Myoglobin Conformational Dynamics studied ESI-MS and H/D Exchange

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Introduction: Proteins in solution exist in equilibrium between the native state and various unfolded conformations. In a partially denaturing solvent, the protein population is a structurally heterogeneous ensemble, undergoing conformational changes on nanosecond to minute time-scales. Hydrogen-deuterium exchange (HDX) is a powerful strategy for monitoring structural dynamics because the kinetics of deuterium uptake by proteins is mediated by conformational fluctuations. Furthermore, the use of ESI-MS to monitor HDX results in the addition of a second, complementary probe of protein structure; ESI is sensitive to protein conformation, and allows monitoring of noncovalent ligand-binding. In this work, two ESI-MS HDX strategies are used to examine the conformation dynamics of myoglobin under partially denaturing conditions. Time-resolved ESI-MS is a continuous-flow technique which allows online monitoring of H/D exchange, at selected times, following the initiation of an isotopic labeling step. This method allows direct correlation of deuterium uptake kinetics with charge state and ligand-binding state. Proteolytic digestion amide HDX ESI-MS involves quenching the exchange reaction, followed by peptic digest and LC-MS analysis. This strategy provides spatially resolved amide HDX data. These complementary techniques, in addition to analytical ultracentrifugation, were used to develop and verify a kinetic model of the equilibrium dynamics of partially denatured myoglobin. This model divides myoglobin into discrete cooperative unfolding units which are sequentially exposed during equilibrium transitions. Proteolytic digest amide HDX ESI-MS results allow partial assignment of the unfolding units to specific regions of myoglobin.

Experimental: *Time-resolved ESI-MS.* A continuous flow mixing device was used to mix partially denatured myoglobin (in 27% CH_3CN v/v adjusted to pH 9.3 with NH_4OH) with a labeling solution (27% CH_3CN v/v in D_2O, adjusted to pD 9.3 with ND_4OD). Two syringes (containing Mb and labeling solutions, respectively) were advanced simultaneously using syringe pumps; H/D exchange was initiated at a mixing tee and allowed to proceed until the solution was electrosprayed into a triple-quadrupole mass spectrometer. Labeling times from 7 ms to 3.1 s were observed, corresponding to labeling capillary lengths of 0.8 to 204 cm. A manually mixed sample with a labeling time of 1.5 hours was also analyzed.

Proteolytic digestion amide HDX ESI-MS was performed by adding a quenching step, reducing the labeling solution to pD 2.5 with 2M HCl prior to sample collection. Quenched samples were subsequently digested in pepsin (E:S = 1:1 and 50:1, 5 min) in an ice bath, and analyzed by LC-MS. Peptic fragments were identified by MS/MS.

Results: The ESI mass spectrum of partially-denatured myoglobin indicates the presence of several heme-binding states: apo-myoglobin (aMb), holo-myoglobin (hMb), and 2-holo-myoglobin (2hMb; myoglobin with 2 heme groups). The majority of these species show a simple mono-exponential EX2 exchange profile with an observed exchange rate constant of 23 s^{-1} (Figure 1, 2). Interestingly, low charge state (presumably native-like) hMb exhibits a complex HDX behavior. Peaks for hMb 7+, 8+, and 9+ each split into a high- and low-mass peak (Figure 2), showing both EX1 and EX2 exchange kinetics. The high-mass peaks exhibited EX2 exchange kinetics (k_{ex} = 23 s^{-1}) identical to those observed for the remainder of the peaks in the mass spectrum (aMb, high charge state hMb, 2hMb) whereas the low mass peaks showed a slower, bi-exponential exchange profile (Figure 1).

A kinetic model was developed to account for the observed HDX behavior (Figure 3). Discussed in detail in ref. (*1*), this model involves the dissection of Mb into three 'unfolding units', corresponding to three populations of exchangeable hydrogens which were identified from the observed kinetics. The protein population is postulated to be in equilibrium between several conformational states which differ in the exchange competency of the three unfolding units (C: closed, O: open). The rate constants, and number of sites in each unit, were taken directly from the exponential fits to observed HDX kinetics (Figure 1). Close agreement between experimental and simulated results (Figure 1, black circles) indicate that the model was successful in describing both the EX2 and EX1 exchange behaviors observed for myoglobin.

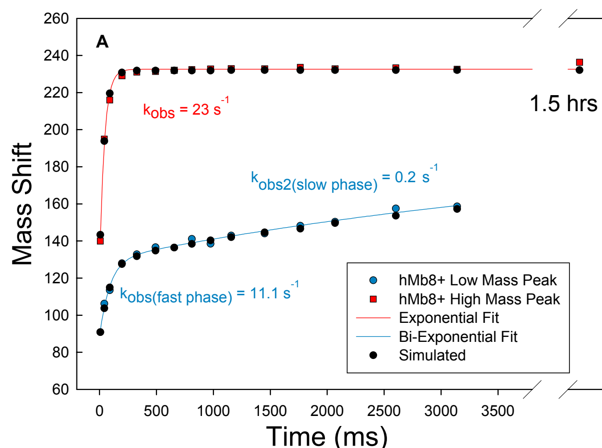


***Figure 1**: Deuterium uptake kinetics observed for the low (blue circles) and high (red squares) mass peaks for hMb8+ (similar results were observed for hMb 7+ and 9+). Results from the computer simulations are also shown (black circles).*

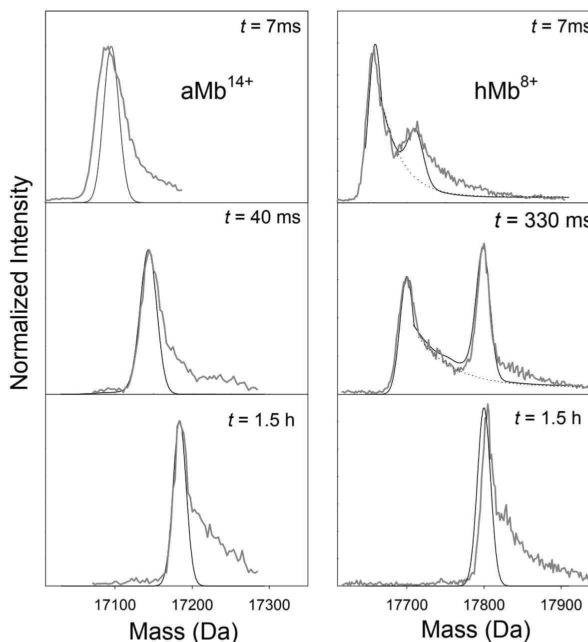


***Figure 2**: (above) Mass spectra of aMb^{14+} (left frame) and hMb^{8+} (right). Peaks corresponding to native hMb showed behavior exemplified by hMb^{8+}; remaining peaks showed EX2 behavior as shown for aMb^{14+}. Simulated peaks are shown in solid black lines.*

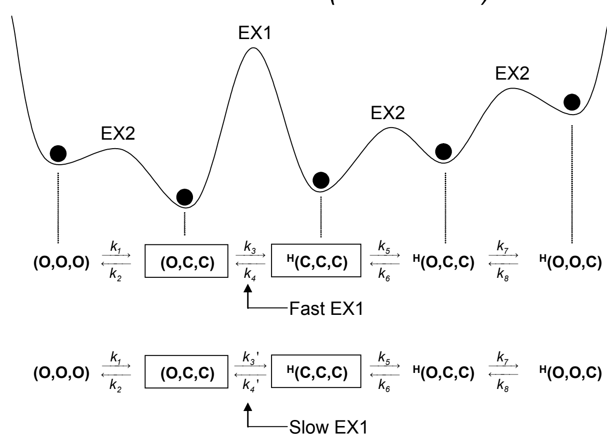
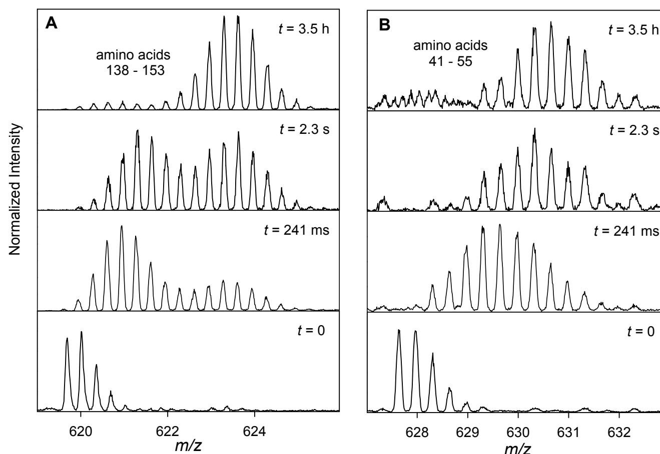


***Figure 3**: (left) Schematic free energy profile of the kinetic model of Mb dynamics. Vector notation for each state (e.g. C,C,C) indicates the exchange competency of unfolding units 1, 2, and 3, respectively.*

Proteolytic digestion amide HDX ESI-MS was used make spatial assignments to unfolding units where possible. HDX behavior observed for helices G and H (amino acids 130 – 153) show EX1 and EX2 kinetics similar to that observed for native-like hMb (Figure 3A). These results indicate that unfolding unit 3 likely corresponds to the myoglobin A-G-H 'folding core'. Conversely, other fragments (e.g. amino acids 41 – 55, comprising helix D) obey simple EX2 exchange kinetics, indicating that units 2 and 3 comprise remaining elements of the protein (helices B – D, E, F). It is not possible to make precise assignments for units 1 and 2.

***Figure 4**: Mass spectra for peptic fragments comprised of amino acids A) 135 – 153, and B) 45 – 55, at various HDX labeling times. The bimodal behavior for fragment 135 – 153 indicates that this portion of the protein corresponds to unit 3, which is only solvent-exposed upon global unfolding. The uni-modal mass shift exhibited by fragment 41-55 indicates that the D helix, and preceding turn, of Mb is included in either unit 1 or 2.*



References:
1) Simmons, DA, Dunn, SD, and L Konermann, 2003, Biochemistry 42:5896 – 5905.