*Supporting Information*

# Submillisecond Protein Folding Events Monitored by Rapid Mixing and Mass Spectrometry-Based Oxidative Labeling

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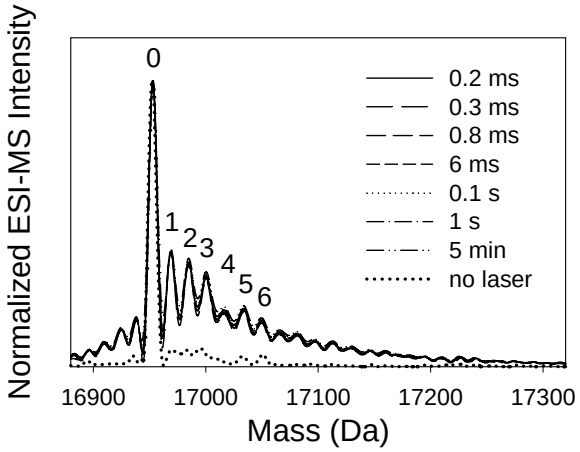


**Figure S1.** Deconvoluted mass spectra of native aMb mixed with buffer and $H_2O_2$ at pH 7 after FPOP at various time points. Data up to $t$ = 6 ms were recorded using the laminar flow mixer of Figure 1. These spectra verify complete and consistent mixing. Spectra for $t$ = 0.1 s and longer were acquired using a conventional mixer, as discussed in the *Experimental* section. Numbers indicate how many oxygen atoms (+16 Da adducts) were incorporated. Also shown are data for a sample exposed to the same conditions as in FPOP experiments, but without laser exposure, demonstrating that background oxidation is minimal.

***$F_u$* Values.** An interesting technical aspect of our work is related to the intact protein data of Figure 2. A key feature of FPOP is the presence of an excluded volume fraction (*EVF*) that remains free of laser exposure.[1] In principle, the fraction of unmodified protein ($F_u$) should not drop below the *EVF*.[2] Simple *EVF* estimates take into account the beam diameter, pulse rate, and flow velocity, assuming a ~1 μs FPOP pulse.[1] Interestingly, these *EVF* considerations tend to underestimate the extent of labeling. The effect is most noticeable for non-native proteins that have a large number of reactive side chains exposed to the solvent. Recent T jump/FPOP experiments by Gross et al. used an *EVF* of 25%.[3, 4] Hence, $F_u$ would not be expected to drop below 0.25.[1, 2] However, the 0.2 ms point in ref.[4] has $F_u \approx 0.07$. Extrapolation to the unfolded state indicates an $F_u$ even closer to zero.[3, 4] The current study reveals a similar phenomenon, where Figure 2A corresponds to $F_u \approx 0.1$ while the estimated *EVF* is about 90%.

The low $F_u$ values observed by different FPOP practitioners point to hidden complexities during the labeling step. The surprisingly large amount of oxidized protein may originate from several factors. Laser scattering at the capillary,[1] radical hopping via H-abstraction from water,[5] and laminar flow effects[6] can widen the effective labeling window. The high quantum yield ($\phi$ = 0.5)[7] for $H_2O_2$ photolysis suggests that perhaps the initial ·OH concentration is larger than assumed in earlier FPOP studies.[2] Reactions of ·OH with the Gln scavenger or with other solutes will produce secondary radicals[8] that could have longer lifetimes and that might still cause protein oxidation.[1] In addition, the quantitation of differentially oxidized protein species can be complicated by differences in ionization efficiency. Detailed studies on all these factors are currently underway in our laboratory, and the results of those investigations will be reported elsewhere.

Despite the potential complications outlined above, the data of this work (and the T jump studies of Gross et al.[3, 4]) demonstrate that FPOP is capable of providing detailed structural information on submillisecond time scales. The fact that the aMb folding data presented here are consistent with earlier HDX data[9] provides additional support for the fidelity of the rapid mixing/FPOP approach. It is important to remember that any "imperfections" of the FPOP protocol will affect all time points to the same extent, such that changes in the oxidation pattern exclusively reflect protein structural transitions.


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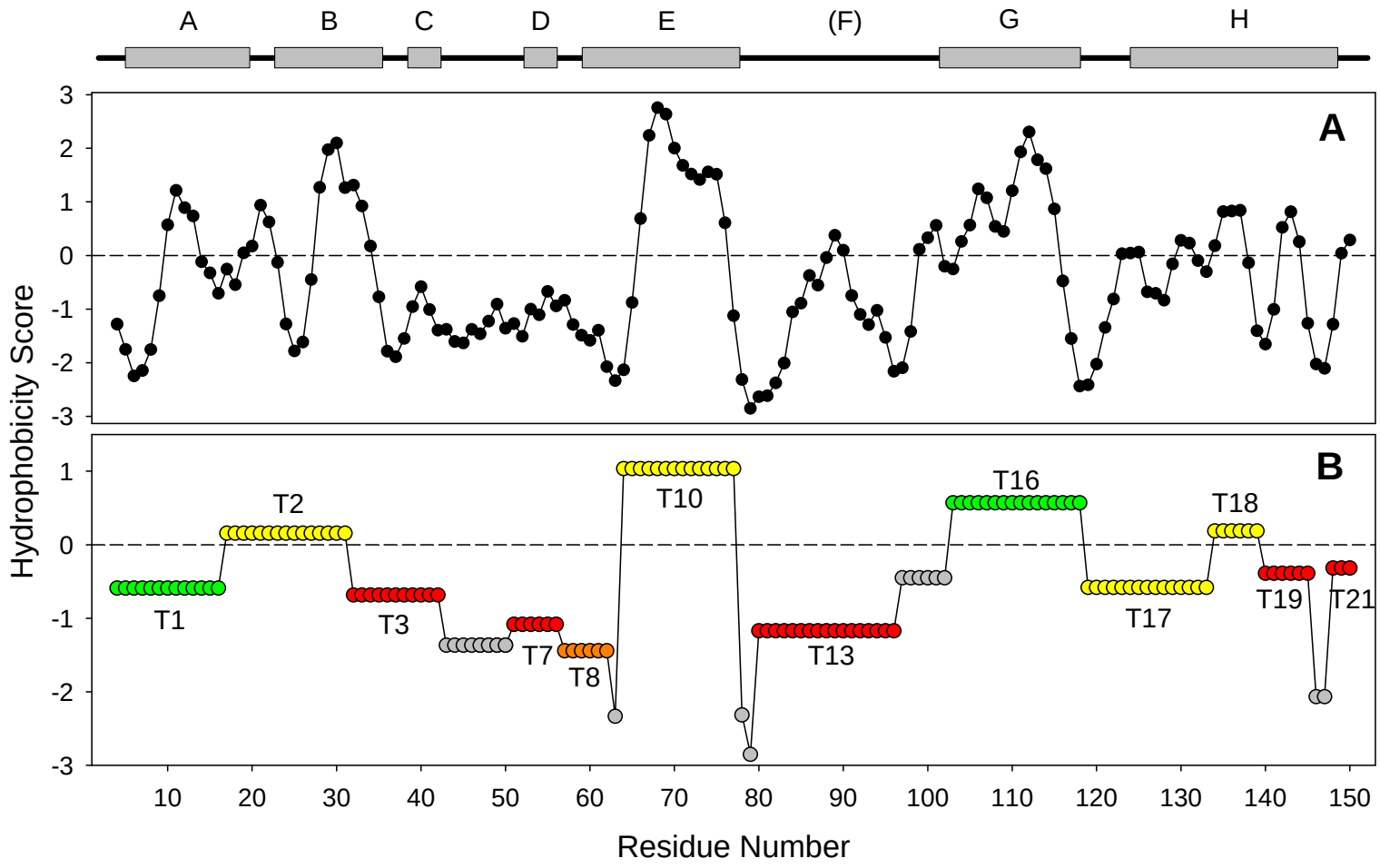


**Figure S2.** (A) Kyte/Doolittle hydrophobicity plot of aMb generated by ExPASy (expasy.org/protscale). A window size of 7 and a triangular weight variation model was applied. (B) Average Kyte/Doolittle hydrophobicity of the 12 aMb tryptic peptides. Positive values represent hydrophobic segments, negative values correspond to hydrophilic regions. The coloring reflects *NOL*s for a folding time of 0.8 ms (from Figure 4D).