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Folding of an Unfolded Protein by Macromolecular Crowding in Vitro

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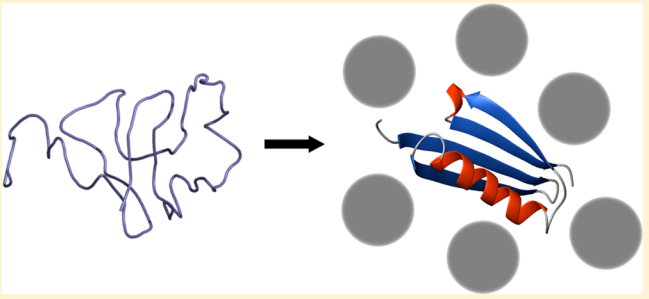
Supporting Information

ABSTRACT: Protein folding in vivo takes place in a highly crowded environment. The resulting excluded volume forces are thought to stabilize folded forms of proteins. In agreement, many in vitro studies have shown that the presence of macromolecular crowding agents increases the stability of folded proteins but often by only a few kJ per mol. Although it should not matter at what position in the transition between folded and unfolded forms the effect of crowding is employed, there have been no studies assessing whether excluded volume forces alone can correctly fold polypeptides that are mostly unfolded. However, some studies have indicated that the effect of crowding becomes larger the more destabilized the protein is (but still being folded), suggesting that the crowding effect may be exaggerated for unfolded proteins. To address this question directly, we turned to a destabilized mutant of protein L that is mostly unfolded in water but can be folded upon addition of salt. We find that the effect of 200 mg/mL Dextran 20 on the folding equilibrium constant for unfolded protein L ($\Delta\Delta G_U \approx 2$ kJ mol^{-1}) matches the crowding effects found on the folded wild type protein and the mutant when prefolded by salt. This result indicates that the excluded volume effect is independent of starting protein stability and that crowding can shift the reaction toward the folded form when the polypeptide is in the transition region between folded and unfolded states.



To function, proteins must fold from extended unfolded states to compact unique structures that are biologically active. For many proteins, folding is a spontaneous process with all information encoded in the amino acid sequence. Through pioneering work during the last three decades, significant progress has been made to pinpoint mechanisms and driving forces important for protein folding. However, in reality, proteins fold inside cells where the environment is very different from the dilute buffer solutions mostly used in in vitro experiments. The intracellular environment is highly crowded due to the presence of large amounts of macromolecules, 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] All macromolecules in physiological fluids collectively occupy between 10 and 40% of the total aqua-based volume.[3] The crowded environment results in excluded volume effects, risk of nonspecific intermolecular interactions, and increased viscosity.

Minton coined the words "macromolecular crowding" in 1981[4] to address the impact of volume exclusion from macromolecules.[5−7] Because of excluded volume effects, any reaction resulting in a volume change will be affected by macromolecular crowding.[5,8] Therefore, macromolecular crowding will provide a stabilizing effect on the folded states of proteins indirectly due to destabilization of the more extended and malleable denatured states.[9,10] The effects of excluded volume on the activity of folded and unfolded states, thereby also on overall protein stability, have been predicted by Minton[5,11,12] using a statistical thermodynamic approach.[13] In the simplest models, the folded and unfolded states are mimicked as effective hard spheres of appropriate sizes and the crowding molecule as a solid sphere[14,15] or solid rod.[16]

To create excluded volume conditions in vitro, one may use so-called macromolecular crowding agents which are inert, noncharged polymers of defined sizes that occupy space (i.e., Dextrans or Ficoll).[7,17,18] Ficoll 70 is a sucrose-based polymer, whereas Dextrans are glucose-based polymers that are available in various sizes. Several in vitro experiments have shown that protein stability can be affected by the presence of macromolecular crowding agents.[6,10,19−23] However, the absolute effects of macromolecular crowding on protein equilibrium stability are often only on the order of 2−3-fold increases in K_{eq} for folding or a few kJ per mol in terms of free energy changes.[24−26] Existing published data for effects of macromolecular crowding agents on thermal and chemical stability of different proteins (T_m and ΔG_U) are summarized in ref 27. These reported data points are most often collected at specific crowder concentrations between 100 and 300 mg/mL, and studies of crowder concentration dependencies within the same system are sparse. We recently demonstrated that simple excluded volume theory (using rods for crowders) could quantitatively capture the observed stability effects of Dextran

Received: February 21, 2014
Revised: March 25, 2014
Published: March 25, 2014

and Ficoll 70 on the protein *Pseudomonas aeruginosa* apoazurin.[28] This finding, together with an extended temperature study,[26] suggests that crowding agents cause mainly excluded volume effects. However, in special cases, synthetic crowding agents may also engage in nonspecific (chemical) interactions.[29,30]

In our early studies of crowding effects on protein stability, we noticed that when *Desulfovibrio desulfuricans* apoflavodoxin was kept in a buffer where it has low intrinsic stability, or when GuHCl was added to horse heart cytochrome c to destabilize the folded state, the effects of crowding agents on the proteins' T_m values were increased.[20,21] This made us speculate that the crowding effect depended on initial stability of the protein, and further, this would predict that a destabilized protein that is mostly unfolded in buffer could be largely stabilized in the presence of crowding agents. However, most crowding agent studies have focused on the increase in stability upon perturbation (heat or chemical denaturation) of proteins that are folded in buffer at room temperature.[27] There have been few studies directly probing the effect of excluded volume forces on proteins that are foldable, but unfolded in physiological pH buffers at room temperature. For cytochrome c at low pH, where the protein is unfolded, addition of crowding induced formation of the more compact, salt-induced molten globule state, suggesting that the molten globule-like state is stabilized relative to the fully unfolded form in crowded environments.[6] For FlgM, which is an intrinsically disordered protein that gains structure in its C-terminal part upon binding the transcription factor σ,[28] its C-terminal part became structured when analyzed inside cells by NMR, although it was not clarified what type of structure was induced.[31] Other studies of crowding effects on intrinsically disordered or unfolded proteins have shown no structural effects at all,[32] or polypeptide compaction but no specific folding.[33−35]

To test explicitly whether macromolecular crowding can fold an unfolded protein at physiological conditions, we studied a variant of the immunoglobin G binding domain of protein L from the mesophile *Streptococcus magnus* that is unfolded in solution due to seven destabilizing (Lys to Glu) mutations making it an obligate halophile.[36] Addition of salt induces a folded structure that matches that of the wild type protein.[36−38] From a combination of NMR and CD experiments on the destabilized variant and wild type protein L, we find that the excluded volume effect due to Dextran 20 is (1) independent of initial protein stability, (2) on the order of a few kJ per mol (at 200 mg/mL Dextran 20), and (3) acts to shift the unfolded population toward the folded state.

MATERIALS AND METHODS

Chemicals. The water used was run through a Milli-Q water purification system (Merck Millipore, Billerica, MA) with a resistivity of ≥18 MΩ cm^{-1}. Dextran 20 of technical grade was purchased from Pharmacosmos A/S (Holbaek, Denmark), and Ultrapure guanidine hydrochloride was from Sigma-Aldrich.

Protein Expression. Genes coding for protein L were ordered from GenScript (NJ, USA), expressed, and delivered in a pET-3a vector using the restriction sites NdeI-*Bam*HI. The mutated protein L variant was subcloned and transferred into a pET-19b vector which carries an N-terminal His-tag and verified using DNA sequencing (MWG Operon, Germany). For protein expression, plasmids were transformed into BL21(DE3) competent cells and grown at 37 °C in 1× LB medium containing 100 μg/mL carbenicillin until $OD_{600} \approx 0.6$. Protein expression was induced by addition of 1 mM IPTG, and the cells were further grown overnight. For production of ^{15}N-labeled protein, $^{15}NH_4Cl$-enriched M9 medium was used instead of LB. The protein purity and protein concentration were verified with SDS-PAGE and the absorption at 280 nm (where ε = 9970 cm^{-1} M^{-1}). Depending on the vector system, different purification methods were used; see below.

Purification of Wild Type Protein L. Cells containing wild type protein L were centrifuged at 5000 rpm for 30 min and resuspended in 50 mM Tris pH 7.5 followed by sonication on ice and centrifugation at 15 000 rpm for 30 min. The supernatant was filtered and loaded on an anion column (Q Sepharose Fast Flow, GE Healthcare) equilibrated with 50 mM Tris, pH 7.5. Protein was eluted using a linear gradient of 50 mM Tris, 2 M NaCl at pH 7.5. Selected fractions were concentrated and finally purified on a gel filtration column (HiLoad 16/60 Superdex 75, GE Healthcare), equilibrated with 20 mM NaP at pH 6.0.

Purification of Mutant Protein L. Cells containing mutant protein L were centrifuged at 5000 rpm for 30 min and resuspended in 50 mM Tris, 2 M NaCl, 10 mM imidazole at pH 7.5 followed by sonication on ice, and centrifugation at 15 000 rpm for 30 min. The supernatant was filtered and loaded on an affinity column (Ni Sepharose 6 Fast Flow, GE Healthcare) equilibrated with 50 mM Tris, 10 mM imidazole, 2 M NaCl, pH 7.5, and eluted with a linear gradient containing 50 mM Tris, 500 mM imidazole, 2 M NaCl, pH 7.5. Selected fractions were finally purified on a gel filtration column (HiLoad 16/60 Superdex 75, GE Healthcare) equilibrated with 20 mM NaP, 2 M NaCl, pH 6.0. For removal of the salt, extensive dialysis or a desalting column (PD-10, GE Healthcare) was used at 4 °C.

Circular Dichroism (CD). Far-UV CD data were recorded on a J-810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier element for temperature control. The protein concentration was 40 or 60 μM, depending on the experiment, using a buffer consisting of 20 mM NaP at pH 6, supplemented with different concentrations of Dextran 20 and/or NaCl. The temperature for all CD experiments was 20 °C, and a 1 mm cell was used. Thermal unfolding was performed using a scan rate of 1 °C min^{-1}, and data were collected between 20 and 95 °C using a fixed wavelength of 214 nm. For all CD spectra shown, buffer, salt, and Dextran backgrounds have been subtracted.

Chemical Unfolding. Equilibrium parameters from CD data can be fitted to eq 1,[39] which gives the free energy of unfolding in absence of denaturant, ΔG_u^0, and the free energy dependence of denaturant (m_{eq}):

$$\begin{aligned} CD_{obs} = \{&CD_N - mN[D] + (CD_U + mU[D]) \\ &\exp((\Delta G_u^0 - m_{eq}[D])/RT)\} \\ &/\{1 + \exp((\Delta G_u^0 - m_{eq}[D])/RT)\} \end{aligned} \quad (1)$$

In the equation CD_{obs} is observed CD signal and [D] is denaturant concentration. The fitted parameters are ΔG_u^0, *mN* and *mU*, corresponding to the denaturant dependence of the folded and unfolded baselines, respectively. m_{eq} is the denaturant dependence of ΔG_u^0 in the direction of unfolding, and CD_N and CD_U are the CD-amplitudes of folded and unfolded states.

Thermal Unfolding. The midpoint of thermal unfolding (T_m) can be obtained by fitting the data to the following equations:[40]

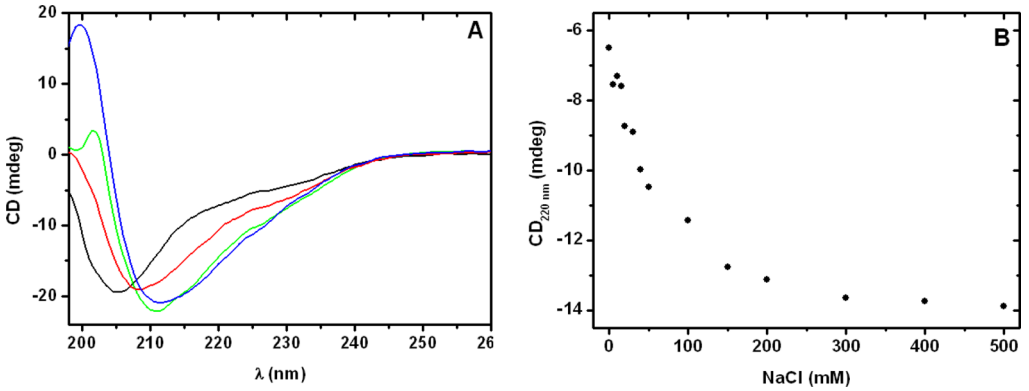


Figure 1. Far-UV CD spectra at 20 °C for mutant protein L in water (black), in 20 mM phosphate buffer (red), in 500 mM NaCl (green), and the wild type protein L CD spectrum (blue) (A). CD signal at 220 nm versus added NaCl concentration for mutant protein L in water (B).

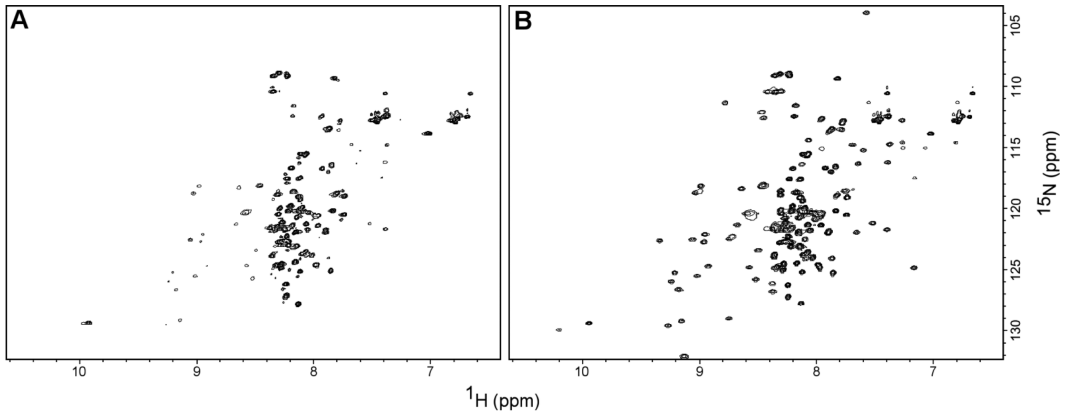


Figure 2. ^{1}H−^{15}N HSQC NMR spectra of mutant protein L in water recorded at 850 MHz (A) and in 20 mM phosphate buffer, recorded at 600 MHz (B).

$$\mathrm{CD}_{\mathrm{norm}}(T) = \frac{S_{\mathrm{f}} + \alpha T + K_{\mathrm{obs}}(S_{\mathrm{u}} + bT)}{1 + K_{\mathrm{obs}}} \tag{2a}$$

where

$$K_{\mathrm{obs}}(T) = \exp\left(\frac{\Delta H_{\mathrm{m}}}{R}\left(\frac{1}{T_{\mathrm{m}}} - \frac{1}{T}\right)\right) \tag{2b}$$

$\mathrm{CD}_{\mathrm{norm}}(T)$ is the normalized CD signal, S_{f}, S_{u}, a, and b are the CD signals for folded and unfolded conditions and the slopes for the folded and unfolded baselines, respectively. ΔH_{m} corresponds to the enthalpy value at T_{m}.

NMR. NMR data were recorded on Bruker DRX 600 MHz and Bruker AVIIIHD 850 MHz spectrometers both equipped with 5 mm triple-resonance cryoprobes, using pulse sequences from the Bruker library. All NMR experiments were recorded at 25 °C, and the temperature was calibrated prior to the experiments by inserting a thermometer into the sample compartment of the cryoprobe. The buffers used were 20 mM NaP with and without 200 mg/mL Dextran 20 at pH 6.0, supplemented with 10% (volume/volume) $^{2}H_2O$. The protein concentration ranged between 100 and 200 μM. Reference spectra of the mutated protein L was recorded in Milli Q-water. All spectra were processed and analyzed in Topspin 3.2 (Bruker).

RESULTS

We selected a crowder (Dextran 20) concentration of 200 mg/mL since it is a biologically relevant concentration. In previous work, we demonstrated that the effects of Dextran on apoazurin stability are independent of Dextran size (in the range 20−70 kDa), and the magnitudes of the effects correlate linearly with Dextran 20 concentration up to 200 mg/mL.[28] The Dextran concentration-dependence for the free energy effect on apoazurin stability in ref 28 can be recalculated to 200 kJ/(mol·M), since 1 M Dextran 20 corresponds to 20 g of Dextran 20/mL. The immunoglobin G binding domain of protein L from the mesophile *Streptococcus magnus* is a well-characterized 7 kDa protein that unfolds in a reversible two-state reaction. Changing seven of its lysine residues to glutamic acids lowers the stability, causing the majority of the molecules to be unfolded in water at room temperature. Upon addition of salt, the variant folds reversibly to the same structure as the wild type protein;[36−38] thus, we reasoned that excluded volume forces due to macromolecular crowding may do the same.

In our initial experiments on mutant protein L, we discovered that the degree of unfolding depended on whether the protein was placed in buffer or water. The variant is mostly unfolded in water, whereas in 20 mM phosphate buffer the magnitude of the CD signal indicates that there is a fraction of folded species (Figure 1A). Upon addition of 300 mM NaCl or more, the protein adopts a CD spectrum indicative of a fully folded structure. As expected, the signal for mutant protein L in high salt matches the CD signal for wild type protein L (Figure 1A). In agreement with a two-state folding transition, salt titration to the variant in water results in a sigmoidal change in the CD signal at 220 nm with a transition midpoint around 30 mM NaCl (Figure 1B). Two-state analysis of the data (not

dx.doi.org/10.1021/bi500222g | *Biochemistry* 2014, 53, 2271−2277

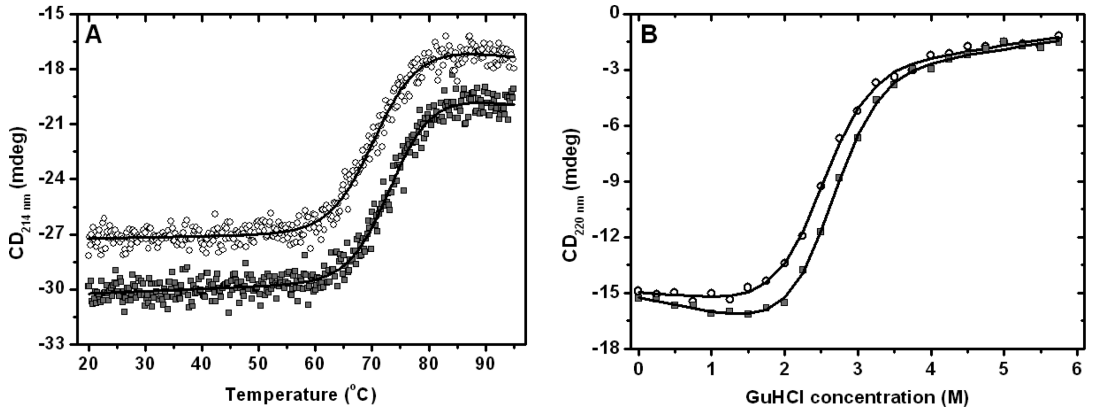


Figure 3. Thermal denaturation (A) and chemical unfolding (B) of wild type protein L with (gray squares) and without (circles) 200 mg/mL Dextran 20 probed by changes in CD at 220 nm as a function of GuHCl concentration. The data sets were analyzed by a two-state model (solid lines), and extracted thermodynamic parameters are reported in the text. In A, the Dextran data set was shifted downward for clarity.

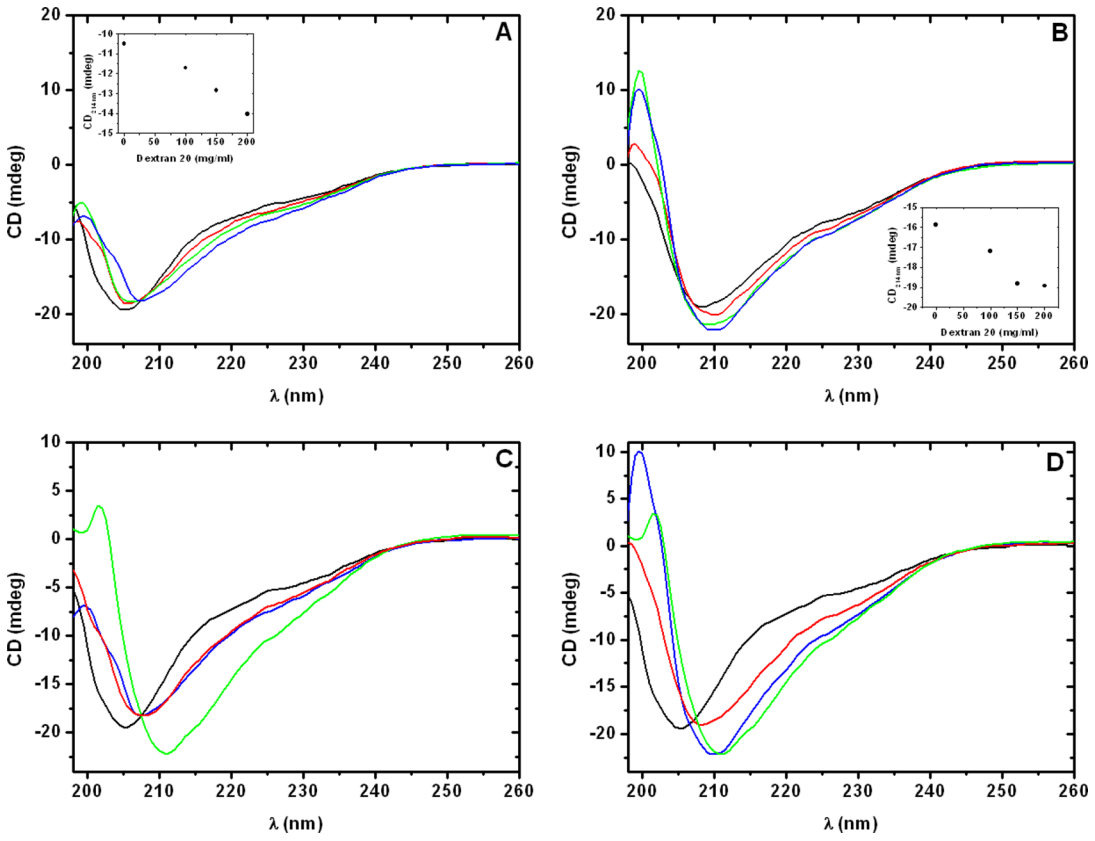


Figure 4. (A) Far-UV CD spectra for mutant protein L water in the presence of 0 (black), 100 (red), 150 (green), and 200 (blue) mg/mL Dextran 20. The inset shows CD at 214 nm as a function of crowder. (B) Far-UV CD spectra for mutant protein L in 20 mM buffer in the presence of 0 (black), 100 (red), 150 (green), and 200 (blue) mg/mL Dextran 20. The inset shows CD at 214 nm as a function of crowder. (C) Comparison of CD spectra of mutant protein L in water (mostly unfolded; black) and in high salt (folded; green) and upon addition of 200 mg/mL Dextran 20 to the water sample (blue). The red line corresponds to a linear combination of the water and salt signals (32% salt and 68% water). (D) Comparison of CD spectra of mutant protein L in water (mostly unfolded; black) and in high salt (folded; green) with the spectrum observed in buffer (partially folded; red) and upon addition of 200 mg/mL Dextran 20 to the buffer sample (blue).

shown) gives an estimate for the folding free energy needed to fold half the molecules in water of 4 kJ mol^{-1}. This value corresponds to 90% of the polypeptides being unfolded in water. Since the salt-induced folding transition for the protein L variant has a midpoint at 30 mM NaCl, our observation that the presence of 20 mM buffer (which can be considered a salt) shifts the folded/unfolded fractions is reasonable. In 20 mM buffer, the CD data of the protein L variant suggest that the fraction unfolded is 0.6.

The structural states of the protein L variants at different conditions were confirmed by NMR (Figure 2). $^{1}H-^{15}N$ HSQC spectra of ^{15}N-labeled mutant protein L in water show a chemical shift dispersion corresponding to a mostly unfolded protein. In buffer, instead, the signal pattern for the mutant indicates a mixture of unfolded and folded molecules in

dx.doi.org/10.1021/bi500222g | *Biochemistry* 2014, 53, 2271−2277

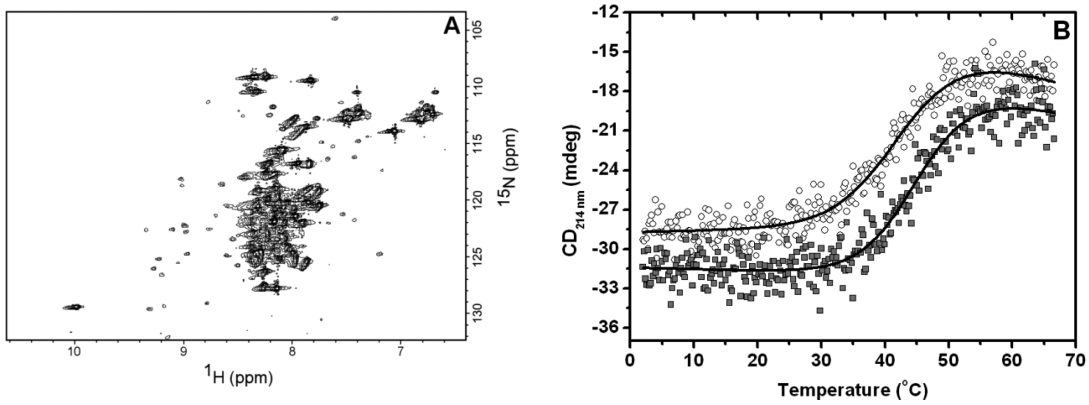


Figure 5. (A) ^{1}H−^{15}N HSQC NMR spectrum of mutant protein L in water with 200 mg/mL Dextran 20, recorded at 850 MHz. A reference experiment without Dextran is shown in Figure 2A. (B) Thermal denaturation of mutant protein L in 500 mM NaCl (to induce full folding at 20 °C) with (gray squares) and without (circles) 200 mg/mL Dextran 20 probed by CD changes at 214 nm. The solid lines are two-state fits to the data, and extracted parameters are given in the text. In B, the Dextran data set was shifted downward for clarity.

solution. The folded signals observed for the mutant in buffer are also found in the NMR spectrum of wild type protein L (see below). The ^{1}H−^{15}N HSQC spectrum of mutant protein L is similar to that reported by Pielak,[38] but in our figure, we have used a different noise threshold so the fraction of folded molecules in the sample is visualized.

First we tested the effect of 200 mg/mL Dextran 20 on wild type protein L. In Figure S1, Supporting Information, we demonstrate that the presence of Dextran 20 has no structural effect on folded protein L, neither as probed by far-UV CD nor by NMR. The NMR cross peaks for the wild type protein L agree with the extra cross peaks found for mutant protein L in 20 mM NaP buffer (Figure 2B). Nonetheless, the presence of macromolecular crowding should stabilize the folded form of protein L toward chemical and thermal perturbations. Thermal unfolding experiments of wild type protein L with and without 200 mg/mL Dextran 20 show that the thermal midpoint (T_m) increases by 2.8 °C, from 70.7 ± 0.5 °C to 73.5 ± 0.5 °C, in the presence of 200 mg/mL Dextran 20 (Figure 3A). Assuming constant $\Delta H(T_m)$ in this temperature range (~260 kJ mol^{-1}), we calculated that the free energy change at T_m(buffer) due to macromolecular crowding (200 mg/mL Dextran 20) is ~1.9 kJ mol^{-1}. In analogy, the stability toward chemical denaturation at 20 °C was also increased by the presence of Dextran 20. In Figure 3B, we show denaturant-induced unfolding curves at 20 °C with and without 200 mg/mL Dextran 20. The midpoint of unfolding shifts from 2.5 to 2.7 M GuHCl when 200 mg/mL Dextran 20 is present in the sample. Two-state analysis of the data shows that wild type protein L is stabilized by ~1.5 kJ mol^{-1} in the presence of 200 mg/mL Dextran (ΔG_u^0 of 18.0 ± 1.4 versus 19.5 ± 1.2 kJ mol^{-1}). We note that a small increase in negative CD signal at 220 nm for protein L in Dextran at low GuHCl concentrations is observed that is not found for the protein in buffer. This may indicate that there is a structural effect on folded protein L due to the combined presence of denaturant and crowder.

Next we investigated the effect of Dextran additions to mutant protein L in water (Figure 4A) and in buffer (Figure 4B). In both cases, the negative CD signals increase upon addition of Dextran. Comparing the signal of mutant protein L in 200 mg/mL Dextran 20 with that found in water and in the presence of high salt, assuming that in water the fraction unfolded is 0.9, we derived that the Dextran signal corresponds to a fraction unfolded of about 0.6 (Figure 4C). Similarly, analyzing the signal of variant protein L in 200 mg/mL Dextran in buffer, with the signal in water ($f_U \approx 0.9$), in buffer ($f_U \approx 0.6$), and in high salt ($f_U \approx 0$), we estimated that the Dextran + buffer condition corresponds to a fraction unfolded of 0.3 (Figure 4D). That the Dextran-induced changes are indeed corresponding to formation of folded species is supported by the ability to reproduce the Dextran + water signal by a linear combination of the water and high salt CD spectra (Figure 4C).

We attempted to confirm the CD data of crowding-induced folding of mutant protein L by NMR experiments. ^{1}H−^{15}N HSQC NMR data for mutant protein L in water with 200 mg/mL Dextran 20 clearly indicate a mixture of folded and unfolded molecules (Figure 5A) and thus qualitatively supports the CD results. However, the expected changes based on CD data in fraction folded protein from 0.1 (water) to 0.4 (dextran) are not easily quantified by NMR cross peak patterns. This is due to the concomitant increase in sample viscosity when Dextran 20 is added, which will weaken the signal intensities in general and affect relaxation rates of folded and unfolded species differently. Moreover, a quantitative comparison of folded cross-peak intensities between wild type and mutant protein samples in 200 mg/mL Dextran 20, that is, when both samples have the same viscosity, was also unreliable since the folded fraction in the mutant protein L sample was very low.

An increase in free energy of around 2 kJ mol^{-1}, as indicated from the wild type experiments, corresponds to a change in $K_{eq,crowding}/K_{eq,buffer}$ of 2.3. Recalculating this to fraction unfolded predicts that if the starting point is $f_U \approx 0.9$ (as in water), addition of 2 kJ mol^{-1} (by crowding) should change the unfolded population to 0.7; if instead the starting point is $f_U \approx 0.6$ (as in buffer), addition of 2 kJ mol^{-1} (by crowding) should change the unfolded population to 0.4. These predictions are in reasonable agreement with the experimental observations. Thus, the macromolecular crowding effect appears independent of starting stability of the protein, and 200 mg/mL Dextran 20 provides about 2 kJ mol^{-1} in additional thermodynamic stability to protein L.

To test the crowding effect another way, we first stabilized the folded state of mutant protein L with salt. Then we measured the thermal stability with and without addition of 200 mg/mL Dextran 20 (Figure 5B). The T_m values found were 42.9 ± 0.5 °C and 45.2 ± 0.5 °C, and this difference can be recalculated to a $\Delta\Delta G$ at T_m (buffer) of 2 kJ mol^{-1}.

dx.doi.org/10.1021/bi500222g | *Biochemistry* 2014, 53, 2271−2277

DISCUSSION

We here show that the thermodynamic effect of 200 mg/mL of Dextran 20 on unfolded mutant protein L matches the effect expected based on studies on the wild type (folded) protein and on the mutant when folded by salt. This finding emphasizes that the crowding effect is independent of initial protein stability which is in agreement with simple excluded volume theory. Moreover, our results show that, at least for small single-domain proteins that exhibit reversible unfolding/refolding, addition of crowding to an unfolded ensemble will shift the population toward the folded state and does not drive the system toward side paths such as aggregation and/or misfolding. Nonetheless, the crowding effect is only a few kJ per mol in terms of free energy (at 200 mg/mL Dextran 20), and thus the shift in K_{eq} for folding is only 2−3 fold. In agreement with the experimental data, a theoretical prediction using excluded volume theory with Dextran modeled as an array of rods with a cylindrical radius of 7 Å[28] reveals a stabilizing effect of ∼4 kJ mol^{-1} for 200 mg/mL Dextran 20 (with $R_{g,U}$ and $R_{g,F}$ of 26 and 16 Å for protein L taken from ref 41).

To change the folded fraction from 10% (ΔG_F = 5.3 kJ mol^{-1}) to 90% (ΔG_F = −5.3 kJ mol^{-1}), a free energy change of ca. 11 kJ mol^{-1} is required. Considering the crowding effect to be 1−4 kJ mol^{-1} for 100−300 mg/mL of crowding agents,[26,28] dramatic effects on folded fractions should not be expected. In a recent paper, Pielak et al. used in-cell NMR to conclude that the same protein L variant as used here could not be folded by the cell milieu, and it was suggested that the favorable (in terms of folding) excluded volume interactions were counteracted by unfavorable (in terms of folding) nonspecific attractive interactions, resulting in a zero net effect.[38] Since our work shows that the excluded volume forces are small, this means that also the nonspecific interactions can be small and still give a net effect of zero.

As mentioned in the introduction, we earlier reported apparent protein-stability dependencies for the absolute crowding effect on two proteins, cytochrome c and apoflavodoxin.[20,21] For apoflavodoxin, the initial stability was modulated by the buffer and presence or absence of salt; for cytochrome c, the protein was destabilized by GuHCl additions. We propose that the different solution conditions affect the unfolded state dimensions of these proteins. For example, in the presence of GuHCl, the unfolded states of proteins will often expand due to better solvation,[42] resulting in a greater difference in size between folded and unfolded states and, therefore, a larger crowding effect. For protein L, however, it has been demonstrated that the unfolded state dimensions are not dependent on GuHCl concentration, at least up to 5 M.[41]

In conclusion, stabilizing forces on proteins due to excluded volume effects in cells are marginal, on the order of 2−3 fold increases in K_{eq} for biologically relevant concentrations of crowders (∼200 mg/mL). Nonetheless, despite being small, these effects may be decisive in vivo for unstable proteins and/or at stress conditions that provoke local unfolding of proteins: changing K_{eq} by a factor of 2 or 3 may then be the turning point between life and death for the cell.[43]

ASSOCIATED CONTENT

Supporting Information

Supplemental figure. (A) Far-UV CD and (B) $^{1}H-^{15}N$ HSQC NMR spectra of wild type protein L in 20 mM NaP, pH 6.0 (blue) and when 200 mg/mL Dextran 20 is added (red), recorded at 850 MHz. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

The Swedish Natural Research Council, the Knut and Alice Wallenberg Foundation, and Göran Gustafsson Foundation provided financial support.

Notes

The authors declare no competing financial interest.

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