J|A|C|S
COMMUNICATIONS

Published on Web 12/22/2005

Dehydration in the Folding of Reduced Cytochrome *c* Revealed by the Electron-Transfer-Triggered Folding under High Pressure

Tetsunari Kimura,[†,§] Koichi Sakamoto,[†,‡] Isao Morishima,[†] and Koichiro Ishimori*[,†,‡]

Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan, and Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

Received July 27, 2005; E-mail: koichiro@sci.hokudai.ac.jp

Characterization of the transition state (TS) is a key to elucidating mechanisms of protein folding. Although the TSs in the folding processes are supposed to have compact and ordered structures,[1] the entropy of the TSs increases compared with the unfolded and collapsed intermediate states.[2] Such an increase in the entropy for the formation of the TSs suggests that the entropy lost in ordering the polypeptide chain must be balanced by that gained from dehydration.[2d] Due to the difficulties in the spectroscopic characterization of water molecules, however, there has been little evidence of dehydration.

One of the promising approaches to characterize dehydration associated with protein folding is to examine the partial molar volume (PMV) change of the protein molecule, because dehydration affects the PMV of proteins.[3] The PMV change associated with protein folding is primarily composed of four processes: (1) acquisition of the void volume (V_V) arising from inefficient packing in the completely folded structure,[4] (2) change of the van der Waals volume (V_W) of the polypeptide chain upon its conformational change,[5] (3) a decrease in the thermal volume (V_T) resulting from mutual vibrations of solute and solvent molecules,[3b,5] and (4) change of the interaction volume (V_I) associated with the dehydration from the amino acid residues.[6,7] In this communication, based on the pressure dependence of the folding rates, we estimated the activation volume ($\Delta V_f^{\ddagger}$), the PMV change for the formation of the TS during protein folding, and clarify the role that dehydration plays in the formation of the TS.

We examined the folding of cytochrome *c* (cyt *c*), because its folding reaction has been extensively investigated.[8] In cyt *c*, heme is covalently bound to the polypeptide chain, offering a good spectral probe to monitor the structural changes. When guanidine hydrochloride (GuHCl) ranges between 3.2 and 4.0 M, oxidized cyt *c* (cyt c^{III}) is unfolded, whereas reduced cyt *c* (cyt c^{II}) is folded. The folding of cyt c^{II} can be triggered by an electron transfer from a photoreductant to cyt c^{III}.[2a,9] Nicotinamide-adenine dinucleotide (NADH) was used as the photoreductant, and we followed the folding of cyt c^{II} by the absorption change at 420 nm after the completion of reduction ($\sim$200 μs).[10] In photoreduction by NADH, it is possible to follow the conformational changes from the "collapsed intermediate" to the native state of cyt c^{II}.[9] We determined $\Delta V_f^{\ddagger}$ for the formation of the TS between the collapsed and native states by measuring the folding rate (k_f) as a function of pressure (10−200 MPa) and GuHCl concentration (3.2−4.0 M) in the high-pressure cell.[11]

A typical time course of the folding of cyt c^{II} in various concentrations of GuHCl is shown in Figure 1A. Observed traces can be fitted by a single-exponential function. As shown in the inset of Figure 1A, ln k_f is linearly proportional to GuHCl concentration, showing that k_f in the absence of the denaturant at ambient pressure would be (12.1 ± 0.8) × 10^3 s^{-1}, which is consistent with a previously reported value.[2a]

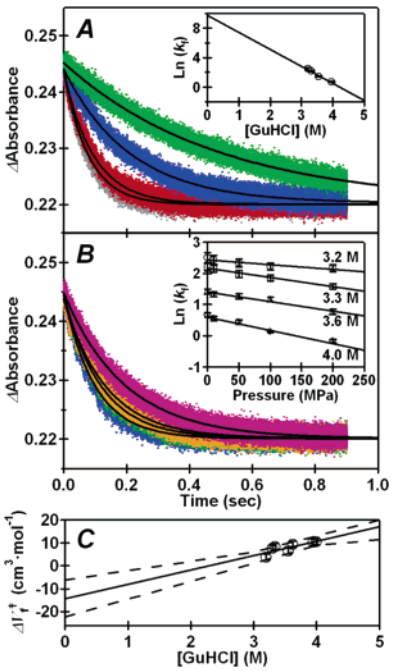

Figure 1. (A) GuHCl-dependent kinetics of cyt c^{II} folding initiated by electron transfer from NADH at ambient pressure and monitored by the absorbance change at 420 nm. Measurements were carried out at 3.2 M (gray), 3.3 M (red), 3.6 M (blue), and 4.0 M (green) GuHCl. Experimental conditions: 50 mM Tris/HCl, pH 8.0, 20 °C. The transients were measured 20 times and then averaged. The continuous lines represent the fit of single-exponential functions to the experimental data. (*Inset*) The folding rate constants (k_f) at various GuHCl concentrations and ambient pressure are indicated on the figure. (B) Pressure-dependent kinetics of cyt c^{II} folding at 3.3 M GuHCl were monitored by the absorbance change at 420 nm. Measurements were carried out at 10 (blue), 50 (green), 100 (orange), and 200 (purple) MPa. Experimental conditions: 50 mM Tris/HCl, pH 8.0, 20 °C. The transients were measured 20 times and then averaged. The continuous lines represent the fit of single-exponential functions to the experimental data. (*Inset*) Pressure dependence of the folding rate constants (k_f) at various GuHCl concentrations indicated on the figure. The lines represent the global fits (Supporting Information) for the rate constants at high pressure. (C) GuHCl dependence of the activation volume for the cyt c^{II} folding. Open circles show the activation volumes for folding from the collapsed intermediate to the native state determined by the global fit of the kinetic data displayed in the inset of Figure 1B. The lines represent the results from the fit (solid line) together with the 2σ confidence intervals estimated according to the experimental errors (dashed lines).

By elevating the pressure, the folding reaction was decelerated (Figure 1B). Under high pressure (up to 200 MPa), the kinetics of cyt c^{II} folding consisted of one phase and k_f was independent of cyt *c* concentration (not shown), suggesting no multimeric com-

[†] Kyoto University.
[‡] Hokkaido University.
[§] Current address: Beckman Institute, California Institute of Technology, Pasadena, CA 91125.

10.1021/ja055084w CCC: $33.50 © 2006 American Chemical Society

ponents in the folding reaction under high pressure. In the presence of 3.3 M GuHCl, the rate constants decrease from 8.6 s^{-1} at 10 MPa to 4.8 s^{-1} at 200 MPa, indicating that $\Delta V_f^{\ddagger}$ is positive at 3.3 M GuHCl. To calculate $\Delta V_f^{\ddagger}$ in the absence of GuHCl at ambient pressure ($\Delta V_f^{0\ddagger}$), we further measured the pressure dependence of the folding rates at different GuHCl concentrations between 3.2 and 4.0 M. As clearly shown in the inset in Figure 1B, ln k_f linearly decreases with pressure at all GuHCl concentrations. We successfully determined $\Delta V_f^{0\ddagger}$ by the global fit for the obtained data set of the folding rates at various pressures and GuHCl concentrations (Supporting Information), and $\Delta V_f^{0\ddagger}$ was estimated to be -14 ± 8 $cm^3 \cdot mol^{-1}$ as shown in Figure 1C. The negative $\Delta V_f^{0\ddagger}$ implies that the PMV of the TS is smaller than that of the "collapsed intermediate" state.

The negative $\Delta V_f^{0\ddagger}$ is rather surprising, because $\Delta V_f^{0\ddagger}$ for other proteins is positive.[3c,d] Although no experimental evidence was reported to show structural voids in the TS of cyt *c*, the Φ-value analysis suggests that, in most proteins, some parts of the peptide chain are already structured in the TSs.[1a] The structured TSs would support the formation of voids and the increased V_V in the TSs compared to those in the "collapsed intermediate" state.

Changes in the V_W would also not significantly shift the $\Delta V_f^{\ddagger}$ for the TSs to a negative value. Some simulations concluded that negative contribution is due to overlaps between the oxygen atoms of C═O groups and the hydrogen atoms of N−H groups in the secondary structures but that these changes in the V_W are much smaller than those caused by the formation of the voids.[5]

In addition, dehydration of charged or polar groups in protein molecules positively contributes to the change in the V_I (ΔV_I), because such hydrated water molecules have a reduced solvent volume due to electrostatic interactions with these groups.[6,7] Consequently the release of hydrated water molecules from charged or polar groups to form the TS would result in an increase of the PMV.

One of the factors to shift the $\Delta V_f^{0\ddagger}$ to a negative value is the change in the V_T (ΔV_T). V_T is directly proportional to the solvent accessible surface area (ASA) of the solute.[3b,5] The positive slope for ln k_f against the concentration of GuHCl, 5.3 ± 0.4 $kJ \cdot mol^{-1} \cdot M^{-1}$ (inset of Figure 1A), as well as the decreased heat capacity for the TS compared to that for the intermediate,[2a] suggests a decrease in the ASA for the TS, leading to a negative shift in the observed $\Delta V_f^{0\ddagger}$.

Another factor is dehydration of hydrophobic groups. It has been assumed that the hydration of hydrophobic residues leads to an increase in the V_I of the PMV of proteins.[13] This assumption is in line with the theoretical calculation that the density of the solvent on the hydrophobic surface is smaller than that of the bulk solvent.[14] Dehydration of hydrophobic groups would negatively contribute to the ΔV_I. In addition to the decrease in the V_I, dehydration of hydrophobic groups would decrease in the ASA, leading to a negative ΔV_T. It is, therefore, likely that water molecules solvating some hydrophobic groups are released in the formation of the TS from the intermediate state during the folding of cyt c^{II}, which shifts both V_T and V_I to negative, resulting in the unusual negative $\Delta V_f^{0\ddagger}$.

Although we have not yet identified which hydrophobic groups are responsible for dehydration in cyt *c* folding, one of the possible candidates is the heme group. In the collapsed state, a hydrophobic core, including the heme group, is formed and is still partially solvated.[2a,15] Upon transition to the native fold, decay kinetics of the zinc-substituted cyt *c* showed that the heme group is dramatically dehydrated,[15c] suggesting that most of the folding free energy is released when the collapsed state evolves into the native state.[15d] The dehydration of the heme group is, therefore, crucial for the transition from the collapsed state to the native state in the folding of cyt *c*.

It should be noted here that dehydration corresponds to an increase in entropy. Despite the decrease in ASA and compact conformation of the polypeptide in the TS,[2c,d] the temperature dependences of the cyt c^{II} folding rate showed a positive change in the entropy for formation of the TS. Subsequently a mechanism in which cyt c^{II} first forms a compact misfolded intermediate followed by expansion to reach the TS was proposed.[2a] Although we cannot exclude the possibility that the protein molecule in the TS is less ordered than the first collapsed state, it is more plausible to assume that the releasing hydrated water molecules associated with formation of the TS increase the entropy of the protein system. We can, therefore, suggest that dehydration entropically facilitates the formation of the TS in the protein folding mechanism.

Acknowledgment. This work was supported by Grants-in-Aids for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan to K.I. (16041226). T.K. was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

Supporting Information Available: Analysis of the data set of the rate constants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Fersht, A. R.; Sato, S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1525−1529. (b) Plaxco, K. W.; Simons, K. T.; Baker, D. *J. Mol. Biol.* **1998**, *277*, 985−994. (c) Sosnick, T. R.; Dothager, R. S.; Krantz, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17377−17382.
(2) (a) Pascher, T. *Biochemistry* **2001**, *40*, 5812−5820. (b) Schuler, B.; Lipman, E. A.; Eaton, W. A. *Nature* **2002**, *419*, 743−747. (c) Akmal, A.; Muñoz, V. *Proteins* **2004**, *57*, 142−152. (d) Parker, M. J.; Lorch, M.; Sessions, R. B.; Clarke, A. R. *Biochemistry* **1998**, *37*, 2538−2545.
(3) (a) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1−63. (b) Chalikian, T. V.; Torov, M.; Abagyan, R.; Bresiauer, K. J. *J. Mol. Biol.* **1996**, *260*, 588−603. (c) Silva, J. L.; Foguel, D.; Royer, C. A. *Trends Biochem. Sci.* **2001**, *26*, 612−618. (d) Royer, C. A. *Biochim. Biophys. Acta* **2002**, *1595*, 201−209.
(4) Frye, K. J.; Royer, C. A. *Protein Sci.* **1998**, *7*, 2217−2222.
(5) (a) Imai, T.; Kovalenko, A.; Hirata, F. *Chem. Phys. Lett.* **2004**, *395*, 1−6. (b) Chalikian, T. V.; Filfil, R. *Biophys. Chem.* **2003**, *104*, 489−499.
(6) (a) Harpaz, Y.; Gerstein, M.; Chothia, C. *Structure* **1994**, *2*, 641−649. (b) Gerstein, M.; Chothia, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10167−10172.
(7) (a) Dill, K. A. *Biochemistry* **1990**, *29*, 7133−7155. (b) Heremans, K. *Annu. Rev. Biophys. Bioeng.* **1982**, *11*, 1−21. (c) Hummer, G.; Garde, S.; Garcia, A. E.; Paulaitis, M. E.; Pratt, L. R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1552−1555.
(8) Winkler, J. R. *Curr. Opin. Chem. Biol.* **2004**, *8*, 169−174.
(9) (a) Telford, J. R.; Wittung-Stafshede, P.; Gray, H. B.; Winkler, J. R. *Acc. Chem. Res.* **1998**, *31*, 755−763. (b) Pascher, T.; Chesick, J. P.; Winkler, J. R.; Gray, H. B. *Science* **1996**, *271*, 1558−1560. (c) Telford, J. R.; Tezcan, F. A.; Gray, H. B.; Winkler, J. R. *Biochemistry* **1999**, *38*, 1944−1949. (d) Kliger, D. S.; Chen, E.; Goldbeck, R. A. *Methods Enzymol.* **2004**, *380*, 308−327.
(10) Orii, Y. *Biochemistry* **1993**, *32*, 11910−11914.
(11) (a) Furukawa, Y.; Ishimori, K.; Morishima, I. *Biochemistry* **2002**, *41*, 9824−9832. (b) Furukawa, Y.; Ishimori, K.; Morishima, I. *J. Phys. Chem. B* **2000**, *104*, 1817−1825.
(12) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Protein Sci.* **1995**, *4*, 2138−2148.
(13) (a) Klapper, M. H. *Biochim. Biophys. Acta* **1971**, *229*, 557−566. (b) Hvidt, A. *J. Theor. Biol.* **1975**, *50*, 245−252.
(14) Wallqvist, A.; Gallicchio, E.; Levy, R. M. *J. Phys. Chem. B* **2001**, *105*, 6745−6753.
(15) (a) Akiyama, S.; Takahashi, S.; Kimura, T.; Ishimori, K.; Morishima, I.; Nishikawa, Y.; Fujisawa, T. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1329−1334. (b) Shastry, M. C.; Roder, H. *Nat. Struct. Biol.* **1998**, *5*, 385−392. (c) Lee, J. C.; Chang, I.; Gray, H. B.; Winkler, J. R. *J. Mol. Biol.* **2002**, *320*, 159−164. (d) Kim, J. E.; Pribisko, M. A.; Gray, H. B.; Winkler, J. R. *Inorg. Chem.* **2004**, *43*, 7953−7960.

JA055084W