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Cooperative Unfolding Units and Metastable States of Cytochrome c_{551} from *Pseudomonas aeruginosa* under Native Conditions

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Abstract

Unfolding pathways of *Pseudomonas aeruginosa* cytochrome c_{551} (Pa cyt c) characterized by native state hydrogen-deuterium exchange (NS H/D) methods in conjunction with multi-dimensional NMR techniques have been reported in the literature. Based on the analyses carried out on the H/D exchange data, it has been shown that there are four submolecular unfolding units or three cryptic intermediates (CIs) of the protein under native conditions. However, OneG, a computational tool for mapping out foldons of proteins, predicted that there is no possible existence of CIs in the protein unfolding but revealed possible existence of metastable states in the protein. The discrepancy in the protein unfolding detected by the NS H/D exchange methods and OneG computational tool has been rationalized through the structural architecture of the protein and differences in the free energy exchange of isotherms representing each cooperative unfolding units identified by the NS H/D exchange methods. Based on comprehensive analyses of data from the two methods, we herein demonstrate that the OneG tool is highly reliable and robust on identifying cryptic intermediates and metastable states that may presumably exist in the unfolding kinetics of proteins under native conditions.

Keywords: Cryptic intermediates, metastable states, H/D exchange, OneG, isotherms and protein folding.

1. INTRODUCTION

Understanding stability, dynamics and folding of proteins is essential to delineate 'structure-function' relationships of proteins and also to design polypeptides with desired functions. The unfolding and refolding of proteins are studied in the presence of chaotropic agents (such as urea, thiourea, guanidinium chloride, sodium dodecyl sulphate etc.) at denaturing concentrations in most of the folding experiments available to date, in general [1,2]. However, the added denaturant may mask some stabilizing interactions between residues in proteins leading to affect the accurate estimation of unfolding stability and folding pathways of the proteins. Fortunately, protein unfolding can be studied under native conditions by using NS H/D exchange methods in conjunction with mass spectrometry and multidimensional NMR techniques [3,4]. Using the methods, energetic landscapes of the proteins can be probed at residue level resolutions. Notwithstanding the uniqueness and exquisite potential of the methods on characterizing the CIs of proteins under native conditions, the methods are time consuming (days to several months) and require sound theoretical and experimental knowledge on the protein dynamics and H/D exchange mechanisms [5]. In these contexts, OneG, a computational tool and an excellent alternative to the H/D exchange methods, identifies CIs of proteins in a fully automated manner using 3 prerequisite inputs - pdb files, ΔG_{HX}, and ΔG_U of proteins and the functions, robustness and unique applications of the tool have been elaborately discussed elsewhere [6].

Pa cyt c is a monomeric and simple helical polypeptide chain consisting of 82 standard amino acids. Lea and Kara have characterized the unfolding kinetics of the protein at pH 6.0, 299 K by using the NS H/D exchange methods and showed that the protein has four submolecular unfolding units or existence of three distinct CIs in the native unfolding of the protein [7]. However, equilibrium and kinetic folding studies performed on the protein supported two-state process of the protein [7,8]. In the present study, we analysed the protein unfolding by OneG computational tool at similar solution conditions (pH 6.0 and 299 K). The tool suggested for possible existence of metastable states of proteins. Structural contexts and energetics of the metastable states of the protein have been discussed at residue level resolutions. Moreover, effect of the metastable states on estimating free energy exchange (ΔG_{HX}) of the protein has also been brought into detail. In addition, the discrepancy observed on the unfolding pathways of the protein detected by the NS H/D exchange and OneG tool is showed to originate from analysis of NS H/D exchange data since each cryptic intermediate proposed from the experimental methods failed to exhibit distinct isotherm for the protein.

2. METHODS

OneG computational tool was used to predict possible existence of cryptic intermediates (CIs) and metastable states in the unfolding pathways of Pa cyt c at pH 6.0, 299 K under native conditions. The tool requires 3 prerequisite inputs: pdb file (351C) of the protein, ΔG_U (free energy of unfolding) and ΔG_{HX} (residue-specific free energy of exchange determined in the absence of denaturant). The program has been discussed in detail elsewhere [6] and the tool can be freely accessed and instantly used at *http://sblab.sastra.edu/oneg.html* Based on the inputs given, the webserver predicted single foldon unit of the protein implying that there is no possible existence of CIs in the protein unfolding. The ΔG_U and ΔG_{HX} of the protein were reported as 5.98 kcal/mol and 8.33 kcal/mol at pH 6.0, 299 K, respectively. The ΔG_X (ΔG_U corrected to effect of *cis-trans* proline isomerization) was calculated as 6.37 kcal/mol The protein consists of 5 proline residues located at 25 (Gly24-Pro25), 58 (Gly57-Pro58), 60 (Ile59-Pro60), 62 (Met61-Pro62) and 63 (Pro62-Pro63) and all the imide bonds are in *trans* conformation, which accounted free

energy corrections of 0.39 kcal/mol. The program listed 16 residues that depicted free energy exchange greater than the ΔG_X and the residues were further subjected to trace possible existence of metastable states of the protein. The computation resulted two distinct metastable states of the protein in terms of distance limit (>7 Å) and free energy coverage limit (> 0.4 kcal/mol).

3. Results and Discussion

The NS H/D exchanges of Pa cyt c have been studied at pH 6.0, 299 K [7]. Of the 82 residues, residue-specific free energy exchanges for more than 50% residues have been reported in the presence of GuHCl (guanidinium hydrochloride ranging from 0M to 1.4M). The free energy values of the residues varied from 2.7 kcal/mol (Ile located at 59 in loop 3 of the protein) to 8.4 kcal/mol (Ala located at 75 in helix 4 of the protein). Based on the analyses of the denaturant-dependent exchange data, four submolecular unfolding units have been proposed: foldons 1, 2, 3 & 4 consisted of regions helix 4, helix 3 & loop 3, helix 2 and loop 1 of the protein, respectively (Table 1). In addition to these 4 foldons, a region comprising of residues from 11 to 16 was found to have stronger stability (showing high protection to amide protons against exchange with solvent deuterium) than that of the foldons identified in the protein. The free energy coverage of the foldon 1, 2, 3 & 4 were found to be ranged from 3.15 to 8.44 kcal/mol, from 2.70 to 8.17 kcal/mol, from 4.13 to 6.21 kcal/mol and from 3.68 to 5.88 kcal/mol, respectively in the 0M denaturant concentration. Cryptic intermediate 1 of the protein was shown to form when the foldons 2, 3 & 4 were unfolded in a concerted manner; similarly, CI2 of the protein was shown to form when the foldon 3& 4 were unfolded together and CI1 was shown to exist when the foldon 4 was unfolded alone. Structural opening of all the 4 foldons would lead to unfolded state of the protein.

In contrary to the above observations, the OneG tool predicted that there was no possible existence of CIs in the unfolding kinetics of Pa cyt c at pH 6.0, 299 K and the results from the computation suggested that there was only one cooperative unfolding unit of the protein. On the other hand, the program identified two metastable states of the protein (Table 1): metastable state 1 was constituted by residues such as Gln72, Leu74, Ala75, Lys76, Trp77, Val78, Leu79 & Ser 80; metastable state 2 was constituted by residues such as Arg47, Ile48, Lys49, Asn50, Gly51 & Ser52. The free energy coverage of the metastable states 1 and 2 were ranged from 8.44 to 6.55 kcal/mol and from 8.17 to 7.27 kcal/mol, respectively. Interestingly, residues of metastable state 1 were from helix 4 segment (CI1 identified by the NS H/D exchange method) and residues of metastable state 2 from part of helix 3 and loop 3 (CI2 identified by the NS H/D exchange method) of the protein. Structural coordinates of the metastable states predicted by the tool can be retrieved from the server and viewed in PyMol, a molecular visualization tool or any tools that read the pdb format.

The free energy of unfolding (ΔG_U) and free energy of exchange (ΔG_{HX}, averaged out to two largest residue-specific free energies) of Pa cyt c at pH 6.0, 299 K were 5.98 kcal/mol and 8.6 kcal/mol, respectively. After accounting the effect of *cis-trans* proline isomerization, the discrepancy between the ΔG_U and ΔG_{HX} of the protein was found to be 2.23 kcal/mol. The discrepancy may be either due to accumulation of CIs or existence of metastable states in the protein unfolding. Since CIs are unstable and sparsely populate in the melting transitions of proteins, they elude analyses of equilibrium unfolding experiments examined by optical probes and consequently the ΔG_U of the protein is underestimated. On the other hand, ΔG_{HX} would be overestimated for residues that are exchanging through metastable states of proteins. Metastable states of proteins are heterogeneous and higher in energy than that of denatured states of the proteins. While existence of CIs are shown through the NS H/D exchange methods of the protein, the OneG predicts only the possible existence of metastable states in the protein unfolding. Residue showing largest free energy exchange from each foldon was selected and denaturant-dependent ΔG_{HX} values (from 0 M to 1.4 M) of the four residues (one residue from each foldon) are shown in the Figure 1A. Obviously, the residues fail to have distinct isotherms implying absence of distinct CIs in the unfolding pathway of the protein. Similar trend was also noticed for residue showing second largest free energy exchange from each foldon as shown in the Figure 1B. These analyses clearly suggest that accumulation of CIs in the unfolding kinetics of the protein is very unlikely under native conditions.

Table 1: Results obtained from Pa cyt c unfolding studied by the NS H/D exchange and OneG computational tool are listed herein in the table.

NS H/D Exchange Results			OneG Results			
***FU*[a]**	***Structural Context*[b]**	***Free energy coverage (kcal/mol)***	***FU*[a]**	***MS*[c]**	***Structural Context*[b]**	***Free energy coverage (kcal/mol)***
4	11-16 [CXXCH region][d] Foldon I: 68-80 [H4] Foldon II: 40-67 [H3 & L3] Foldon III: 17-26 [L1] Foldon IV: 27-33 [H2]	3.66-8.05 3.15-8.44 2.70-8.17 4.13-6.21 3.68-5.88	0	2	MS1: 72 74-80 [H4] MS2: 47-52 [H3 & L3]	6.54-8.43 7.26-8.17

[a] **Foldon Units.**
[b] **Residues numbers and their respective structural contexts are given; Helices, strands and loops are denoted by 'H', 'S' and 'L' respectively.**
[c] **Metastable states.**
[d] **Cys-X-X-Cys-His motif depicting residual protections.**

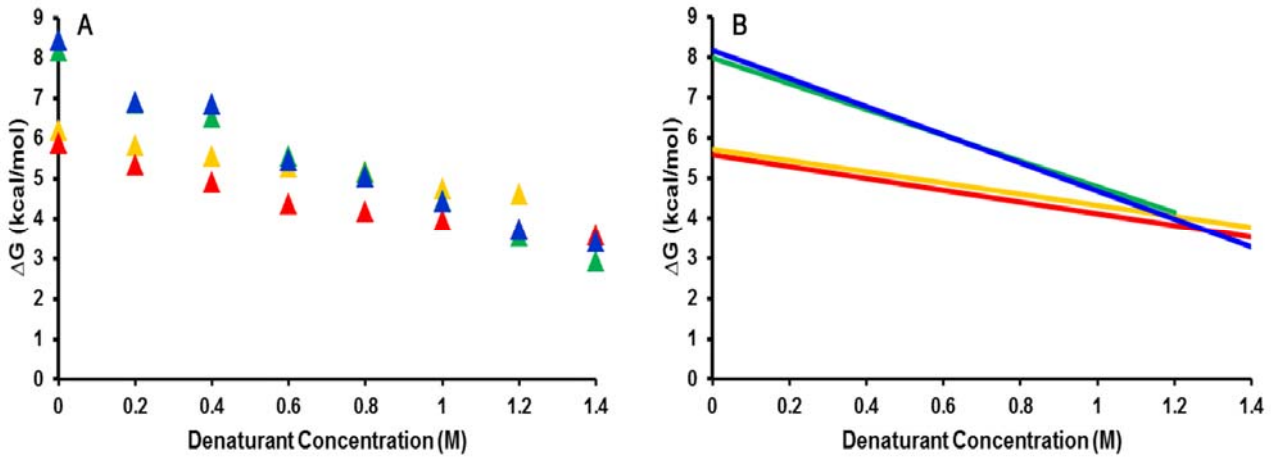

Figure 1: Free energy exchange of residues that (A) are most highly protected and (B) second most protected from each foldon of Pa cyt c are shown with respect to denaturant concentration. (A) Ala75 (Blue triangle), Ile48, (Green triangle), Ala17 (Yellow triangle) and Ala31 (Red triangle) are from foldons 1, 2, 3 and 4, respectively. (B) Lys76 (Blue line), Gly51, (Green line), Gly24 (Yellow line) and Ala32 (Red line) are from foldons 1, 2, 3 and 4, respectively. Linear fit of the ΔG_{HX} vs. denaturant concentrations for the 4 residues are shown in the figure.

The ΔG_U and C_m (denaturant concentration wherein ΔG_U is zero) of the Pa cyt c as estimated by optical probes are 5.98 kcal/mol and 1.92 M, respectively. However, the ΔG_{HX} and C_m of foldon 1 of the protein as estimated by H/D exchange method is 8.6 kcal/mol and 2.25 M, respectively. Since the C_m values for free energy of unfolding of protein and free energy of exchange of foldon 1 of the protein are remarkably different from each other, residues in the foldon 1 are presumably not exchanging through global unfolding events of the protein. Interestingly, most of the residues of foldon 1 (as shown by NS H/D exchange studies) constituted metastable state 1 of the protein as predicted by the OneG program. Moreover, the program predicted that metastable state 2 of the protein are composed of residues from helix 3 and loop 3, which represented as foldon 2 of the protein as shown by NS H/D studies. From a quick inspection to the Fig. 1B, it is obvious that the isotherms of foldons 1 & 2 are indistinguishable from each other suggesting that foldon 1 and 2 are very unlikely to be distinct units from each other. On the other hand, metastable states 1 and 2 of the protein as predicted by the OneG are distinct units in terms of structural contexts and energetic factors (Table 1). Moreover, steady-state and kinetic folding studies of Pa cyt c strongly favour two-state folding-unfolding process for the protein [7,8]. It should also be mentioned that unfolding pathway of equine cytochrome c, a homologous protein to Pa cyt c, has been unambiguously characterized by using NS H/D exchange methods and the study uncovered that accumulation (about 16%) of three distinct CIs in the unfolding kinetics of the protein [9,10]. Though one can expect similar trend for proteins, as Pa cyt c in the present study, that are homologous to the equine cytochrome c, there is no straightforward correlations between the structural similarities and folding events of the proteins. Moreover, the equine cytochrome c and Pa cyt c are different from each other in their size (former and later are made up of 104 and 82 amino acids, respectively), stability (ΔG_U of the former and later are 10.0 kcal/mol and 5.98 kcal/mol, respectively) and folding kinetics (multistate vs. two-state) [7-10]. Recently, using the OneG computational tool, we have also shown possible existence of a CI and metastable state in the unfolding pathways of cardiotoxin III (CTX III) and cobrotoxin (CBTX), respectively. The CTX III and CBTX are homologous (paralogous) proteins and their three-dimensional structures are very similar to each other [11]. However, the unfolding stability, biological functions and kinetic folding of the proteins are drastically different from each other [12-14]. Strikingly, the OneG predictions on the unfolding kinetics of the proteins under native conditions and folding studies on the proteins studied by experimental methods were in excellent agreements as we demonstrated elsewhere [6,12-14]. By tie together, it is very apparent that the discrepancy between the ΔG_U and ΔG_{HX} of Pa cyt c is due to existence of metastable states of the protein. As discussed above, possible existence of CIs seems to be highly unlikely in the unfolding kinetics of the protein.

4. Concluding Remarks

Unfolding pathways of Pa cyt c examined by the NS H/D exchange methods and OneG computational tool have been analysed and compared. While the former method suggests existence of cryptic intermediates in the unfolding kinetics of the protein, the later method favours for possible existence of metastable states of the protein under native conditions. Based on the comprehensive analyses of the data from the two methods, discrepancy between the ΔG_U and ΔG_{HX} (about 2.2 kcal/mol) of Pa cyt c is attributed to the metastable states of the protein under native unfolding conditions. Moreover, to our best knowledge, OneG is only available computational tool to date for predicting CIs and metastable states of proteins under native conditions and the tool is also very useful to address the discrepancy between the ΔG_U and ΔG_{HX} of proteins in a systematic manner.

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