)** absence and **(B)** presence of 1 M NaCl; pD = 7.8.

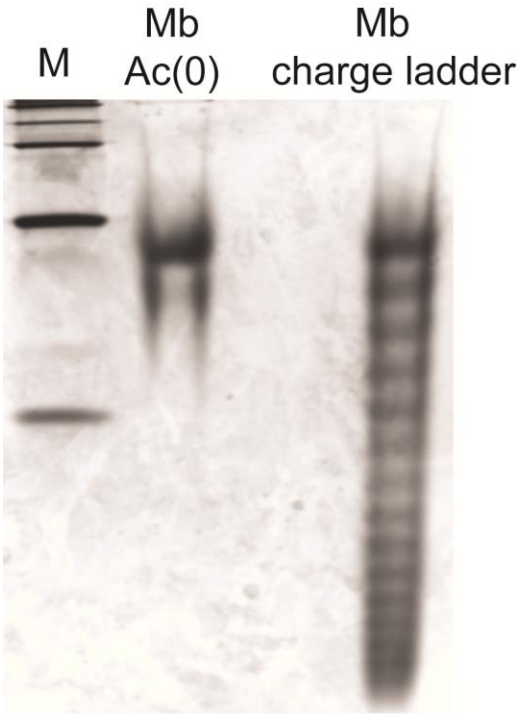


**Figure S4.** Native PAGE (15 %) of unacetylated Mb and a Lys-acetyl charge ladder of Mb. The mobility of each rung of the ladder increases with acetylation. Electrophoresis was performed with constant voltage (90 V) at 4 °C. M represents the native PAGE standard marker.

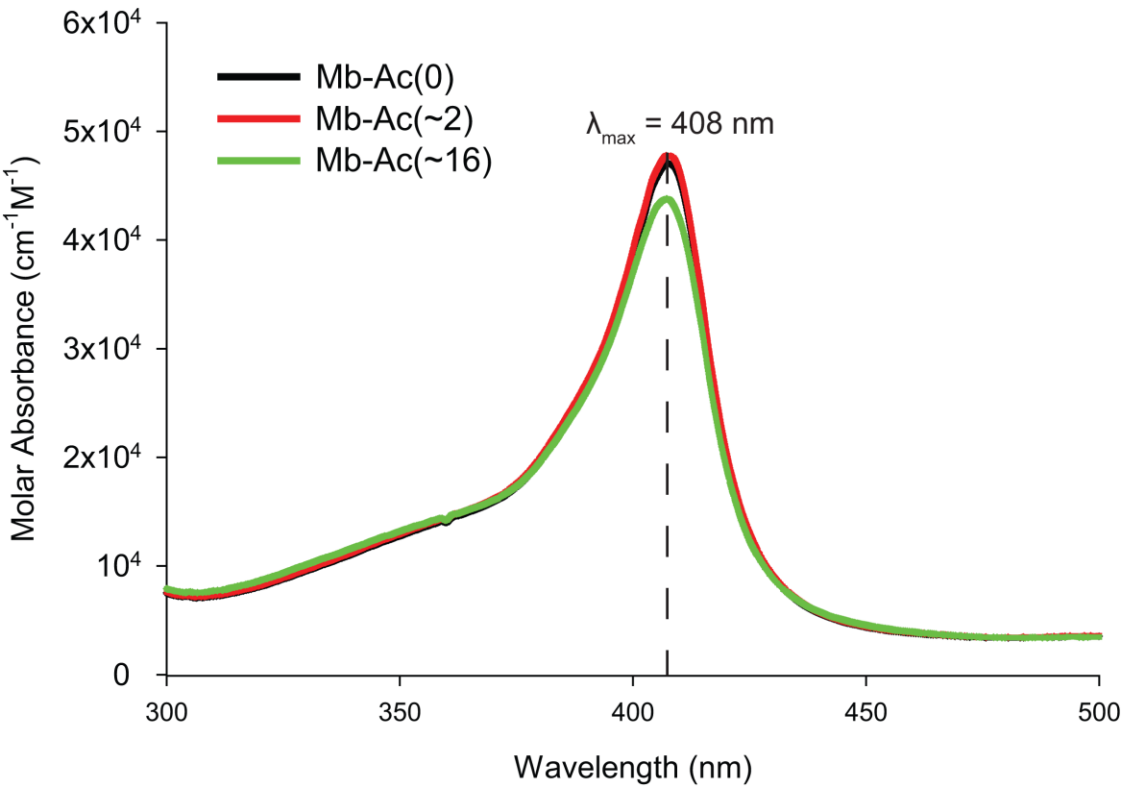


**Figure S5.** UV-Vis spectra of Mb-Ac(0), Mb-Ac(~2), and Mb-Ac(~16). The peak at $\lambda_{max}$ = 408 nm confirmed the presence of the prosthetic heme group in Mb, and that acetylation did not cause the heme group to dissociate from Mb.

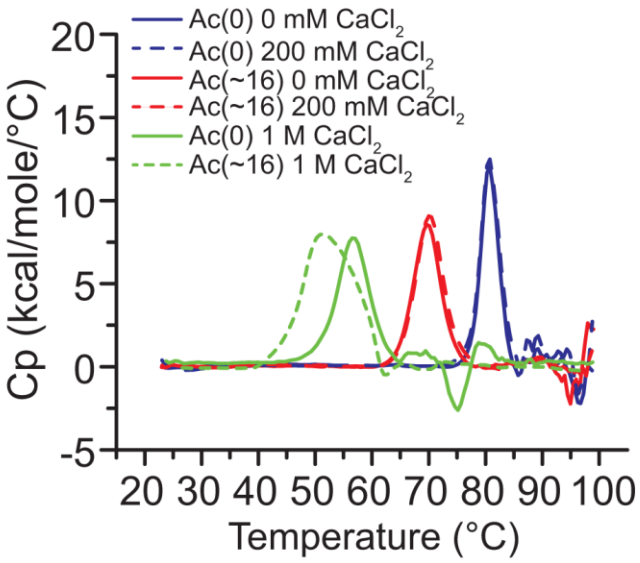


**Figure S6**. Differential scanning calorimetry of unacetylated and acetylated (i.e., Ac(~16)) Mb in the presence of 0 M, 200 mM, and 1 M $CaCl_2$.

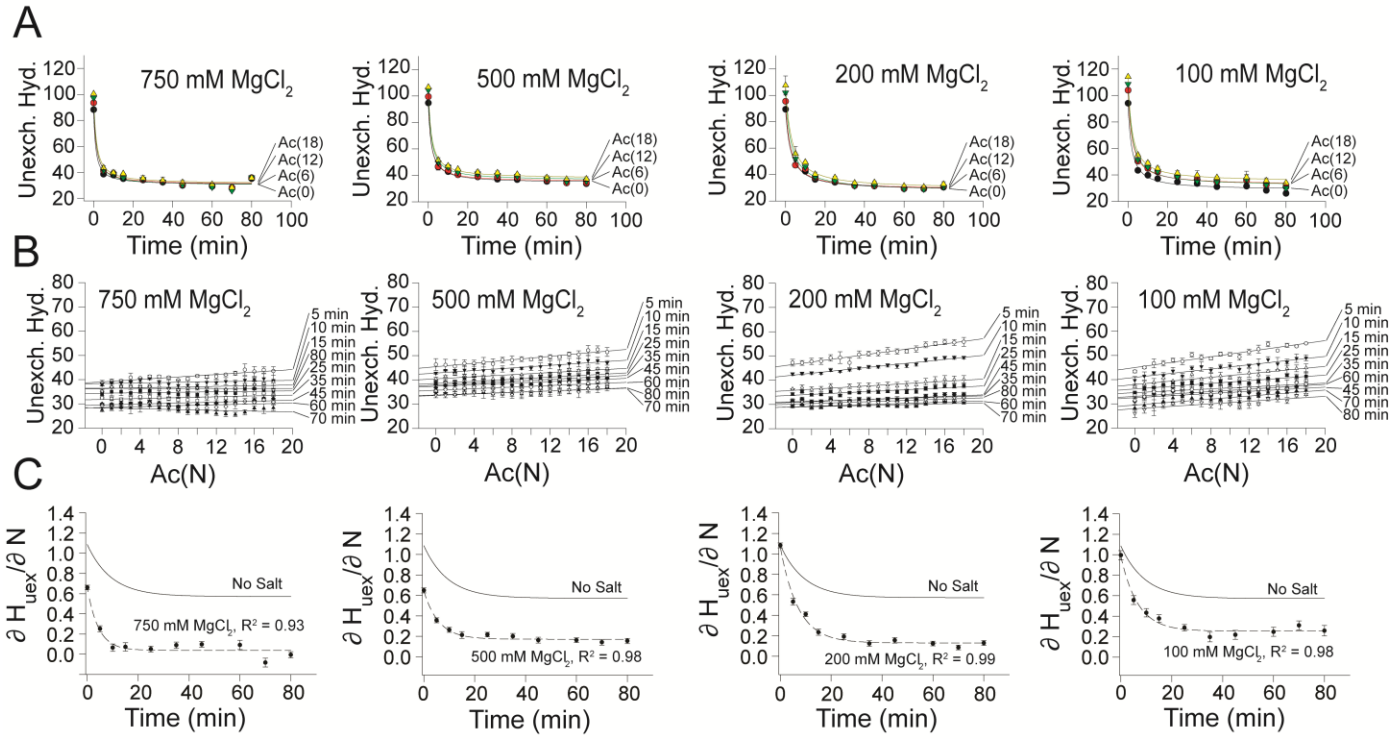


**Figure S7. (A)** Amide H/D exchange of Mb-Lys-Ac(N) charge ladder at diminishing concentrations of $MgCl_2$. Error bars represent standard deviation from three separate measurements. **(B)** Partial derivatives of plots in part (A); error bars represent the standard deviation of three separate runs. **(C)** Plots of partial derivative as a function of time; in each panel, solid line represents partial derivative of Mb-Lys-Ac(N) charge ladder in 0 mM $Mg^{2+}$ shown for reference. Error bars are the standard errors associated with the linear fit of $\partial H_{uex}/\partial N$ points from panel (B).

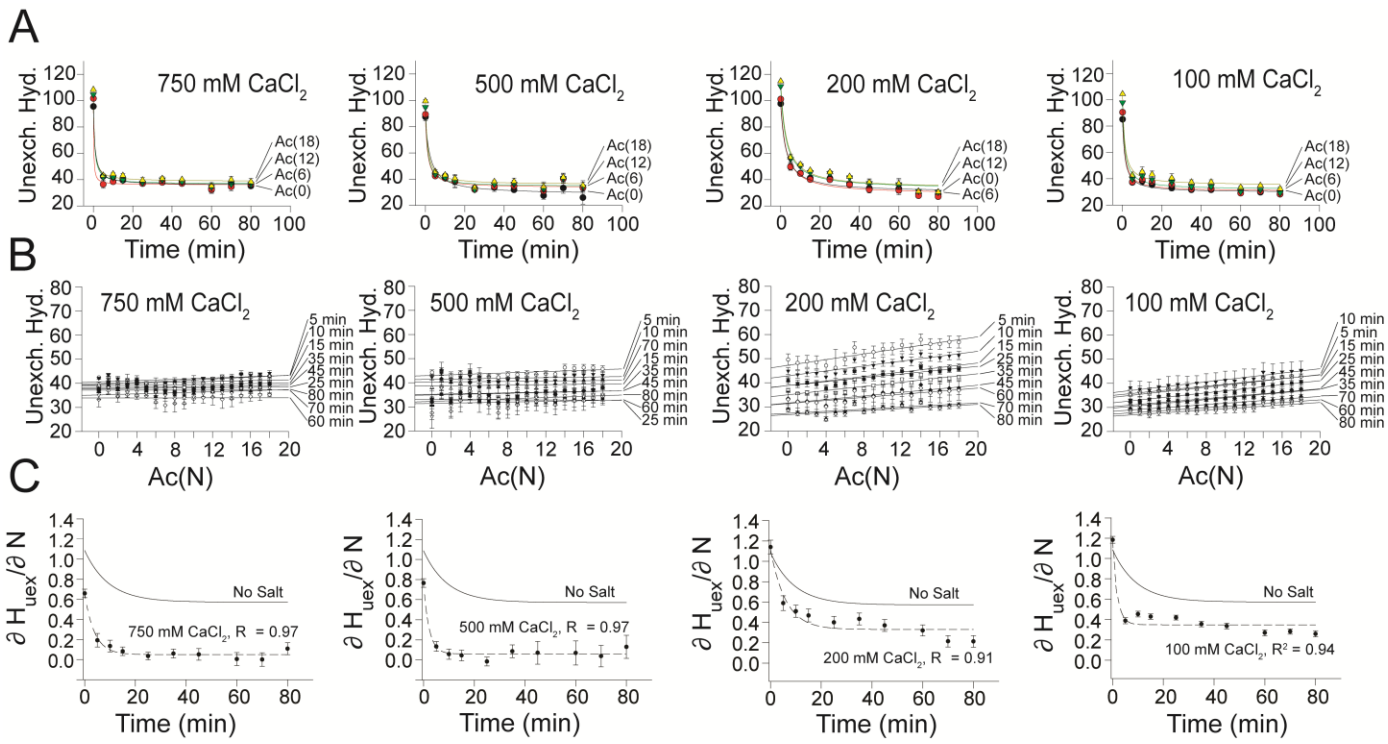


**Figure S8. (A)** Amide H/D exchange of Mb-Lys-Ac(N) charge ladder at diminishing concentrations of $Ca^{2+}$. Error bars represent standard deviation from three separate measurements. **(B)** Partial derivatives of plots in part (A); error bars represent the standard deviation of three separate runs. **(C)** Plots of partial derivative as a function of time; solid line in each panel represents partial derivative of Mb-Lys-Ac(N) charge ladder in 0 mM $Ca^{2+}$ shown as reference. Error bars represent standard error from the linear fit in part (B).