389/21-3+16

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Title of Thesis: Role of Extra N-terminal Residues (-2 & -1) in the Folding and Stability of the Yeast Cytochrome- *c*

Understanding the mechanism of protein folding is a central problem of structural biology. One of the most intriguing questions in the biophysics is how do protein sequences determine their unique three-dimensional structure? This question, known as the protein folding problem, is of great importance because understanding protein folding mechanisms is a key to successful manipulation of protein structure and, consequently, functions. Understanding the mechanisms of protein folding is also crucial for deciphering the imprints of evolution on protein sequence and structural spaces.

Cytochrome-*c* (cyt-*c*) is considered as a model protein to study the mechanism of folding/unfolding. A direct comparison of primary sequence of cyt-*c* from horse heart (h-cyt-*c*) and that from yeast (y-cyt-*c*) shows that the latter protein has additional five amino acid at the amino terminus. Moreover, y-cyt-*c* was found to be less stable in comparison to the h-cyt-*c* because of significant differences in their sequences. These extra residues are referred to as residues -5 to -1 when using the eukaryotic numbering system for cyt-*c*. To understand the role of these extra N-terminal residues in y-cyt-*c* folding/stability, we need a precise knowledge of thermodynamic parameters such as changes in Gibbs free energy, enthalpy, entropy, heat capacity, etc., which can either be measured by heat-induced denaturation and/or chemical-induced denaturation.

In this study we are interested in understanding the role of only two residues (-1 and -2) out of five extra residues at the N-terminus, in the structure, stability and folding of

y-cyt-*c*. For this, we have used *in silico* and *in vitro* methods to answer the question whether these two residues at N-terminal extension are required for the stability of the protein and proper folding of the protein.

Three dimensional crystallographic structure of the wild-type (WT) y-cyt-*c* is known. From this study, we determined the interactions of each extra residue at the N-terminus with rest of the protein, and predicted the stability of the WT protein and its deletants, Δ(-5/-3) and Δ(-5/-2). The order of stability that we observed is WT > Δ(-5/-3) > Δ(-5/-2). In order to understand the role of extra N-terminal residues in y-cyt-*c*, we sequentially deleted the extra residues and successfully expressed and purified and performed secondary and tertiary structure analysis of each deletant and found no considerable difference in the structure. We also compared thermal stabilities of all the proteins, using equilibrium method and differential scanning calorimetry (DSC) and chemical stability (Urea and GdmCl) using equilibrium method. Our *in vitro* data are in excellent agreement with those from *in silico* prediction of protein stability.

Equilibrium denaturation curves induced by the weak salt denaturant, LiCl monitored by $\Delta\varepsilon_{405}$, $\Delta[\theta]_{409}$ and $[\theta]_{222}$, are biphasic (N ↔ X ↔ D, where X is intermediate state) transitions in case of WT as well as deletants. This observation provides evidence that there exists a thermodynamically stable intermediate at pH 6.0 and 25 °C. Intermediate state, X, obtained during LiCl-induced denaturation at pH 6.0 and 25 °C, was structurally characterized with various spectroscopic techniques (far-UV and near UV-CD, ANS binding, and dynamic light scattering). It was found that the X state possesses all the structural properties of premolten globule (PMG) state.