J|A|C|S
COMMUNICATIONS

Published on Web 08/01/2006

Cold Denaturation of Encapsulated Ubiquitin

Maxim S. Pometun, Ronald W. Peterson, Charles R. Babu,‡ and A. Joshua Wand*

Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059

Received April 25, 2006; E-mail: wand@mail.med.upenn.edu

It has been recognized for a very long time that proteins of even modest size have an astronomical number of potential conformations.[1] Subsequent statistical mechanical[2] and thermodynamic[3,4] treatments of this conformational landscape have emerged to illuminate roles for non-native states in phenomena ranging from protein folding to allostery. The experimental exploration of the ensemble of states accessible to protein molecules has however proven to be difficult. Recently we introduced an approach that seeks to differentiate cooperative substructure of proteins on the basis of fundamental thermodynamic parameters and to do so with the potential of resolving them from each other.[5] The approach employs a simple prediction of the temperature dependence of the various types of interactions that stabilize protein structure. For a two-state equilibrium with a temperature independent change in heat capacity (ΔC_p), a familiar form of the Gibbs−Helmholtz equation is applicable:

$$\Delta G(T) = \Delta H(T_{ref}) + \Delta C_p(T - T_{ref}) - T\,\Delta S(T_{ref}) + T\,\Delta C_p \ln(T/T_{ref})$$

where T_{ref} is an arbitrary reference temperature and the remaining symbols have their usual meanings. Proteins generally have a large positive ΔC_p and are predicted to unfold at both high and low temperatures.[6−8] This two-state treatment is easily generalized to the ensemble view of proteins.[5]

The key is that proteins are dominated by two types of interactions that have quite distinct changes in heat capacity associated with their disruption.[5] Hydrophobic interactions generally have a large ΔC_p while polar interactions generally have a ΔC_p near zero associated with their disruption in water. Importantly, states with different ΔC_p values underlying their stability can potentially be distinguished and significantly populated during cold-induced disassembly.[5] This is in distinct contrast to high-temperature unfolding, where *effective* two-state behavior is generally observed and careful analysis can only infer the presence of intermediates.[9] The idea then is to employ cold-induced unfolding to dissect and characterize the cooperative substructure of proteins using NMR.[5]

Unfortunately, the thermodynamic parameters of most proteins are such that cold-induced unfolding is not expected to occur until well below the freezing point of water.[7] Historically, destabilizing perturbations such as mutations, chemical denaturants,[10] and hydrostatic pressure[11] have been employed to promote cold denaturation at higher temperatures where standard NMR methods can be employed. However, these adjunct perturbations tend to distort the energy landscape of the native state and can potentially obscure features unique to cold denaturation.

To largely avoid these issues, we have turned to a technology initially introduced as a means to overcome the "slow tumbling" problem presented by large proteins to solution NMR spectroscopy.[12] The basic idea is to encapsulate a single protein molecule in a reverse micelle dissolved in a low viscosity fluid. Low viscosity promotes faster molecular reorientation and slower spin−spin relaxation that in turn improves spectroscopic performance. The first example of employing NMR spectroscopy of encapsulated proteins dissolved in low viscosity fluids to examine the process of cold denaturation was that of recombinant human ubiquitin in bis(2-ethylhexyl)sulfosuccinate (AOT) reverse micelles.[5] This system allowed cold denaturation of the protein to be monitored by high-resolution NMR methods at temperatures approaching −35 °C. The cold-induced unfolding of ubiquitin was found to be highly noncooperative, in distinct contrast to its apparent two-state thermal unfolding.

AOT is a negatively charged surfactant. Though the structure of encapsulated ubiquitin has been shown to be virtually identical to the free solution structure[13] and the chemical shifts of the protein are very similar,[12] there remains a possibility that electrostatic interactions between the slightly positively charged protein and the surfactant may significantly influence the results obtained. In this respect, our earlier work examined the cold denaturation of ubiquitin at pHs above and below the pI of the protein.[5] The cold-induced unfolding qualitatively followed the same highly noncooperative unfolding process.[5]

In an effort to further explore this issue, we have developed a surfactant mixture ($C_{12}E_4$/AOT/DTAB) that is largely composed of the neutral $C_{12}E_4$ species (70%) and is qualitatively charge balanced with respect to the charge of the protein.[14] Solutions of encapsulated ubiquitin are stable to temperatures below −25 °C. We have followed the cold-induced unfolding of ubiquitin encapsulated in this surfactant mixture using both ^{15}N and ^{13}C HSQC spectroscopy. As we noted previously for ubiquitin encapsulated in AOT,[5] ubiquitin containing $C_{12}E_4$/AOT/DTAB reverse micelles gradually lose water to the bulk. Spectra were obtained after an equilibrium water loading was attained. It is important to note that the charge balanced reverse micelles used here are much larger than AOT reverse micelles. To avoid artifacts due to inordinately long T_1 relaxation arising from slow tumbling, the low viscosity of propane was required.

As before,[5] the backbone amide NH correlations and side chain methyl correlations observed in HSQC spectra are in slow exchange between states. Cross-peaks of the native structure lose intensity with decreasing temperature and new cross-peaks are not observed. This is consistent with the redistribution of the ensemble toward a family of states, none of which is dominant.

As was the case for ubiquitin encapsulated in AOT, in the neutral surfactant mixture the protein shows highly noncooperative cold-induced denaturation. As before,[5] to express the process of cold denaturation structurally, we employ stringent quantitative criteria to interpret the spectral perturbations brought about by a reduction in temperature. On a ribbon representation of the native structure, we have colored red (non-native) those sites that retain less than a

‡ Present address: Analytical Development, AstraZeneca Pharmaceuticals, Wilmington, DE 19850.

10.1021/ja0628654 CCC: $33.50

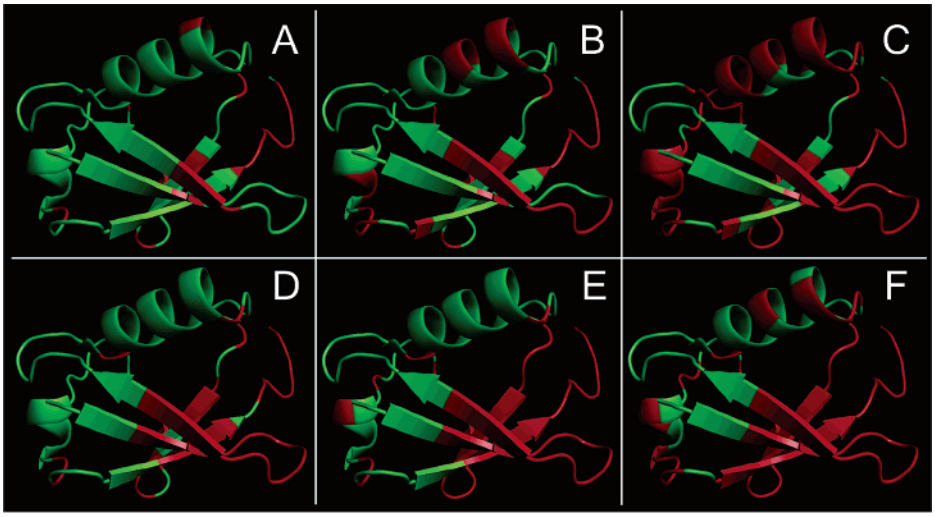

Figure 1. Summary of an intermediate state of cold-induced unfolding of encapsulated recombinant ubiquitin. The ribbon representations of the structure of encapsulated ubiquitin[13] are color-coded according to whether a given amide N−H correlation in the ^{15}N HSQC spectrum is less than (red) or more than (green) a given fraction of its intensity at +20 °C. Panels A, B, and C are of ubiquitin encapsulated in 70% C_4E_{12}/25% AOT/5% DTAB at −20 °C with an equilibrium water loading of 7 and employ intensity criteria of 5%, 10%, and 15%, respectively. Total surfactant concentration was 100 mM. The encapsulation buffer was 50 mM Na acetate, pH 5. Solutions were prepared in liquefied propane, which provided significantly narrower resonance lines than preparations in higher viscosity pentane. Panels E, F, and G are of ubiquitin encapsulated in 100 mM AOT in pentane with a target water loading of 40 and at −30 °C. Panels E, F, and G employ intensity criteria of 5%, 10%, and 15%, respectively. The buffer was 50 mM Na acetate pH 5 and 1.5 M NaCl. Spectra were collected at 750 MHz (^{1}H). Protein was prepared as described[15] and further purified by HPLC. The figure was drawn with PyMol (DeLano Scientific).

given fraction of their initial intensity at +20 °C else they are colored green (native). The state observed at −20 °C is summarized in Figure 1 (top panels). The pattern of non-native sites is striking both for its localized nature and for its close correspondence to that observed for ubiquitin encapsulated with low ionic strength buffer in AOT reverse micelles.[5]

Flynn and co-workers report that a different result is obtained if a high salt buffer is used to screen AOT-protein charge−charge interactions.[16] They claim that cold-induced unfolding of encapsulated ubiquitin occurs in a two-state manner in the presence of 1.5 M NaCl and imply that multistate unfolding is an artifact of protein−surfactant interactions.[16] We have repeated these experiments and have found the opposite result (Figure 1, bottom panels). Overall, the *progression* of cold-induced structural changes is remarkably similar for all three surfactant−buffer systems. For the preparations used here, this seems to rule out any significant influence of the charge state of the reverse micelle (i.e., neutral versus anionic surfactant; low or high salt encapsulation buffer; pH above or below the pI of the protein[5]) on the fundamental nature of the cold-induced unfolding of the protein. The differences in structure of AOT (short, branched) and $C_{12}E_4$ (long polyether) would also seem to rule out interactions of the surfactant tails with the protein. In addition, such hydrophobic interactions are disfavored by low temperature for the same reasons that promote cold denaturation of the protein. In summary, all evidence points to the cold-induced unfolding of encapsulated ubiquitin being inherently multistate.

The apparent disagreement with conclusions of Flynn and co-workers can be traced to at least two issues. First, in comparing the previous low salt study[5] to their high salt work, the quantitative criteria outlined above were not considered. Second, ubiquitin has at least one chloride binding site.[17] High chloride concentration will stabilize the protein against cold-induced unfolding. Indeed, ubiquitin encapsulated in high ionic strength buffer in AOT cold-denatures at a slightly lower temperature than when encapsulated in the neutral surfactant (Figure 1). In addition, the C-terminal region of the long α helix suspected[17] of harboring a chloride binding site is stabilized under high NaCl concentrations (Figure 1).

In summary, we have reexamined the low-temperature behavior of ubiquitin under a variety of conditions and confirmed our previous findings that the cold-induced unfolding process is highly noncooperative and decidedly multistate. We conclude that protein−surfactant interactions play a minor role and do not interfere significantly. This would seem to reinforce the appropriateness of employing NMR spectroscopy of encapsulated proteins for the study of protein cold denaturation.

Acknowledgment. This work was supported by NIH Grant GM 35940.

References

(1) Levinthal, C. *J. Chim. Phys. Phys.-Chim. Biol.* **1968**, *65*, 44.
(2) Onuchic, J. N.; Luthey-Schulten, Z.; Wolynes, P. G. *Annu. Rev. Phys. Chem.* **1997**, *48*, 545−600.
(3) Freire, E. *Adv. Protein Chem.* **1998**, *51*, 255−279.
(4) Liu, T.; Whitten, S. T.; Hilser, V. J. *Proteins* **2006**, *62*, 728−738.
(5) Babu, C. R.; Hilser, V. J.; Wand, A. J. *Nat. Struct. Mol. Biol.* **2004**, *11*, 352−357.
(6) Becktel, W. J.; Schellman, J. A. *Biopolymers* **1987**, *26*, 1859−1877.
(7) Privalov, P. L. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 281−305.
(8) Privalov, P. L.; Gill, S. J. *Adv. Protein Chem.* **1988**, *39*, 191−234.
(9) Oliva, F. Y.; Munoz, V. *J. Am. Chem. Soc.* **2004**, *126*, 8596−8597.
(10) Ibarra-Molero, B.; Makhatadze, G. I.; Sanchez-Ruiz, J. M. *Biochim. Biophys. Acta* **1999**, *1429*, 384−390.
(11) Nash, D. P.; Jonas, J. *Biochem. Biophys. Res. Comm.* **1997**, *238*, 289−291.
(12) Wand, A. J.; Ehrhardt, M. R.; Flynn, P. F. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15299−15302.
(13) Babu, C. R.; Flynn, P. F.; Wand, A. J. *J. Am. Chem. Soc.* **2001**, *123*, 2691−2692.
(14) Peterson, R. W.; Pometun, M. S.; Shi, Z. S.; Wand, A. J. *Protein Sci.* **2005**, *14*, 2919−2921.
(15) Wand, A. J.; Urbauer, J. L.; McEvoy, R. P.; Bieber, R. J. *Biochemistry* **1996**, *35*, 6116−6125.
(16) Van Horn, W. D.; Simorellis, A. K.; Flynn, P. F. *J. Am. Chem. Soc.* **2005**, *127*, 13553−13560.
(17) Makhatadze, G. I.; Lopez, M. M.; Richardson, J. M.; Thomas, S. T. *Protein Sci.* **1998**, *7*, 689−697.

JA0628654