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Title of Thesis: Cloning, Expression, Purification, and NMR Characterization of the Molten Globule State and Pre-Molten Globule State of the L94G Mutant of Horse Cytochrome-*c*

Abstract

Cytochrome-*c* (cyt-*c*) is widely used as model protein to study folding and stability aspects of the protein folding problem, structure-function relationship from the evolutionary point of view. The sequences databases of cyt-*c* now contain around 285 cyt-*c* sequences from different organism (**Website:** http://pir.georgetown.edu/). A sequence alignment of all these proteins with respect to horse cytochrome *c* (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*. Structurally, Leu94 is situated in the middle of the C-terminal helix, and is a key residue forming the folding nuclei. We have tried to question: what changes would one observe at the atomic resolution in Leu94Gly mutant? To answer this question, we have created a mutant of Leu94Gly of horse cyt-*c* using site-directed mutagenesis and determined their effects on the 3D structure. We successfully mutated, cloned, expressed, and purified WT-h-cyt-*c* (labelled and unlabeled) and its mutant L94G (labelled and unlabeled). To understand the effect of mutation on various biophysical properties, we carried out the spectroscopic characterization of WT-h-cyt-*c* (labelled and unlabeled) and its mutant L94G (labelled and unlabeled) under native conditions. We have observed that replacement of Leu94 with Gly disturbs structure, stability and folding of horse cyt-*c* with spectroscopic and hydrodynamic properties different from native WT-h-cyt-*c* under physiological conditions. The labelled and unlabeled L94G mutant of h-cyt-*c* has identical optical and hydrodynamic properties. To determine the role of mutation on the stability of WT protein, we carried out thermal denaturation of WT-h-cyt-*c* (labelled and unlabeled) and its mutant L94G (labelled and unlabeled). Thermal denaturation was monitored by following changes in spectral properties ($[\theta]_{222}$) as a function of temperature. These studies showed that both T_m (midpoint of thermal denaturation) and ΔG_D^0 of labelled and unlabeled L94G mutant are less than those of WT labelled and unlabeled protein.

Biophysical techniques *viz.* CD, fluorescence, absorbance, dynamic light scattering etc. unequivocally suggest that the L94G mutant of cyt-c exists in a molten globule state. In the absence of 3D structure of L94G mutant we cannot determine how many interactions in the native WT-h-cyt-*c* are actually perturbed? To answer this question, we are moving at the atomic level resolution. We have determined the structure of this molten globule using NMR. The NMR sample was stable when left at room temperature for long periods. Comparison of the mutant structure with WT-h-cyt-*c* crystal *structure (PDB ID: 1hrc)* suggest, our structure is very similar to wild type with slight differences. These structural differences are as follows: (i) The overall native secondary structure and their crude mutual positions in three dimensional space are conserved, but differ from the native state by a variable loop region. (ii) (a) Most of the interactions of the side chain of Leu 94 (C-terminal) with side chain of Tyr 97 (C-terminal), Gly 6 and Phe 10 (N-terminal) are lost, and as a result, the two helices move slightly apart; (b) the interaction between Hδ2 of L94 and bridge-2 CH_3 atom of heme is lost, and the latter interaction plays an important role in holding the heme near Cys14; (c) the interaction between Hγ of Leu94 and CH_3 of heme ring 1 is lost; (d) *Met 80*does not ligate to the heme iron; (e) the conformation of heme is notably different in our structure; and (f) h-cyt-*c* has a single Trp residue at position 59, which is at a distance of ~10 Å from the heme propionate, in our structure heme propionate are far away from Trp 59. (iii) Structure analysis using the 3D coordinates suggested that our molten globule would be larger than that of the WT-h-cyt-c. Clearly, the backbone conformation of the L94G mutation is conserved, but side chains/heme contacts are different. Our L94G mutant fulfills all the aforementioned criteria and therefore can be classified as a molten globule state.