Rakesh Krishnan et al /J. Pharm. Sci. & Res. Vol. 7(11), 2015, 978-980

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Computational Characterization of a Cryptic Intermediate in the Unfolding Kinetics of An All β-Sheet Protein

Rakesh Krishnan, Anand Srinivasan and Thirunavukkarasu Sivaraman*

Structural Biology Lab, Department of Bioinformatics,
School of Chemical and Biotechnology, SASTRA University,
Thanjavur - 613401, India.

Abstract

Free energy of unfolding (ΔG_U) and free energy of exchange (ΔG_{HX}) of cardiotoxin III (CTX III), an all β-sheet protein isolated from *Naja atra*, were reported as 4.9 kcal/mol and 6.7 kcal/mol, respectively. Herein, we have demonstrated that the discrepancy between the ΔG_U and ΔG_{HX} of the CTX III could be qualitatively attributed to the possible existence of a cryptic intermediate (CI) in the folding kinetics of the protein as probed by OneG-Vali computational tool. However, quantitative analyses revealed that the CI could account only about 50% of the discrepancy observed in the thermodynamic stabilities of the protein. In these contexts, various structural factors affecting precise estimations of ΔG_U and ΔG_{HX} of the proteins have been discussed in detail.

Keywords: CTX III, cryptic intermediate, folding pathway, H/D exchange and OneG-Vali.

INTRODUCTION

Cardiotoxin III (CTX III) is an all β-sheet protein consisting of 60 amino acids and belongs to three-finger toxin superfamily. The three-dimensional structures, stabilities, folding pathways and dynamics of the CTX III have been extensively characterized as reported in the literature [1-3]. Particularly, the structural stabilities and refolding kinetics of the protein have been analyzed at residue-level resolution by using NMR hydrogen-deuterium (H/D) exchange methods and quenched-flow H/D exchange techniques, respectively [4-6]. In the present study, the folding kinetics of the CTX III have been mapped out by using OneG-Vali computational tool and the data revealed that unfolding kinetics of the CTX III is a three-state process under native conditions. On the basis of results obtained from quantitative analyses of various states in the unfolding/folding pathway of the protein, we have herein shown that the possible existence of a cryptic intermediate (CI) of the protein could account about 50% of the discrepancy observed between ΔG_U (4.9 kcal/mol) and ΔG_{HX} (6.7 kcal/mol) for the protein as reported in the literature. In addition, uniqueness of the OneG-Vali in addressing contributions of various structural factors on estimating thermodynamic stabilities of the protein has been clearly brought into fore.

MATERIALS AND METHODS

The 3D structure of CTX III (2CRT) was retrieved from PDB [7]. The structure was subjected to energy minimization using steepest descent algorithm down to a maximum gradient of 1000 kJ/mol/nm and a resultant 3D conformation was used for further analysis of the present study. The ΔG_U of CTX III, estimated using Guanidine hydrochloride (GdnHCl) and urea-induced denaturation studies and residue-specific free energies (ΔG_{HX}) of the protein were obtained from literature reports [4,5,8]. Free energy changes for the protein and as well for residues of the protein were considered in kcal/mol with two decimal resolutions throughout the calculations of the present work.

The OneG-Vali computational tool is being routinely used to characterize folding kinetics of proteins under native conditions in terms of qualitative and quantitative manner. The tool requires four prerequisite inputs: 3D structures of proteins (in PDB format), ΔG_U (in kcal/mol), ΔG_{HX} (in kcal/mol), and C_m (in molarity), the denaturant concentration wherein ΔG_U is zero. Algorithms, validations and uniqueness of the OneG-Vali have already been well documented [9-11]. The OneG-Vali is useful to systematically address the discrepancy between ΔG_U (free energy of unfolding determined by using optical probes) and ΔG_{HX} (free energy of exchange determined by using NMR-assisted H/D exchange methods) and as well as to characterize CIs presumably existing in the unfolding kinetics of proteins. Overall functions of the computational tool can be broadly divided into two stages: detections and validations of CIs. In the first stage, number of foldon(s) and possible existence of CIs of proteins are qualitatively defined on the basis of their hydrogen bonds (H-bonds) patterns and strengths. In the second stage, effect of CIs on estimating free energy of unfolding by optical probes will be quantitatively examined. The program also facilitates to carry out the quantifications by taking into account of *cis-trans* proline isomerization of proteins [9-11].

RESULTS AND DISCUSSION

CTX III is a basic (pI > 9.0), highly stable and monomeric protein consisting of 60 amino acids and it belongs to simple β-sheet folds. Interestingly, the primary structure of the protein depicts negligible percentage of helical propensities implying that the protein is a typical all β-sheet protein [12]. Chaotropic agents such as GdnHCl and urea have been used in order to estimate structural stabilities of protein in steady state experiments. The GdnHCl-induced and urea-induced unfolding studies carried out for the protein at pH 3.2, 298 K revealed that the ΔG_U values of the protein were 4.85 kcal/mol and 4.88 kcal/mol, respectively. However, C_m and cooperative constants (m) for the protein from the two different denaturant studies were found to be different from each other: the 'C_m' values were 4.08 M and 5.81 M and 'm' values were 1.19 kcal/mol/M and 0.84 kcal/mol/M for GdnHCl-induced and urea-induced unfolding processes of the protein [8]. Interestingly, the total population of CI that may presumably accumulate in the unfolding kinetics of the CTX III was estimated to be 6% irrespective of the denaturants as explained below herein.

Primary sequence of CTX III consists of 5 proline residues positioned at 8, 15, 30, 33 and 43 and all the five Pro-Xaa peptide bonds are in *trans* conformation. Of the 55 backbone amide protons (NHs), residue-specific free energy of exchange values for 31 NHs of proteins dissolved in D_2O containing low ionic strength were determined at pH 3.2, 298 K. The ΔG_{HX} of the CTX III was calculated to be 6.66 kcal/mol [13, 14]. These results obviously suggest that the ΔG_U estimated by using optical biophysical methods and ΔG_{HX} estimated by using NMR H/D exchange methods are not in agreement to each other. The discrepancy between ΔG_U and ΔG_{HX} of the CTX III is about 1.8 kcal/mol. After accounting the effect of *cis-trans* proline on estimating ΔG_U by using optical methods, the discrepancy between ΔG_U and ΔG_{HX} of the CTX III was still found to be about 1.5 kcal/mol, as the five *trans* prolines of the protein accounted only 0.3 kcal/mol to the higher energy denatured states that may be populated in the denaturants-induced unfolding of the protein. These results suggest that the discrepancy is probably due to possible existence of cryptic intermediate(s) or metastable states in the unfolding kinetics of the protein under native conditions.

In order to understand the discrepancy explained above, the folding pathways of CTX III were examined by using OneG-Vali computational tool. The tool predicted two foldons: foldon I was consisting of residues such as Cys21, Tyr22, Lys23, Met24, Phe25, Met26, Val27, Val34, Lys35, Ile39, Val52 and Cys54 in the triple-stranded β-sheet; foldon II was consisting of residues such as Cys3, Lys5 and Cys14 in double-stranded β-sheet. The free energy coverage for the foldon I and II were 2.77 – 6.72 kcal/mol and 4.23 – 5.09 kcal/mol, respectively. Similarly, the free energy changes for the foldon I and II were 6.70 kcal/mol and 4.84 kcal/mol, respectively. The CI of CTX III is considered to have intact foldon I only in its 3D structure, whereas unfolded CTX III has no defined structural interactions. The unfolding kinetic pathway of the CTX III as predicted by the OneG-Vali is depicted in Figure 1. Interestingly, the kinetic folding pathways of CTX III have been characterized to proceed through an intermediate accumulating in the burst phase (< 5 ms) of the protein. Based on the refolding rate constants of NHs of CTX III obtained from quenched-flow H/D exchange experiments, it has been shown that the triple-stranded β-sheet was formed before the double-stranded β-sheet segment in the refolding kinetics of the protein [6]. Moreover, it has also been demonstrated that the triple-stranded β-sheet segment of the protein was persistently found in the intermediate states identified along the acid-induced and alcohol-induced unfolding pathways of CTX III [15, 16]. To this extent, the predictions of OneG-Vali on the possible existence of a cryptic intermediate of CTX III under native conditions are consistent with the data reported from equilibrium and kinetic studies of the protein.

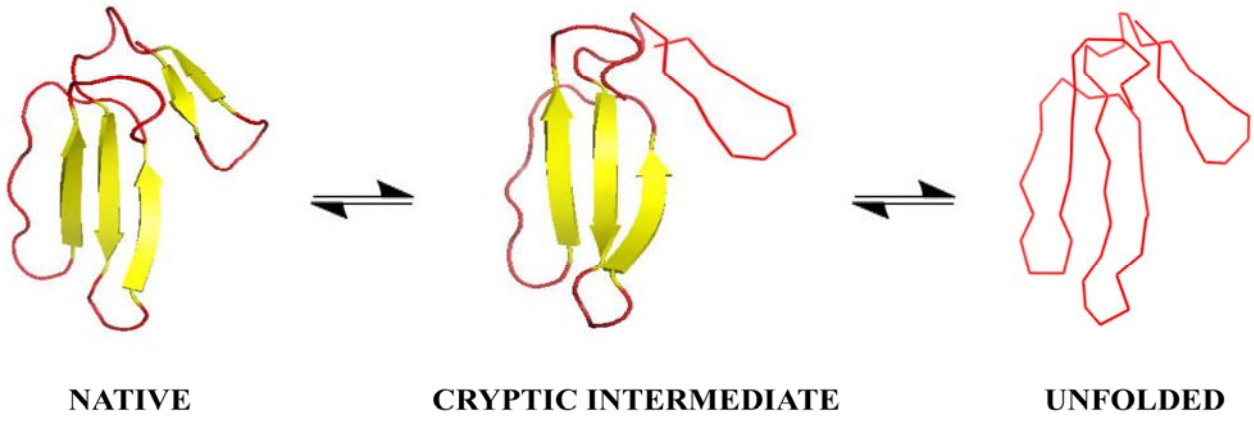

Figure 1: Figurative representations of unfolding kinetics of CTX III under native conditions as predicted by using OneG-Vali computational tool.

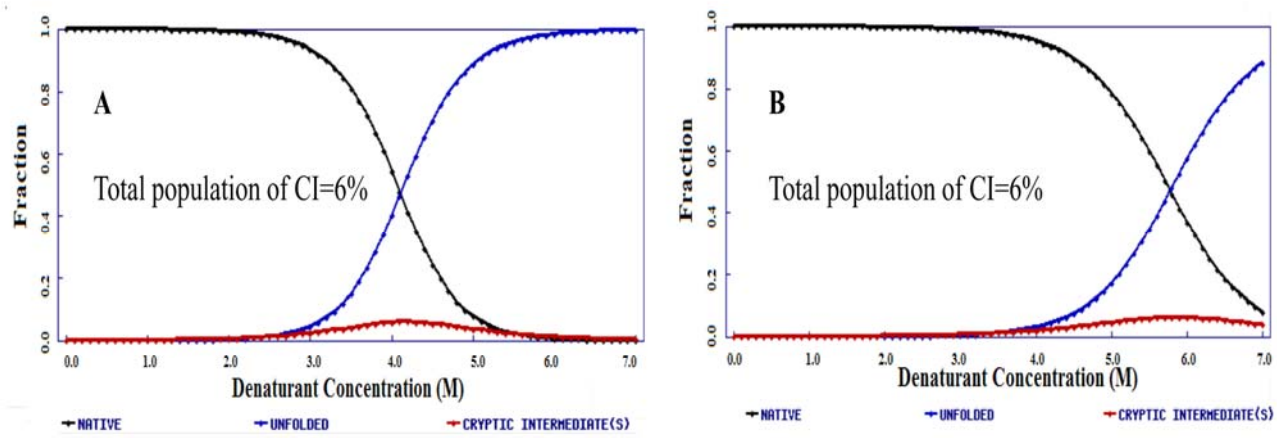

Figure 2: Fractional population of folded, unfolded and cryptic intermediate of CTX III calculated by using C_m values obtained from A) GdnHCl-induced and B) urea-induced steady state unfolding experiments of the protein under similar solution conditions.

Using the C_m values derived from urea-induced denaturation (5.8 M) and GdnHCl-induced denaturation (4.1 M) studies, total population of the CI detected in the CTX III unfolding was calculated. Interestingly, both calculations resulted same percentage of CI population, which was 6 % (Figure 2). Moreover, the free energies of unfolding predicted (ΔG_{UPred}) for the CTX III by the OneG-Vali were 6.2 kcal/mol in both calculations, wherein effect of *cis-trans* proline isomerizations on the protein folding was not considered. Of the 5 prolines, 4 prolines were present in foldon I and 1 proline located at position 8 was present in foldon II. After taking into consideration of the proline isomerisation, the ΔG_{UPred} for the CTX III was 5.85±0.05 kcal/mol, which accounts only about 50% of discrepancy between ΔG_U (4.9 kcal/mol) and ΔG_{HX} (6.7 kcal/mol) of the protein. At this present juncture, the disagreement between ΔG_{UPred} and ΔG_U of the CTX III could be rationalized due to anyone or combinations of following reasons: (i) inaccuracy in *cis-trans* proline calculations (ii) over estimation of ΔG_{HX} (iii) under estimation of ΔG_U and (iv) over estimation of secondary structural contents of CI detected by the OneG-Vali for the protein. Since the ΔG_{HX} and ΔG_U of the CTX III have been unambiguously authenticated in the literature and as well CI detected by the OneG-Vali is in excellent agreement with kinetic intermediates of the protein characterized by various biophysical techniques, the disagreement between ΔG_{UPred} and ΔG_U of the CTX III is seemed to be originated chiefly from uncertainties in the calculations of accounting effect of *cis-trans* proline isomerisation on the folding pathways of the protein.

CONCLUDING REMARKS

The folding pathways of CTX III, an all β-sheet protein, have been characterized by using OneG-Vali computational tool and the analyses suggested that possible existence of a cryptic intermediate in the unfolding kinetics of the protein. Total population of the CI was found to be 6% and the CI has also been characterized in terms of structural contexts and stabilities. Using the CI of the CTX III, we have herein demonstrated that about 50% of the discrepancy between the ΔG_{HX} and ΔG_U of the protein could be well addressed. Moreover, we have also shown that the data derived from GdnHCl-induced and urea-induced steady state experiments of the protein in conjunction with OneG-vali computations could suggest a unique and same unfolding kinetics of the protein under native conditions.

REFERENCES

1. Kini, R.M., Acta Chim Slov. 2011, 58, 693–701.
2. Kini, R.M., Doley, R., 2010, Toxicon 56, 855 – 867.
3. Kumar, T.K.S., Jayaraman, G., Lee, C.S., Arunkumar, A.I., Sivaraman, T., Samuel, D., Yu, C., 1997, J. Biomol. Struct. Dyn. 15, 431–463.
4. Sivaraman, T., Kumar, T.K.S., Hung, K.W., Yu, C., 2000, Biochemistry, 39, 8705–8710.
5. Sivaraman, T., Kumar, T.K.S., Yu, C., 1999, Biochemistry, 38, 9899–9905.
6. Sivaraman, T., Kumar, TKS., Chang, DK., Lin, WY., Yu, C., 1998, J. Biol. Chem. 273, 10181–10189.
7. Bhaskaran, R., Huang, CC., Chang, DK., and Yu, C. (1994) J. Mol. Biol. **235,** 1291–1301.
8. Sivaraman, T., Kumar, T.K.S., Tu, Y.T., Peng, H.J., Yu, C., 1999, Arch.Biochem.Biophys. 363, 107–115.
9. Richa T, Sivaraman T. PLoS ONE 2012, 7: e32465.
10. Richa T and Sivaraman T. RSC Adv. 2014, 4(68): 36325-36335.
11. Richa T and Sivaraman T., Biologia, 2015, 70(4), 420-427.
12. Sivaraman, T., Kumar, TKS., Hung, KW, Yu, C., J. Prot. Chem. (1999), 18, 481-488.
13. Richa, T., Ph.D., thesis, SASTRA University, Tamil Nadu, India, 2014.
14. Kumar, TKS, Sivaraman, T., Yu, C., ACS Symposium Series (Natural and selected synthetic toxins), 2000, 745, 222-248.
15. Kumar, TKS., Jayaraman, G., Lee, CS., Sivaraman, T., Lin, WY., Yu, C., Biophys. Res. Commun. 1995, 207, 536-543.
16. Sivaraman, T., Kumar, TKS., Jayaraman, G., Han, CC., Yu, C., Biochem. J. (1997), 321, 457-464.