JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

Macromolecular Crowding Tunes Folding Landscape of Parallel α/β Protein, Apoflavodoxin

Loren Stagg,[†,‡] Alexander Christiansen,[§] and Pernilla Wittung-Stafshede*[,†,§]

[†]Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77005, United States

[‡]Department of Biochemistry and Molecular Biology, University of Texas MD Anderson, Houston, Texas 77030, United States

[§]Department of Chemistry, Chemical Biological Center, Umeå University, 901 87 Umeå, Sweden

ABSTRACT: Proteins normally fold in crowded cellular environments. Here we use a set of *Desulfovibrio desulfuricans* apoflavodoxin variants to assess—with residue-specific resolution—how apoflavodoxin's folding landscape is tuned by macromolecular crowding. We find that, under crowded conditions, initial topological frustration is reduced, subsequent folding requires less ordering in the transition state, and β-strand 1 becomes more important in guiding the process. We propose that conditions more closely mimicking the cellular environment make the ensemble of unfolded conformations less expanded, resulting in a folding funnel that is smoother and narrower.

It is most often assumed that protein biophysical and structural properties observed in dilute buffer solutions *in vitro* also represent the *in vivo* scenario. However, the intracellular environment is highly crowded due to the presence of large amounts of soluble and insoluble biomolecules, including proteins, nucleic acids, ribosomes, and carbohydrates. This means that a significant fraction of the intracellular space is not available to other macromolecular species. It has been estimated that the concentration of macromolecules in the cytoplasm ranges from 80 to 400 mg/mL.[1,2] The term `macromolecular crowding'[3] implies the nonspecific influence of steric repulsions on specific reactions that occur in highly volume-occupied media. Due to excluded volume effects,[4] any reaction that increases the available volume will be favored by macromolecular crowding.[5] It is proposed that crowding provides a stabilizing effect to the folded state of proteins indirectly due to compaction (i.e., increase in free energy) of the more extended and malleable denatured states.[6,7] Macromolecular crowding in solution can be mimicked experimentally by inert synthetic or natural macromolecules, termed crowding agents, to the systems *in vitro*. Many experimental and theoretical studies have demonstrated effects of macromolecular crowding on protein stability and folded- and unfolded-state structures.[7−12] Despite this, data about the effects of macromolecular crowding on protein folding kinetics *in vitro* are sparse.

D. desulfuricans apoflavodoxin is an excellent model system for such folding studies for several reasons: (1) it is a small single-domain protein (148 residues) with an α/β-repeat fold involving a parallel β-sheet (strand order: 21345) surrounded by four helices (Supporting Information (SI) Figure S1); (2) it unfolds reversibly (chemically or thermally) in a two-state equilibrium reaction in vitro;[11,13] (3) equilibrium stabilizing effects of crowding have been reported[10,11] (4) the kinetic folding landscape for apoflavodoxin has been probed: it involves an initial misfolded species that rearranges in a rate-limiting process to the native structure.[14] We recently combined *in vitro* and *in silico* experiments on a set of 13 *D. desulfuricans* apoflavodoxin variants, with mutations in different secondary structure elements, to reveal molecular details of apoflavodoxin's complex kinetic folding reaction. Our study[14] revealed that competition between the two sides of apoflavodoxin's central β-sheet (i.e., $\beta2\beta1\beta3$ side versus $\beta3\beta4\beta5$ side of the same β-sheet; SI Figure S1) directs initial misfolding. In contrast, interactions between both sides of the central β-strand 3 (i.e., $\beta1\beta3\beta4$ aligned) is necessary for productive folding (probed by ϕ-value analysis of the overall folding barrier). Notably, the extent of heterogeneity in the folding nuclei growth correlated with the size of the *in vitro* burst phase amplitude in the far-UV circular dichroism (CD) signal at 222 nm.[14]

We here use the same set of apoflavodoxin variants (SI Figure S1) to probe how the time-resolved folding reaction is affected by crowded conditions *in vitro*. Equilibrium- and stopped-flow folding/unfolding experiments were performed (see SI and ref 14) in the absence[14] and presence of 100 mg/mL Ficoll 70. The sugar-based polymer Ficoll 70 (average MW of ~74 kDa) adopts a semirigid spherical shape[15−18] and was selected since it is believed to affect proteins via excluded volume effects, has low absorption above 200 nm, does not interact with proteins, and is inert toward heat and chemicals. Ficoll 70 (100 mg/mL) corresponds to a volume occupancy of about 35−40%. First, in agreement with Ficoll-induced thermal stabilization,[11] the apoflavodoxin variants are also mostly stabilized toward urea-induced unfolding in presence of 100 mg/mL Ficoll 70 as compared to that in buffer (SI Table S1). Time-resolved CD-detected reactions in the presence of Ficoll 70 showed the same trends as found in buffer for all variants (SI Table S2, key kinetic parameters at buffer conditions): an initial burst is followed by a slower single-exponential folding step. This was also reported earlier for wild-type apoflavodoxin[19] where computer simulations further supported the proposed folding mechanism. Analysis of m_{unf}/m_{eq} (Tanford β value) ratios for all the variants in buffer versus those in crowding show good agreement, implying that the mechanism is the same at both conditions (SI Figure S2). Brönstedt plots of $\Delta(RT \ln k_{unf})$ versus $\Delta\Delta G_{eq}$ for the set of variants show linear trends in both

Received: August 24, 2010
Published: December 22, 2010

ACS Publications © 2010 American Chemical Society

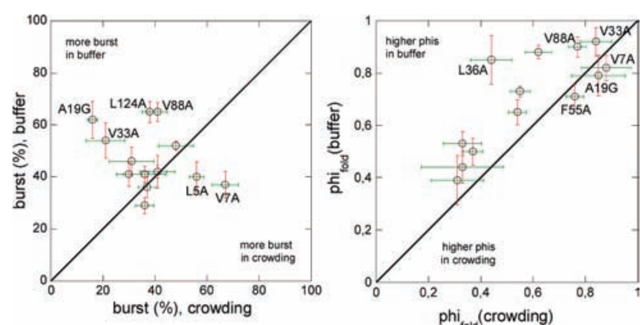

Figure 1. (Left) Burst phase amplitudes (% of total CD change at 222 nm) found in the first millisecond of refolding for all apoflavodoxin variants in buffer (y-axis) versus in 100 mg/mL Ficoll 70 (x-axis). (Right) ϕ-values for productive folding for all apoflavodoxin variants in buffer (y-axis) versus in 100 mg/mL Ficoll 70 (x-axis). Key variants mentioned in the text are marked. Error bars come from standard error propagation analysis.

conditions (SI Figure S2). Analysis of the Chevron plots using linear and curved fits are reported in Table S3 and Figures S3 and S4 of the SI. Direct comparisons between buffer and Ficoll 70 data for the variants are shown in Figures S5 (T_m, ΔG_{eq}) and S6 (Chevron plots) of the SI.

In Figure 1A, we show the correlation between burst phase amplitude, which reports on initial unproductive bias toward one side of the sheet or the other, in Ficoll 70 versus buffer conditions. It is clear that for most variants, the burst phase is reduced in Ficoll 70; exceptions are V7A(β1) and L5A(β1) variants that cause larger bursts. Particularly for the A19G, V33A, V88A, and L124A variants, with mutations at residues previously identified to be important in hindering misfolding (i.e., mutation gives large burst),[14] their burst amplitudes are significantly reduced in crowded conditions (SI Figure S7A). These observations imply that macromolecular crowding reduces topological frustrations in the early stages of apoflavodoxin folding. In Figure 1B, we show the correlation between ϕ-values for overall folding of apoflavodoxin in Ficoll 70 versus in buffer. It emerges that many residues have significantly lower ϕ-values in Ficoll 70 than in buffer, implying a less native-like folding-transition-state structure (or more unfolded-like), as compared to that in buffer (SI Figure S7B). Notably, three residues exhibit similar or higher ϕ-values in Ficoll 70 than in buffer: F55(β3), A19(α1), and V7(β1). Control experiments using sucrose at the same mg/mL as in the Ficoll experiments, and probing the Ficoll 70 concentration dependence are described in the SI (Figures S8 and S9). Sucrose stabilizes apoflavodoxin variants: the Chevron plots are shifted to the right (giving higher k_f and lower k_u) as compared to those in buffer although the ϕ_f values are not changed.

This is the first time-resolved study of protein folding in a crowded condition *in vitro* that involves *residue-specific information* and a *frustrated folding mechanism*. Previous studies have implied that folding speeds (most often for two-state folders) will be faster in crowded conditions[7,12,20,21] although retardation of folding rates has been noted.[22] For wild-type apoflavodoxin, we have reported that, while unfolding speed is roughly the same, the final refolding step becomes faster in the presence of Ficoll 70.[19] It emerges from our current data on the variants that the presence of Ficoll 70 does not change the apparent mechanism but tunes both the first (topological frustration) and second (productive folding) steps. In the presence of Ficoll 70, there is, in general, less early misfolding (i.e., smaller burst phase); this is apparently achieved by β1 interactions as mutations in this β-strand (i.e., positions 5 and 7) result in larger bursts than for the same mutations in buffer. Although the subsequent productive folding requires less average ordering in the transition state at the crowded condition, a few residues exhibit somewhat higher ϕ-values in Ficoll 70 than in buffer.

Folding can be explained using energy landscape theory, assuming minimal frustration and a funneled-shaped energy surface.[23] The shape and ruggedness of the funnel surface are dictated by protein sequence and topology as well as surrounding conditions.[24] Our work here implies that macromolecular crowding alters the folding funnel for apoflavodoxin; this effect was previously suggested from simulations of dimer formation.[25]

The observed reduced burst phases and lower ϕ-values for the folding-transition state under crowded conditions suggest that apoflavodoxin's folding funnel will be narrower at the top (reduced conformational entropy) with smoother walls (i.e., less misfolding and traps) in crowded as compared to that in dilute buffer conditions. This can be explained by the excluded volume effect causing a more compact distribution of unfolded states, which results in less topological frustration early on in the folding landscape. In other words, crowding alters the unfolded population such that correct folding is favored and the search for native contacts is reduced (manifested in faster k_f in Ficoll). In accord, computer simulations have shown unfolded-state compaction of apoflavodoxin under crowded conditions.[11] Thus, lower ϕ values do not necessarily mean less order but that the change from the unfolded to the transition state is smaller at crowded conditions. Both *in vitro* (here and in ref 11) and *in silico*[11] experiments reveal native-state stabilization of apoflavodoxin in Ficoll 70 which can be explained by unfavorable (in terms of energetics, but mechanistically helpful) compaction of the unfolded state. Unfolded-state compaction in Ficoll 70 has been demonstrated *in vitro* for another, unrelated protein.[26] Clearly, our study is only one approach toward a better understanding of how folding reactions may occur in cell-like conditions. We note that other crowding agents, weak interactions with other macromolecules, proteins with different folding mechanisms, as well as chaperones and cotranslational processes, must be considered in future work.

■ ASSOCIATED CONTENT

Supporting Information. Materials and Methods, Figures S1−S9, Tables S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
pernilla.wittung@chem.umu.se

■ ACKNOWLEDGMENT

P.W.S. acknowledges funds from the Kempe and Wallenberg foundations, the Swedish Research Council, and Umeå University. We thank M. S. Cheung and M. Wolf-Watz for helpful discussions and suggestions.

■ REFERENCES

(1) Rivas, G.; Ferrone, F.; Herzfeld, J. *EMBO Rep.* **2004**, *5* (1), 23–27.
(2) Ellis, R. J.; Minton, A. P. *Nature* **2003**, *425* (6953), 27–28.

(3) Minton, A. P.; Wilf, J. *Biochemistry* **1981,** *20* (17), 4821–4826.
(4) Laurent, T. C.; Ogston, A. G. *Biochem. J.* **1963,** *89*, 249–253.
(5) Minton, A. P. *Biophys. J.* **2005,** *88* (2), 971–985.
(6) Zhou, H. X. *Acc. Chem. Res.* **2004,** *37*, 123–130.
(7) Cheung, M. S.; Klimov, D.; Thirumalai, D. *Proc. Natl. Acad. Sci. U.S.A.* **2005,** *102* (13), 4753–4758.
(8) Dedmon, M. M.; Patel, C. N.; Young, G. B.; Pielak, G. J. *Proc. Natl. Acad. Sci. U.S.A.* **2002,** *99* (20), 12681–12684.
(9) Sasahara, K.; McPhie, P.; Minton, A. P. *J. Mol. Biol.* **2003,** *326* (4), 1227–1237.
(10) Perham, M.; Stagg, L.; Wittung-Stafshede, P. *FEBS Lett.* **2007,** *581* (26), 5065–5069.
(11) Stagg, L.; Zhang, S. Q.; Cheung, M. S.; Wittung-Stafshede, P. *Proc. Natl. Acad. Sci. U.S.A.* **2007,** *104* (48), 18976–18981.
(12) Homouz, D.; Perham, M.; Samiotakis, A.; Cheung, M. S.; Wittung-Stafshede, P. *Proc. Natl. Acad. Sci. U.S.A.* **2008,** *105* (33), 11754–11759.
(13) Muralidhara, B. K.; Rathinakumar, R.; Wittung-Stafshede, P. *Arch. Biochem. Biophys.* **2006,** *451*, 51–58.
(14) Stagg, L.; Samiotakis, A.; Homouz, D.; Cheung, M. S.; Wittung-Stafshede, P. *J. Mol. Biol.* **2010,** *396* (1), 75–89.
(15) Bohrer, M. P.; Patterson, G. D.; Carroll, P. J. *Macromolecules* **1984,** *17*, 1170–1173.
(16) Davidson, M. G.; Dean, W. M. *Macromolecules* **1988,** *21*, 3474–3481.
(17) Ohlson, M.; Sorensson, J.; Lindstrom, K.; Blom, A. M.; Fries, E.; Haraldsson, B. *Am. J. Physiol. Renal. Physiol.* **2001,** *281* (1), F103–F113.
(18) Oliver, J. D., III; Anderson, S.; Troy, J. L.; Brenner, B. M.; Deen, W. H. *J. Am. Soc. Nephrol.* **1992,** *3* (2), 214–228.
(19) Homouz, D.; Stagg, L.; Wittung-Stafshede, P.; Cheung, M. S. *Biophys. J.* **2009,** *96* (2), 671–680.
(20) van den Berg, B.; Wain, R.; Dobson, C. M.; Ellis, R. J. *Embo J.* **2000,** *19* (15), 3870–3875.
(21) Zhou, B. R.; Liang, Y.; Du, F.; Zhou, Z.; Chen, J. *J. Biol. Chem.* **2004,** *279* (53), 55109–55116.
(22) Ladurner, A. G.; Fersht, A. R. *Nat. Struct. Biol.* **1999,** *6* (1), 28–31.
(23) Bryngelson, J. D.; Onuchic, J. N.; Socci, N. D.; Wolynes, P. G. *Proteins* **1995,** *21* (3), 167–195.
(24) Onuchic, J. N.; Wolynes, P. G. *Curr. Opin. Struct. Biol.* **2004,** *14* (1), 70–75.
(25) Wang, W.; Xu, W. X.; Levy, Y.; Trizac, E.; Wolynes, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **2009,** *106* (14), 5517–5522.
(26) Hong, J.; Gierasch, L. M. *J. Am. Chem. Soc.* **2010,** *132* (30), 10445–10452.