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Title of Thesis: Role of the Conserved Residue L94 in the Stability and Folding of Horse Cytochrome-*c*

Abstract

Sequences of 285 cytochrome *c* (cyt *c*) from all kingdoms/phyla are deposited on **www.uniprot.org**. A sequence alignment of all these proteins with respect to horse cyt *c* led to several conclusions. Two of them are: (1) Sequence similarity is 28 - 99%. (2) Leu94 is always conserved in all 30 mammalian cyts *c*. interestingly, some lower eukaryotes and prokaryotes have either Val or Ile or Phe at position 94 of their cyts *c*. Furthermore, all 11 known crystal structures of cyts are super impossible on one another. We have asked a question: What would happen in terms of folding and stability, if Leu94 of the mammalian cyt *c* is substituted by Val or Ile or Phe? To answer this question we created natural substitutes of Leu94 of horse cyt *c* with Val, Ile, Phe using site-directed mutagenesis. Characterization of all purified mutants in the native buffer (30 mM cacodylate buffer, pH 6.0) by far-UV, near-UV and Soret circular dichroism, visible absorbance, Trp and ANS (1-anilino-8-napthaline sulphonate) fluorescence and dynamic light scattering measurements (DLS) at 25 ^{o}C led us to conclude that a substitution by Val/Ile/Phe at position 94 of horse cyt *c* leads to misfolding, and all mutants exist as a molten globule. Stability parameters T_m (mid-point of thermal denaturation) and ΔG_D^0 (standard Gibbs free energy change at 25 ^{o}C) measured by spectroscopic and differential scanning calorimetric methods led us to conclude that mutants are significantly less stable than the wild type protein and their order of stability (in terms of Gibbs free energy change (ΔG_D^0) obtained from thermal denaturation is as, WT h-cyt *c* > L94I > L94V > L94F.

Moreover, we also carried out chemical (Urea and GdmCl)-induced denaturation of WT and its L94V, L94I and L94F mutant proteins at pH 6.0 and 25 ± 0.1 ^{o}C by use of four different probes, i.e., $[\theta]_{222}$, $[\theta]_{405}$, $[\theta]_{416}$ and $\Delta\varepsilon_{405}$ to follow the denaturation of each protein. We observed that that urea-induced denaturation of WT and its L94V and L94I mutants is a cooperative two-state (N ↔ D) process. For L94F mutant, a non-coincidence behaviour of normalized transition curves

was observed although the values of thermodynamic parameters obtained from all different probes are within experimental error comparable that suggests urea-induced denaturation of L94F mutant may be a two-state process. The thermodynamic parameters obtained from thermal denaturation monitored by $[\theta]_{222}$ and $\Delta\varepsilon_{405}$ in presence of different concentrations of urea are with experimental error, identical, that suggest denaturation of L94F mutant is a two-state process. The order of stability in terms of ΔG_D^0 is same as that obtained previously by thermal denaturation measurements.

To further verify that ΔG_D^0 is property of the protein only and is independent of the denaturant, we carried out GdmCl-induced denaturation of all proteins under the same experimental conditions as those used for urea-induced denaturation measurements. We observed normalized transition curves obtained from different probes are not coincident with each other for all proteins that suggest GdmCl-induced denaturation of all studied proteins is not a two-state process. Thus, it can be concluded that the mechanisms of denaturation induced by urea and GdmCl are different.

Denaturation of these proteins was followed by observing changes in $[\theta]_{222}$, $\Delta[\theta]_{409}$ and $\Delta\varepsilon_{405}$. It was observed that in each protein, LiCl induces a biphasic transition, N state ↔ X (intermediate) state ↔ D state. We designated reaction, N state ↔ X (intermediate) state as transition I, and reaction, X state ↔ D state as transition II. The intermediate (X) state of each protein characterized structurally and thermodynamically at pH 6.0 and 25 ± 0.1 °C. ΔG_I^0 (value of ΔG_I in the absence of LiCl) associated with transition I of each mutant was significantly lower than that of WT protein, which suggests Leu94 is important for the stability of WT protein. And mutation also affects the stability of X state as observed ΔG_{II}^X (ΔG_{II} at X M LiCl at which intermediate state exists) associated with transition II of each mutant, was slightly lower than that of the WT protein. Structural characterization of X state of all proteins by the far-UV, near-UV and Soret CD, protein intrinsic and ANS fluorescence, and DLS measurements at pH 6.0 and 25 ± 0.1 °C led us to conclude that X state of the WT protein corresponds to molten globule state, whereas this state of all mutants L94V, L94 and L94F corresponds to pre-molten globule state. On the basis of the results obtained in this study we conclude that (i) there exists a thermodynamically stable intermediate on the reversible folding ↔ unfolding pathway of WT and its mutants L94V, L94I and L94F at pH 6.0 and 25 ± 0.1 °C, and (ii) mutation of Leu94 by Val, Ile and Phe significantly affect the folding pathway of WT protein.