J|A|C|S
COMMUNICATIONS

Published on Web 03/02/2006

^{15}N NMR Spin Relaxation Dispersion Study of the Molecular Crowding Effects on Protein Folding under Native Conditions

Xuanjun Ai,† Zheng Zhou,‡ Yawen Bai,‡ and Wing-Yiu Choy*,†

Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1, and National Cancer Institute, Laboratory of Biochemistry, Building 37, Room 6114E, Bethesda, Maryland

Received November 17, 2005; E-mail: jchoy4@uwo.ca

In vitro studies of protein structure, dynamics, folding, and ligand binding are usually performed using low concentrations of purified protein in dilute buffer solutions. These environments, however, are dramatically different from that within cells where proteins carry out their functions. The presence of high concentrations of macromolecules, such as protein and RNA, occupy a significant fraction (typically 20−30%) of cellular volume and create a crowded environment inside a cell.[1−6] The excluded volume effect brought on by the high volume occupancy of solution by macromolecules can affect the kinetics and thermodynamics of biochemical reactions by orders of magnitude.[1−6] In particular, recent studies have shown that the presence of high concentrations of macromolecules can significantly alter the stabilities and folding rates of proteins.[7−10] This strongly suggests that the macromolecular crowding effects may play a crucial role in promoting the folding of protein into functional form in cells.

Macromolecular crowding effects on protein folding processes are usually studied by conventional spectroscopic techniques such as circular dichroism or fluorescence spectroscopy. Highly soluble and inert polymers such as dextran, poly(ethylene glycol) (PEG), and Ficoll are added to protein solutions to mimic the intracellular environments.[5] Even though these methods can be used to detect global structural changes of proteins under different environments, detailed structural and dynamical information at atomic resolution cannot be obtained. Another disadvantage of using conventional spectroscopic techniques to study the macromolecular crowding effects is that, on top of the crowding agents, a high concentration of denaturant needs to be added to the sample to generate the protein unfolded state. In this work, we demonstrated the feasibility of using the NMR relaxation dispersion technique to investigate the macromolecular crowding effects on protein folding under native conditions without involving any denaturant. The method has been applied to an engineered mutant of apocytochrome b_{562}.

Due to advancements in technology and methodology, NMR is a unique tool for protein dynamics studies. Protein motions over a broad range of time scales can now be probed by various spin relaxation techniques. Recent studies demonstrated that protein folding transitions can also be studied using the newly developed relaxation dispersion techniques.[11] These experiments measure the contribution to transverse relaxation rates of nuclei from the chemical/conformational exchange process. Useful information such as rates of exchange and the chemical shift differences between states can be extracted from the relaxation dispersion profiles. In a recent work, we have used the ^{15}N relaxation dispersion technique to extensively characterize the kinetics and thermodynamics of the folding transitions of two mutational variants of a redesigned four-helix bundle protein (Rd-apocyt b_{562}) under native conditions.[12] Temperature-dependent studies of the folding and unfolding rates of the mutants has allowed thermodynamics parameters, including enthalpy, entropy, and heat capacity of the proteins' native and transition states to be derived. This methodology provides a new and unique approach to obtain valuable kinetic and structural information on protein folding under native conditions at atomic level.

To gain a better understanding of how protein stability and folding are affected by the environment inside living cells, in this work, we extended the application of the ^{15}N relaxation dispersion technique to the study of macromolecular crowding effects on protein folding kinetics. Relaxation dispersion data of the F61A/I72A mutant of Rd-apocyt b_{562} were collected at 600 and 800 MHz (^{1}H frequency) as described in the previous study.[12] PEG 20K (MW $\approx$ 20 000) was added to the protein sample to mimic the crowded intracellular environment. To investigate the temperature dependence of the macromolecular crowding effects, dispersion profiles were also recorded for the protein at different temperatures (20, 25 and 30 °C). Relaxation dispersion data was then fitted to a two-state exchange model as established in our previous work.[12] Thermodynamics and kinetics of the folding transition process of Rd-apocyt b_{562} F61A/I72A in the presence and the absence of the crowding agent were obtained. Figure 1 illustrates typical fits of dispersion profiles for a representative residue of the F61A/I72A mutant. The solid lines correspond to the best fits obtained from global analysis of all residues at the two magnetic fields and at specific temperatures. The parameters obtained from the two-state-folding model fitting of ^{15}N relaxation dispersion data are summarized in Table 1. Note that data collected at different temperatures were fitted individually. Our result clearly demonstrates that useful information about macromolecular crowding effects on protein folding kinetics and thermodynamics can be extracted.

The ^{1}H−^{15}N correlation spectrum of the protein obtained in the presence of 85 mg/mL of PEG 20K is similar to the corresponding spectrum obtained without the crowding agent (Supporting Information), except that there are increases in peak line widths due to the change in viscosity. This strongly suggests that the addition of crowding agent causes a minimal change to the protein structure, at least with the conditions used.

Significant increases in the protein folding rates, however, were observed at all three temperatures in the presence of a mild concentration of crowding agent. At 20 °C, the effect of macromolecular crowding causes the folding rate (k_f) of the protein to increase by 80%, while the unfolding rate (k_u) remains unchanged within experimental error. As the temperature increases, the increases in the folding rate due to the crowding effect seem to become less (∼33% increase at 30 °C).

The changes in the folding rates of the protein indicate that the unfolded state of the protein is destabilized by macromolecular crowding. This is reflected in the changes of the population of unfolded state (pU) and free energy difference (ΔG) between the

† University of Western Ontario.
‡ National Cancer Institute.

10.1021/ja057832n CCC: $33.50 © 2006 American Chemical Society

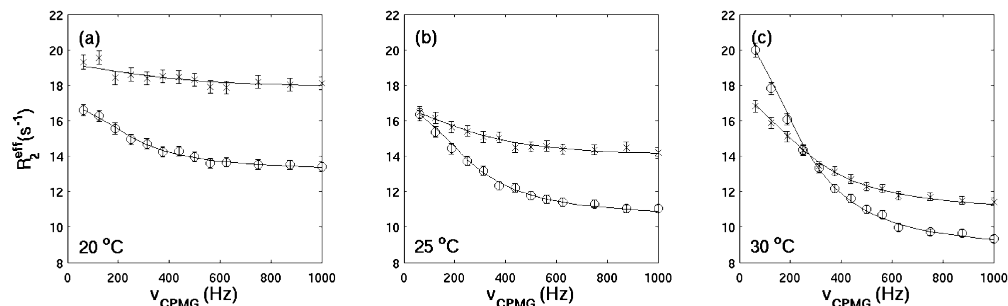

Figure 1. Typical fits of dispersion data at 800 MHz from a single residue of F61A/I72A mutant of Rd-apocyt b_{562} at (a) 20 °C, (b) 25 °C, and (c) 30 °C, using a global two-state model in the absence (o) and in the presence (x) of 85 mg/mL of PEG 20K. R_2^{eff} and υ_{CPMG} are the effective transverse relaxation rate and the CPMG field strength, respectively.

Table 1. Summary of Thermodynamics and Kinetics Parameters in the Presence and Absence of Crowding Agent[a]

temp (°C)	k_f (s^{-1})	k_u (s^{-1})	*pU* (%)	ΔG (kcal/mol)
Without Crowding Agent				
20.0	1215 ± 67	8.9 ± 0.4	0.73 ± 0.02	−2.86 ± 0.02
25.0	1182 ± 34	13.9 ± 0.4	1.16 ± 0.02	−2.63 ± 0.01
30.0	1133 ± 15	28.0 ± 0.4	2.41 ± 0.02	−2.22 ± 0.01
With 85 mg/mL PEG 20K				
20.0	2180 ± 253	10 ± 1	0.48 ± 0.02	−3.10 ± 0.03
25.0	1718 ± 107	13 ± 1	0.75 ± 0.02	−2.89 ± 0.02
30.0	1511 ± 36	25 ± 1	1.65 ± 0.04	−2.46 ± 0.01

[a] k_f folding rate, k_u unfolding rate, *pU* population of the unfolded state, and Δ*G* the free energy difference between the folded and unfolded state.

folded and unfolded states upon additions of PEG 20K. Table 1 shows that the protein stability increases by ~0.25 kcal/mol under the crowding conditions. This result agrees quite well with what Qu and Bolen observed in their study of crowding effects on the stability of TCAM.[8] If the increase in stability of the F61A/I72A mutant is linearly proportional to the concentration of crowding agent as observed for TCAM,[8] the stability of the F61A/I72A mutant will increase by 1.1 kcal/mol inside a cell, where the concentration of macromolecules is approximately 400 mg/mL. Assuming that the unfolding rate of the protein is unaffected by the crowding effects, the increase in stability will translate into a 5.5-fold increase in the folding rate of the protein at 30 °C.

Another advantage of using NMR relaxation dispersion techniques to study protein folding transitions is that structural information about the protein unfolded state under native conditions can be derived from the chemical shift difference between states extracted from the relaxation dispersion profiles. Figure 2 shows the ^{15}N chemical shift differences ($\Delta\omega$) between the folded state and the unfolded state of F61A/I72A mutant of Rd-apocyt b_{562} in the presence and the absence of crowding agent. The good agreement of $\Delta\omega$ values under these two conditions strongly suggests that the structure in the unfolded state is not perturbed significantly by the crowding agent, at least not at the concentration used.

In summary, here we report a new and unique way to investigate the macromolecular crowding effects on protein folding processes under native conditions. By applying the ^{15}N relaxation dispersion technique to an engineered mutant of Rd-apocyt b_{562}, the thermodynamics and kinetics parameters of the folding process, both in the presence and the absence of crowding agent, can be extracted.

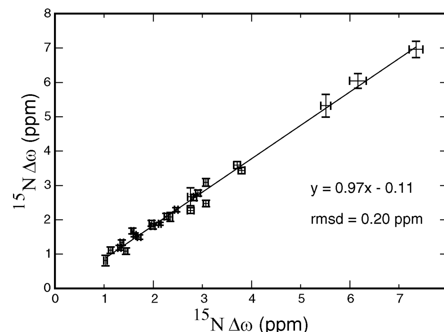

Figure 2. ^{15}N chemical shift differences between residues in the folded state and unfolded state in the presence (*y*-axis) and the absence (*x*-axis) of PEG 20K.

Studying the effects of crowding agent of different molecular sizes and at different concentrations on the thermodynamics and kinetics of the folding transition process will follow.

Acknowledgment. We thank Dr. Lewis E. Kay (University of Toronto) for the 800 MHz spectrometer time and many helpful discussions. W.-Y.C. is a recipient of a Senior Research Fellowship (phase 2) from the Canadian Institutes of Health Research (CIHR). This work was supported by a grant from CIHR.

Supporting Information Available: Detailed materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org

References

(1) Zimmerman, S. B.; Minton, A. P. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, 22, 27−65.
(2) Wenner, J. R.; Bloomfield, V. A. *Biophys. J.* **1999**, 77, 3234−3241.
(3) Minton, A. P. *Curr. Opin. Struct. Biol.* **2000**, 10, 34−39.
(4) Minton, A. P. *J. Biol. Chem.* **2001**, 276, 10577−10580.
(5) Ellis, R. J. *Trends Biochem. Sci.* **2001**, 26, 597−604.
(6) Sasahara, K.; McPhie, P.; Minton, A. P. *J. Mol. Biol.* **2003**, 326, 1227−1237.
(7) van den Berg, B.; Wain, R.; Dobson, C. M.; Ellis, R. J. *EMBO J.* **2000**, 19, 3870−3875.
(8) Qu, Y.; Bolen, D. W. *Biophys. Chem.* **2002**, 101−102, 155−165.
(9) Tokuriki, N.; Kinjo, M.; Negi, S.; Hoshino, M.; Goto, Y.; Urabe, I.; Yomo, T. *Protein Sci.* **2004**, 13, 125−133.
(10) Ghaemmaghami, S.; Oas, T. G. *Nat. Struct. Biol.* **2001**, 8, 879−882.
(11) Korzhnev, D. M.; Salvatella, X.; Vendruscolo, M.; Di Nardo, A. A.; Davidson, A. R.; Dobson, C. M.; Kay, L. E. *Nature* **2004**, 430, 586−590.
(12) Choy, W.-Y.; Zhou, Z.; Bai, Y.; Kay, L. E. *J. Am. Chem. Soc.* **2005**, 127, 5066−5072.

JA057832N