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## Early Events in the Refolding of Cytochrome-c Evaluated by Time-Resolved FTIR Microscopy

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We are interested in the evolution of structure during the early stages of protein folding and the timescales for the hydrophobic collapse and secondary structure formation. At present, we have developed a rapid-mixing continuous flow cell that is suitable for acquiring time-resolved FTIR spectra in the sub-millisecond and single millisecond time regimes. The fast refolding kinetics of cytochrome-c (cyt-c) has been studied extensively in the literature using a variety of optical techniques (UV/fluorescence, CD, and Raman) as structural probes. Thus, we chose cyt-c as a model alpha-helical protein to further evaluate the performance of our rapid-mixing continuous flow apparatus. Using time-resolved FTIR microscopy, structural changes in acid-denatured cyt-c (cyt-c<sup>U</sup>) were evaluated by monitoring the amide I absorption profile (1600-1700 cm<sup>-1</sup>) as a function of refolding time. Cyt-c<sup>U</sup> refolding to the native (N) state was examined initially by two pH jump methods (pD<sub>f</sub> = 4.5 and 7.5) to discern the impact of histidine deprotonation and consequent heme misligation. Under conditions where the native heme ligations can form (pD<sub>f</sub> = 4.5), solvated and buried alpha-helical formation was found to be complete within 0.32 ms and 0.70 ms, respectively, at the expense of solvent-exposed and buried coil or unordered structure. Yet, it has been reported that the initial collapsed state at 50 microseconds occurs in absence of secondary structure formation. Moreover, we found that promotion of heme misligation (pD<sub>f</sub> = 7.5) impeded the sub-millisecond formation of native helical structure by merely 2-fold, rather than 100- to 1000-fold decrease reported for the formation of tertiary structure. This observation suggests that cyt-c secondary structure formation does not depend on the formation of native tertiary contacts. In a second pH jump to pD<sub>f</sub> = 7.5 using the exogenous heme ligand, imidazole, heme misligation was prevented and the sub-millisecond rates of solvated and buried alpha-helix formation reverted to values similar to those obtained for the pH jump to pD<sub>f</sub> = 4.5. In a final experiment, cyt-c<sup>U</sup> was refolded to the KCl-induced molten globule (MG) state. Charge-shielding the excess positive charge on cyt-c<sup>U</sup> by Cl<sup>-</sup> appeared to occur on a slower timescale than deprotonation by the pH jump to pD<sub>f</sub> = 4.5, since at 5.7 ms the solvated helix content was in excess by ca., 20% over that for the MG at equilibrium, while ca., 20% of the buried alpha-helix content had yet to form. Nevertheless, the formation of solvated helix and the majority of buried alpha-helix were still fast and complete within 0.5 ms and 1.0 ms, respectively. The cumulative data for the formation of native cyt-c and MG cyt-c, appears to be consistent with a sequential refolding scheme, whereby unordered structure ↔ solvated helix ↔ native alpha-helix. Although this work provides further insight on the formation of cyt-c secondary structure, our rapid-mixing technology needs improvement in order to investigate the formation of the initial collapsed state. Thus, we need to improve the mixing efficiency of our rapid-mixing continuous flow cell and reduce the dead time (ca., 200 microseconds) to access the timescale of the hydrophobic collapse.