Conformational and Thermodynamic Characterization of the Molten Globule State of Horse Cytochrome c.

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A central issue in protein folding is to understand how a protein can fold quickly and efficiently to a unique native structure, despite astronomically large number of possible conformations available to a structureless polypeptide chain. This dilemma led to postulate presence of a specific folding pathway, in which a protein has to go through a sequence of intermediates to fold into the native structure. Thus, in order to understand protein folding, it is crucial to characterize the intermediate state on these pathways. Since stable molten globule state of many protiens are now identified as general intermediates in protein folding, it is then useful to characterize molten globule state of a protein observed under various denaturing conditions. One of the best methods to detect the presence of intermediates is the non-coincidence of denaturation transition curves observed at different wavelengths or by different optical probes.

In our studies cytochrome-c denaturation was carried out by various chemical denaturants, namely, guanidine hydrochloride (GdnHCl), urea, lithium chloride (LiCl), calcium chloride ($CaCl_2$), lithium perchlorate ($LiClO_4$) and lithium bromide (LiBr). The unfolding transition was followed by monitoring changes in molar absorbance coefficient near 400 nm, at 530 and 695 nm and changes in mean residue ellipticity, ([q]) at 222 nm and at different wavelengths in the region 405-416 nm.

It has been observed that GdnHCl and urea induced a cooperative single transition curve at pH 6.0 and 25 $\pm$ 0.1 ^{o}C. It has also been observed that denaturation of cytochrome-c by GdnHCl is not a two-state process. This conclusion was reached from the non-coincidence of GdnHCl-induced transition curves monitored by absorbance at 405, 530, and 695 nm and circular dichroism (CD) measurements at 222, 405 and 416 nm. These measurements on cytochrome-c at lower concentrations of the denaturant (0.75-2.0 M) suggest that the protein retains all the native far-UV CD but has slightly perturbed tertiary interactions as measured by $[q]_{416}$. The characterizations of the intermediate by near-UV CD and 8-Anilino-1-naphthalene sulfonic acid (ANS)-binding florescence measurements reveal that the intermediate state does not possess structural characteristics of the molten globule state. We have also carried out thermal denaturation of cytochrome-c in the presence of increasing concentrations of the GdnHCl monitored by absorbance at 405 nm and far-UV CD at 222 nm. The heat-induced denaturation measurements in the presence of GdnHCl reveal that the denaturartion process in the presence of low denaturant concentrations (0.6-0.8 M) is not a two-state process, for T_m and D $H_m$values obtained from absorbance and CD

measurements do not match with one another. However, heat-induced denaturation in the presence of more than 0.8 M GdnHCl follows a two-state process.

The estimation of D G_D^o, the Gibbs free energy change in buffer at 25 ^oC of cytochrome-c from GdnHCl-induced measurements yielded a value in the range of 6.8-8.8 kcal mol^{-1} for D G_D^owhich is lower than the value of 10.13 $\pm$ 0.5 kcal mol^{-1} obtained from the thermal denaturation measurements of the protein in the presence of GdnHCl above 0.8 M.

Contrary to the GdnHCl-induced denaturation of cytochrome-c, urea-induced denaturation of the protein is a simple two-state process, as coincident transition curves are obtained from different optical technique. The urea-induced denaturation yields a value of 9.7 $\pm$ 0.4 kcal mol^{-1} for D G_D^o, which is in good agreement with that obtained from measurements of the denaturation by heat in the presence of GdnHCl concentration > 0.8 M (D G_D^o = 10.13 $\pm$ 0.5 kcal mol^{-1}). This again supported that urea-induced denaturation of cytochrome-c is a two-state process.

We have measured the denaturation of cytochrome-c by weak salt denaturants (LiCl, LiBr, $CaCl_2$ and $LiClO_4$) at 25 ^oC by monitoring change in molar absorption coefficient at 400 nm (De_{400}) and CD at 222 nm and 409 nm. Measurements of De_{400} and mean residue ellipticity at 409 nm ($[q]_{409}$) gave a biphasic transition for both modes of denaturation of cytochrome-c. It has been observed that the first denaturation phase, N (native) conformation « X (intermediate) conformation and the second denaturation phase, X conformation « D (denatured) conformation are reversible. Conformational characteristics of X state by the far-UV CD, near-UV CD, ANS binding, tryptophan florescence and intrinsic viscosity measurements reveal that (1) X state retains all native secondary structure, (2) specific tertiary interactions are lost in the intermediate state, (3) hydrophobic clusters are accessible to the aqueous solvent, (4) state of heme-tryptophan domain is maintained, and (5) radius of gyration (R_g) of X-state increases only by 9-11%. Characterization of the intermediate state by far-UV CD and florescence measurements could not be done as bromide shows absorption in far-UV region and acts as a quencher of tryptophan florescence. Owing to these reasons, the characterization of the intermediate state was done only by viscosity measurements. All the characteristics of the X state led us to conclude that the intermediate state observed in presence of moderate concentrations of LiCl, LiBr and $CaCl_2$ is a molten globule (MG) state comparable to the acid molten globule of cytochrome-c reported by other workers and that the molten globule state observed in the presence of $LiClO_4$ is more extended than that of the MG state observed in the presence of LiCl and $CaCl_2$.

For a protein coincidence of all the normalized denaturation curves obtained in the presence of LiCl, $CaCl_2$, and LiBr suggested that the transition N « X and X « D are two-state mechanism. The analysis of denaturation transition curves for the stability of different states in terms of Gibbs energy change at pH 6.0 and 25 ^oC suggested that

heme interaction with the globin stabilizes the native state of cytochrome-c by 9.0 ± 0.5 kcal mol^{-1} and the contribution of the secondary structure towards stability of the protein is only 1.4 ± 0.2 kcal mol^{-1}. However, values of Gibbs free energy change estimated from $LiClO_4$ denaturation gave a value of 7.3 ± 0.3 kcal mol^{-1} for N « X transition.

The other important question about the molten globule state that we have addressed in this work is whether the molten globule state is a specific thermodynamic state of a protein, or whether it is thermodynamically similar to the denatured state. To understand this question we have studied the effect of temperature on the equilibria, N conformation « X conformation and X conformation « D conformation using two different optical probes, namely, mean residue ellipticity at 222 nm and molar absorption coefficient around 400 nm. These measurements yielded T_m , the midpoint of denaturation and D H_m, enthalpy change at T_m as a function of denaturant concentration. A plot of D H_m versus T_m was used to determine the constant-pressure heat capacity change, DC_p (=$(D H_m / T_m)_p$). It should be noted that thermodynamic parameters associated with X « D transition observed in the presence of $LiClO_4$ were not estimated, as the process does not follow a two-state mechanism.

The values of DC_p obtained for both, N « X and X « D transition suggested that about 30% of the hydrophobic groups are present in the molten globule state that are not accessible to the aqueous solvent and that the molten globule is stabilized by hydrophobic interactions.