# Supplemental Information

## Neutralizing positive charges at the surface of a protein lowers its rate of amide hydrogen exchange without altering its structure or increasing its thermostability.

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## Supplemental Materials and Methods

*H/D exchange measured by ESI-MS.*

Immediately prior to hydrogen exchange, protein aliquots were thawed and concentrated to approximately 20 mg/mL in a Microcon centrifugal filtration device (MW 10,000; Millipore). Concentrated protein samples were transferred to PCR tubes and incubated at 15 ºC for 5 minutes and then quickly diluted 1:10 (v/v) into a deuterated phosphate buffer (10 mM, pD 7.4, 15 ºC; prepared as previously described [10]). The pD value is approximated by adding 0.4 units to the value that is measured by a pH electrode. [25] During isotopic exchange, the temperature was maintained at 15 ºC with a Peltier-effect device (MJ Research). At various time points, aliquots (5µL) were removed and isotopic exchange was quenched for later analysis with ESI-MS. Quenching was done by diluting deuterated protein samples (1:10; v/v; 5 µL:50 µL) into cold, low pH buffer (100 mM

phosphate; pH 2.4, 0 ºC) followed by immediate flash freezing in $N_2$ (l) and storage for < 24 hr at -80 ºC.

Immediately prior to mass spectral analysis, each protein sample was thawed and loaded into an ice-chilled Rheodyne injector that was attached to an ice-chilled column for desalting (MacroTrap, Michrom Inc.). The injector was connected to the electrospray ion source and HPLC pump. The desalting column was washed with ice-chilled 0.3 % formic acid/$H_2O$ (v/v) prior to sample loading. For the purpose of desalting an additional 500 µL of 0.3 % formic acid/$H_2O$ (v/v) was passed through the MacroTrap immediately after sample loading. The injector was then immediately turned to the "inject" position and proteins were eluted from the trap with an ionization solution consisting of formic acid/$H_2O$/acetonitrile (0.3/49.8/49.8 % v/v). This solution was delivered to the electro-spray ion source by an HPLC pump (running at 80 µL/min). Mass spectra were consistently acquired less than 2 minutes after thawing of protein samples. Mass spectrometric data was collected with a Q-STAR hybrid quadrupole-time of flight mass spectrometer (Applied Biosystems). Calibrations were performed daily using the +2 and +3 charges state of angiotensin.

The rate of deuterium incorporation was monitored as a function of time. The value "number of unexchanged hydrogen" ($H_{unex}$) was calculated by subtracting the mass of the protein after a given time, t, in deuterated buffer and under conditions that favor the native state (denoted $M[D]_{Native,t}$) from the mass of the thermally unfolded (and therefore perdeuterated) protein ( denoted $M[D]_{Unfolded}$).

$$H_{unex} = M[D]_{Unfolded} - M[D]_{Native, t}$$

The following sum of three exponentials was fit to the hydrogen exchange data for all 19 rungs of the charge ladder:

$$M_{D,t} = M_{D, \infty} - Ae^{-k_1 t} - Be^{-k_2 t} - Ce^{-k_3 t}$$

Where $M_{D,t}$ = mass at each time point in deuterated buffer; $M_{D, \infty}$= mass of completely exchanged protein; A, B and C denote the number of amide hydrogens undergoing exchange with fast ($k_1 > 1$ min$^{-1}$), intermediate ($0.01$ min$^{-1}< k_2 < 1$ min$^{-1}$) and slow ($k_3 < 0.01$ min$^{-1}$) rate constants. The presence of the slow-exchanging amide hydrogen atoms proved troublesome in the curve fitting. In order to make the fittings converge, an artificial endpoint time and mass value (t = 43,200 min, mass = $M_{D, \infty}$) was added to each data set.

Back exchange controls were performed as previously described.[10] The back-exchange control samples are necessary because 1) it accounts for the loss of incorporated deuterium that occurs during quenching, desalting and mass spectral analysis and 2) it accounts for the increased number of exchangeable amide protons that can be expected as a result of lysine acetylation. Back-exchange controls were done by heating the same BCA II protein solutions that were used to collect mass-time points at 80 ºC for 10 min. These back-exchange control samples were then cooled and incubated at 15 ºC for 10 min before being quenched and frozen as described above.

*HSQC and TROSY enhanced NMR analysis of carbonic anhydrase II.*
The spectral widths were 16.033 ppm for proton (centered on the water resonance at 4.690 ppm) and 36 ppm for nitrogen (centered at 118 ppm). A recycling delay of 0.9 s was used and 16 scans were accumulated per increment; 512 ($^1$H) × 128 ($^{15}$N) complex

points were recorded. The total measuring time was 1 hour for each experiment. The data was processed with the NMRPipe package. The $^{15}N$ dimension was linear predicted and zero filled to a final size of 1024 ($^1H$) × 1024 ($^{15}N$). The resulting NMR spectrum was analyzed using the software CARA (available from http://www.nmr.ch/) and intensities of the peaks were measured using the program Sparky.

Separately, the spectral widths were 16.033 ppm for proton (centered on the resonance of water at 4.70 ppm) and 5 ppm for nitrogen (centered at 128 ppm). A recycling delay of 0.9 s was used and 16 scans were accumulated per increment; 512 ($^1H$) × 24 ($^{15}N$) complex points were recorded. The total measuring time was (15 min) for each experiment. The data was processed with the NMRPipe package. The $^{15}N$ dimension was linearly predicted and zero filled to a final size of 1024 ($^1H$) × 128 ($^{15}N$)

*Preparation of model lysine and -ε-NH₃ acetylated lysine molecules.*
Reagent **1** (below) was purchased from Acros, Inc., and PS-DIEA and MP-TsOH were obtained from Biotage, Inc. All other reagents were purchased from Sigma-Aldrich and were used as received. To a solution of 30 mg Ac-Lys($\varepsilon$-$NH_3^+$)-NHMe (0.15 mmol, 1.0 eq) in 1.5 mL anhydrous DMF was added 150 mg of PS-DIEA (4 mmol/g loading, 0.6 mmol, 4 eq) and 34 uL acetic anhydride (0.36 mmol, 2.4 eq). The reaction was stirred for 3 hours, and 133 mg MP-TsOH was added (4.5 mmol/g loading, 0.6 mmol, 4 eq) to scavenge any unreacted amine. The reaction mixture was filtered, and the solids were rinsed with DMF. The combined filtrate was evaporated to dryness to provide an analytically pure sample of **2** in 83% yield (30.3 mg), a white solid (mp: 183-184). $^1H$ NMR (CD₃OD, 300 MHz): 4.22 (dd, $J_1$=8.8 Hz; $J_2$= 6.4 Hz, 1H), 3.15 (t, J= 6.9 Hz, 2H),

2.72 (s, 3H), 1.98 (s, 3H), 1.92 (s, 3H), 1.75 (m, 1H), 1.63 (m, 1H), 1.50 (m, 2H), 1.35 (m, 2H). $^{13}$C NMR (CD$_3$OD, 125 MHz): 173.8, 172.2, 172.0, 53.7, 39.0, 31.5, 28.8, 25.1, 23.1, 21.3, 21.2. HRMS (MH$^+$): Calculated: 244.1661; Found: 244.1668.
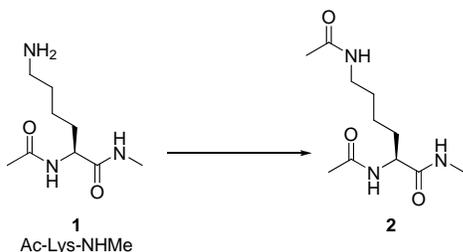


*Measuring the rate of amide H/D exchange of model lysine amino acids with $^1$H NMR spectroscopy.*

HPLC purified compounds 1 and 2 were dissolved separately in 200 mM acetate-d$_3$ buffer (pH 3.95) at a concentration of 10 mg/mL. After each compound was dissolved, the pH of each solution was measured again and adjusted to pH = 3.95. Hydrogen exchange was initiated by diluting an aliquot of each solution (45 μL) into 450 μL of D$_2$O. Analysis with NMR spectroscopy began immediately after dilution and the first data point was typically collected at 4 min 53 s after dilution into D$_2$O. The exchange of the backbone amide hydrogen with deuterium was monitored by the disappearance of amide signals at approximately 8.1 and 7.8 ppm. The final pH of each solution was measured after analysis and found to be pH=4.08 (pD ≈ 4.5). Each time point was an average of 16 scans collected over 60 s. A zero time point was collected by diluting an identical aliquot into 95% H$_2$O, 5% D$_2$O; 5 % of the integrated signal intensity was added back to the measured integral of signal intensity in order to account for decreased signal that arises from 5 % D$_2$O.

**Supplemental Results**


*Kinetic analysis of hydrogen exchange data derived from LC-ESI-MS.*

The global exchange of amide hydrogen in proteins, when measured by ESI-MS, is typically analyzed by fitting a tri-exponential function to the kinetic data; this analysis yields parameters in terms of "fast", "intermediate", and "slow" exchanging amide hydrogen. We fit the kinetic data for all 19 rungs of the charge ladder to the following tri-exponential function:


$$M[D]_{native} = M[D]_{unfolded} - Ae^{-k_1 t} - Be^{-k_2 t} - Ce^{-k_3 t} \quad (S1)$$


In equation S1, $M[D]_{native}$ equals the measured mass of folded BCA II per time in deuterated buffer; $M[D]_{unfolded}$ equals the mass of the perdeuterated protein (i.e., the mass of the unfolded protein in deuterated buffer). The pre-exponential factors A, B and C denote the number of amide hydrogen that are undergoing exchange at a relatively fast ($k_1 > 1$ min$^{-1}$), intermediate ($0.01$ min$^{-1} < k_2 < 1$ min$^{-1}$) or slow ($k_3 < 0.01$ min$^{-1}$) rate. For BCA-Ac(0), $k_1 = 5.8$ min$^{-1}$, $k_2 = 1.9 \cdot 10^{-1}$ min$^{-1}$, and $k_3 = 1.1 \cdot 10^{-3}$ min$^{-1}$ (Supplemental Table 2). The pre-exponential coefficients for BCA-Ac(0) are: A =36.2, B =24.3 and C =96.4 (Supplemental Table 2). These three values are interpreted to mean that: approximately 36 hydrogen underwent exchange at a fast rate ($k_1 > 1$ min$^{-1}$), approximately 24 hydrogen exchange at an intermediate rate ($0.01$ min$^{-1} < k_2 < 1$ min$^{-1}$) and approximately 96 are undergoing slow exchange ($k_3 < 0.01$ min$^{-1}$). We consider the 96 slowest exchanging hydrogen to be "protected" from solvent exchange. The sum of A,

B, and C, for BCA-Ac(0) is 156.9 and this is the number of amide hydrogen whose exchange can be measured using our mass spectrometric apparatus and methods.[49] These six kinetic parameters express the effects of lysine acetylation on the rate of H/D exchange for BCA II (Supplemental Table 2 lists these parameters for all 19 rungs of the charge ladder). For example, the three rate constants, $k_1$, $k_2$, and $k_3$ are equal for all rungs of the charge ladder. Two of the pre-exponential coefficients (A and B) are also similar, meaning that each rung of the charge ladder has a similar number of "fast" and "intermediate" exchanging hydrogen. The value of C, however, increases by approximately 1 for each higher rung of the charge ladder, meaning that the addition of 1 acetyl group results in the addition of 1 hydrogen that exchanges with a first order rate constant of $k \leq 1 \cdot 10^{-3}$ $min^{-1}$.
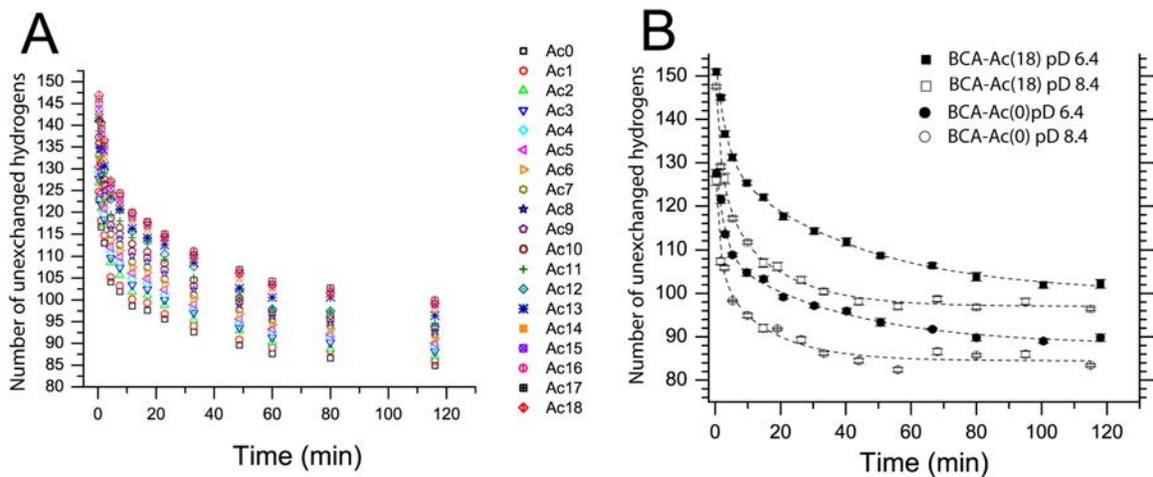
**Supplemental Figure 1. Lysine acetylation decreases the rate of H/D exchange of BCA II as measured by ESI-MS.** A) H/D exchange kinetics of the BCA II charge ladder (90 % $D_2O$, pD 7.4, 15 °C). Data for all 19 rungs of the charge ladder are shown here. BCA-Ac(0) retained ~85 unexchanged hydrogen after 100 minutes in $D_2O$. B) Lysine acetylation does not alter the mechanism of hydrogen exchange in BCA II. H/D exchange kinetics for BCA-Ac(0) and BCA-Ac(18) at pD 6.4 and 8.4 (15 °C). The rate of exchange of both proteins was similarly dependent upon the pD of solvent which suggests that both proteins exchange similarly via an EX2 mechanism. Error bars represent the standard deviation of average mass values calculated from seven charge states for each protein.
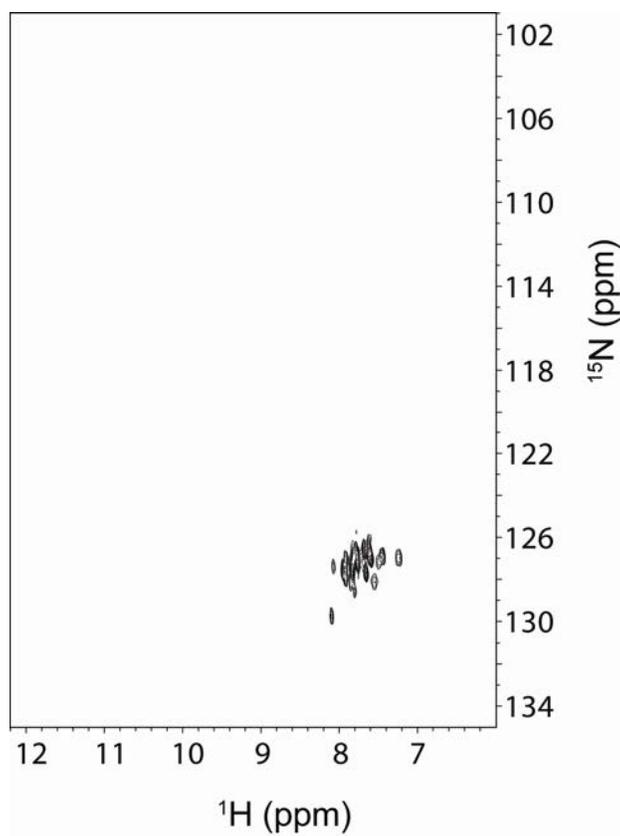
**Supplemental Figure 2.** The N-H plane(2D-HN-HNCO) of a TROSY-HNCO spectrum of peracetylated HCA II. Protein was acetylated with ^{13}C labeled acetic anhydride. Each signal in this spectrum corresponds to an [15]N-^{1}H group that is coupled to a [13]C=O.
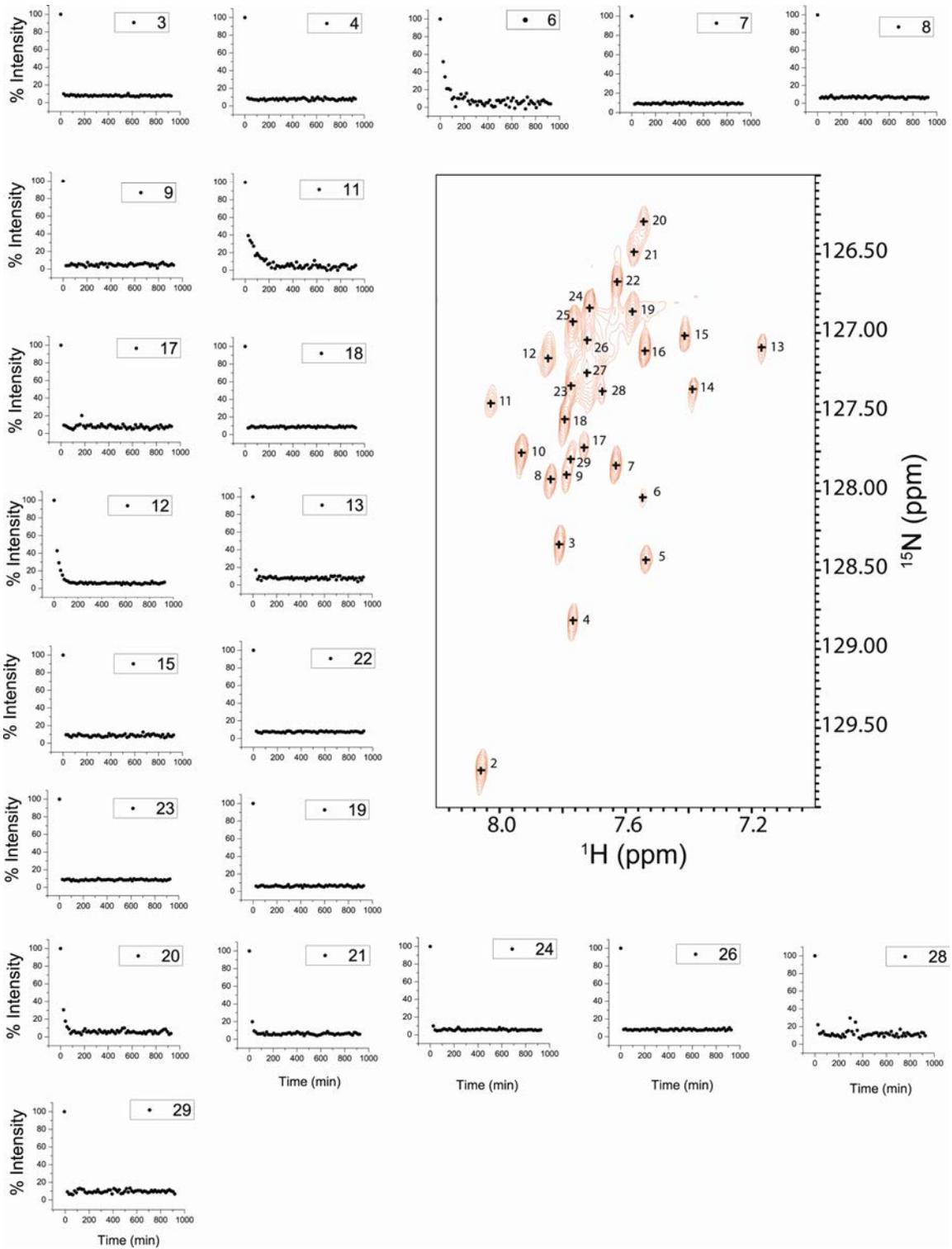
**Supplemental Figure 3.** Amide H/D exchange of acetyl groups in peracetylated HCA II measured by TROSY (15 °C, pD 7.4, 90 % $D_2O$, 10 mM $PO_4^{3-}$). The intensity of most peaks decreases to 5-10 % intensity after $< 27$ minutes in $D_2O$ suggesting that the amide groups of acetyl functionalities are undergoing H/D exchange rapidly.
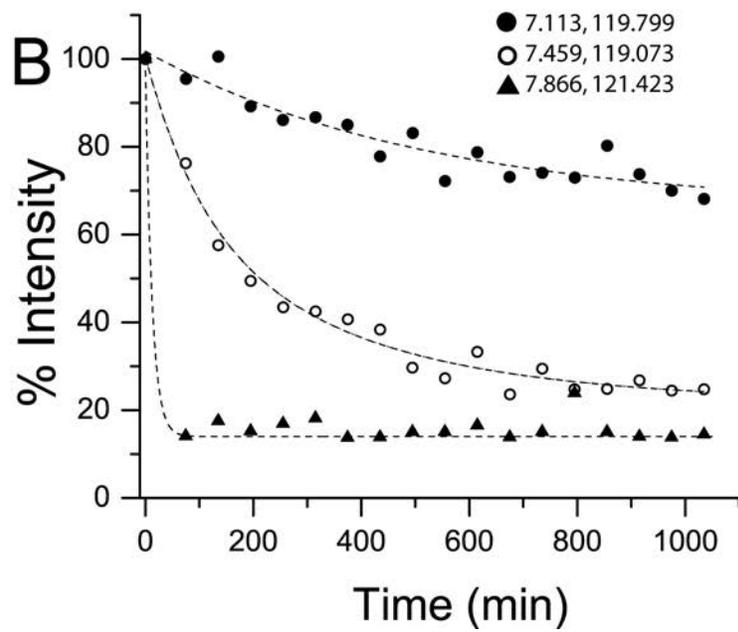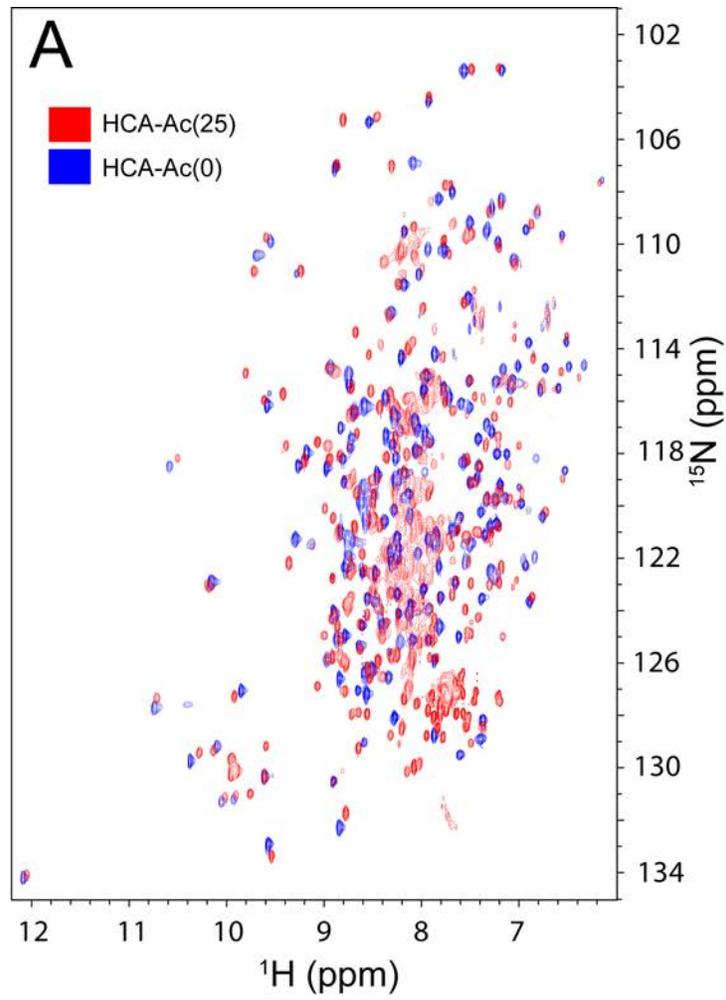
**Supplemental Figure 4.** H/D exchange of backbone amides in peracetylated HCA II measured by HSQC NMR. A. TROSY-HSQC spectra of unmodified and peracetylated HCA II. B) Three amide signals were chosen from A based upon their observed exchange (e.g., "fast", "medium", "slow").

| Ac | M[H] (mass in $H_2O$) | M[D]$_{Native}$ (mass in $D_2O$, 80 min, 15 ºC) | M[D]$_{Native}$ - M[H] (# in-exchanged deuterons, 80 min, 15 ºC) | M[D]$_{Unfolded}$ (mass in $D_2O$, 80 ºC) | M[D]$_{Unfolded}$ - M[D]$_{Native}$ (# unexchanged hydrogen, 80 min, 15 ºC) | M[D]$_{Unfolded}$ - M[H] (maximum # exchangeable hydrogen) |
|---|---|---|---|---|---|---|
| 0 | 29028.9 | 29100.0 | 71.1 | 29185.8 | 85.8 | 156.9 |
| 1 | 29070.4 | 29143.8 | 73.4 | 29231.0 | 87.2 | 160.6 |
| 2 | 29112.1 | 29185.5 | 73.4 | 29273.7 | 88.1 | 161.6 |
| 3 | 29153.7 | 29226.0 | 72.3 | 29315.2 | 89.3 | 161.5 |
| 4 | 29195.2 | 29267.6 | 72.4 | 29358.1 | 90.5 | 162.9 |
| 5 | 29237.7 | 29309.6 | 71.9 | 29400.9 | 91.3 | 163.2 |
| 6 | 29280.2 | 29351.8 | 71.6 | 29444.1 | 92.3 | 163.9 |
| 7 | 29321.9 | 29393.5 | 71.6 | 29486.7 | 93.2 | 164.8 |
| 8 | 29363.6 | 29435.4 | 71.8 | 29529.4 | 94.0 | 165.8 |
| 9 | 29405.2 | 29477.2 | 72.0 | 29572.0 | 94.8 | 166.8 |
| 10 | 29446.9 | 29519.2 | 72.3 | 29614.9 | 95.7 | 168.0 |
| 11 | 29488.4 | 29561.1 | 72.7 | 29657.6 | 96.4 | 169.2 |
| 12 | 29530.0 | 29603.5 | 73.5 | 29700.9 | 97.4 | 170.9 |
| 13 | 29571.3 | 29645.2 | 73.9 | 29744.7 | 99.5 | 173.4 |
| 14 | 29613.5 | 29686.8 | 73.3 | 29787.7 | 100.9 | 174.2 |
| 15 | 29655.1 | 29728.4 | 73.3 | 29829.6 | 101.2 | 174.5 |
| 16 | 29696.8 | 29770.4 | 73.6 | 29872.1 | 101.6 | 175.3 |
| 17 | 29738.6 | 29813.0 | 74.4 | 29914.9 | 101.9 | 176.3 |
| 18 | 29780.1 | 29855.3 | 75.2 | 29956.5 | 101.2 | 176.4 |

**Supplemental Table 1. Experimentally determined mass values.** "M[H]" is the measured mass of BCA-Ac(N) in $H_2O$. "M[D]$_{Native}$" is the mass of folded BCA-Ac(N) after 80 min in deuterated buffer (pD 7.4, 15 ºC). The difference between "M[D]$_{Native}$" and "M[H]" yields the number of deuterons that are incorporated into BCA-Ac(N) at 80 min, pD 7.4, 15 ºC. "M[D]$_{Unfolded}$" is the mass of each rung in deuterated buffer under thermally denaturing conditions and expresses the highest possible mass of each rung under the experimental conditions. The difference between "M[D]$_{Unfolded}$" and "M[D]$_{Native}$" yields the number of unexchanged protons in BCA-Ac(N) at 80 min (i.e. the number of hydrogens that are protected from exchange with solvent after 80 min). The difference between "M[H]" and "M[D]$_{Unfolded}$" yields the maximum number of deuterons that can be incorporated into each rung, as determined by our methods and apparatus. All mass values are in Daltons (Da). Mass values were determined from the average of at least 7 different charge states in each mass spectrum, resulting in errors that are typically < 1 Da.

| Ac | A | B | C | $k_1$ (min$^{-1}$) | $k_2$ ($10^{-1}$min$^{-1}$) | $k_3$ ($10^{-3}$min$^{-1}$) |
|---|---|---|---|---|---|---|
| 0 | 36.2 (3.6) | 24.3 (2.9) | 96.4 (2.7) | 5.8 (1.9) | 1.9 (0.5) | 1.1 (0.3) |
| 1 | 37.9 (4.0) | 24.4 (3.2) | 98.2 (2.9) | 5.9 (2.2) | 1.9 (0.6) | 1.3 (0.3) |
| 2 | 37.0 (3.7) | 25.1 (3.1) | 99.4 (3.2) | 5.9 (1.9) | 1.7 (0.6) | 1.3 (0.2) |
| 3 | 37.1 (3.6) | 24.3 (2.9) | 100.1 (3.2) | 5.4 (1.7) | 1.4 (0.4) | 1.2 (0.3) |
| 4 | 37.7 (3.4) | 24.5 (2.9) | 100.7 (3.4) | 5.1 (1.4) | 1.3 (0.4) | 1.1 (0.3) |
| 5 | 36.8 (3.5) | 24.5 (2.9) | 101.9 (3.4) | 5.0 (1.5) | 1.3 (0.4) | 1.1 (0.3) |
| 6 | 36.6 (3.4) | 24.7 (3.0) | 102.7 (3.6) | 4.8 (1.3) | 1.1 (0.4) | 1.1 (0.3) |
| 7 | 36.4 (3.3) | 24.9 (2.9) | 103.4 (3.6) | 4.7 (1.3) | 1.1 (0.3) | 1.1 (0.3) |
| 8 | 36.2 (3.3) | 24.7 (3.0) | 104.8 (3.6) | 4.6 (1.2) | 1.1 (0.3) | 1.1 (0.4) |
| 9 | 36.1 (3.3) | 25.2 (3.0) | 105.5 (3.7) | 4.6 (1.2) | 1.1 (0.3) | 1.1 (0.4) |
| 10 | 36.2 (3.3) | 25.4 (3.2) | 106.5 (3.9) | 4.4 (1.2) | 1.0 (0.3) | 1.1 (0.4) |
| 11 | 37.2 (3.3) | 27.7 (4.3) | 104.3 (5.2) | 4.0 (1.0) | 0.7 (0.2) | 0.8 (0.5) |
| 12 | 37.4 (3.1) | 27.4 (4.3) | 106.0 (5.3) | 3.8 (0.9) | 0.7 (0.2) | 0.9 (0.5) |
| 13 | 38.9 (3.3) | 26.8 (4.0) | 107.6 (4.9) | 3.9 (0.9) | 0.8 (0.3) | 0.9 (0.5) |
| 14 | 36.9 (2.9) | 25.4 (2.9) | 111.8 (3.6) | 4.6 (1.1) | 1.0 (0.2) | 1.2 (0.3) |
| 15 | 35.4 (3.1) | 26.0 (2.9) | 113.1 (3.5) | 4.7 (1.2) | 1.1 (0.3) | 1.2 (0.3) |
| 16 | 35.3 (3.2) | 26.5 (3.1) | 113.5 (3.7) | 4.5 (1.2) | 1.0 (0.3) | 1.2 (0.3) |
| 17 | 35.1 (3.1) | 27.4 (2.9) | 113.8 (3.6) | 4.5 (1.2) | 1.0 (0.3) | 1.2 (0.3) |
| 18 | 34.8 (3.0) | 29.2 (2.9) | 112.5 (3.6) | 4.3 (1.2) | 1.0 (0.2) | 1.1 (0.3) |

**Supplemental Table 2.** Kinetic parameters of hydrogen exchange data for the BCA II charge ladder generated from a least squares fit of the tri-exponential function: $M[D]_{native} = M[D]_{unfolded} - Ae^{-k_1 t} - Be^{-k_2 t} - Ce^{-k_3 t}$ where $M[D]_{folded}$ = mass of protein at each time point in deuterated buffer. $M[D]_{unfolded}$ = the mass of the perdeuterated protein (i.e. the mass of the unfolded protein in 90 % $D_2O$). The pre-exponential factors A, B, and C denote the number of amide hydrogens that are undergoing exchange at fast ($k_1 > 1$ min$^{-1}$), intermediate (0.01 min$^{-1} < k_2 < 1$ min$^{-1}$) and slow ($k_3 < 0.01$ min$^{-1}$) rates. Standard errors for all values are listed in parentheses.