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Protein Crowding Tunes Protein Stability

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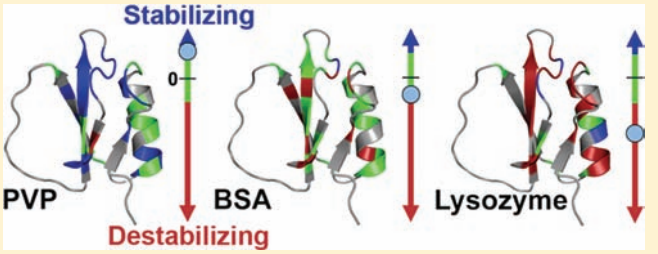
Supporting Information

ABSTRACT: Thirty percent of a cell's volume is filled with macromolecules, and protein chemistry in a crowded environment is predicted to differ from that in dilute solution. We quantified the effect of crowding by globular proteins on the equilibrium thermodynamic stability of a small globular protein. Theory has long predicted that crowding should stabilize proteins, and experiments using synthetic polymers as crowders show such stabilizing effects. We find that protein crowders can be mildly destabilizing. The destabilization arises from a competition between stabilizing excluded-volume effects and destabilizing nonspecific interactions, including electrostatic interactions. This competition results in tunable stability, which could impact our understanding of the spatial and temporal roles of proteins in living systems.



INTRODUCTION

Over 70 years ago, Krebs stated that dilute solution data may fail to capture a full picture of protein chemistry in cells.[1] One important protein property is stability, which is quantified as the global free energy of denaturation, $\Delta G^{0'}_{den}$. For proteins that follow two-state unfolding, this quantity equals $-RT \ln(f_{denatured}/f_{native})$, where R is the gas constant, T is the absolute temperature, and f is the fraction of each state. The value of $\Delta G^{0'}_{den}$ is expected to change when moving from dilute solution to crowded conditions.

Specifically, the crowded cytoplasm is predicted to stabilize globular proteins compared to dilute solution[2] because the denatured state is larger than the native state and crowding favors smaller species over larger ones. There are, however, few quantitative tests of this prediction, and the few studies of protein stability in cells report a surprising result; proteins are either unaffected or destabilized.[3−5] One reason for the paucity of results is that the intracellular environment is difficult to manipulate, hampering systematic efforts to unravel the effects of crowder concentration, size, and shape. Reductionist stability studies have been performed with synthetic polymers as crowders,[6−8] but these "artificial" crowding agents are nonbiological and may not reveal physiologically relevant information.[9]

In vitro experiments with proteins as crowders can bridge the gap between in-cell studies and studies using synthetic polymers. The use of proteins as crowders, however, has been hampered by the difficulty in detecting a test protein under crowded conditions where its mass is only a few percent of the total protein mass. Another difficulty is that globular proteins have dilute solution stabilities of 2−10 kcal/mol at room temperature, which means the native state of even the least stable proteins represents >99% of the population. Few methods can detect such low concentrations of the denatured state. Perturbants, including heat and denaturing agents, facilitate detection by increasing the population of the denatured state. Extrapolation to zero denaturant concentration, or lower T, is then used to obtain the stability in the absence of perturbation. This approach is not reasonable for protein crowders because denaturants and heat perturb the properties of both the test protein and the crowder.

NMR-detected amide proton exchange allows detection of a test protein in a high concentration of other proteins, while eliminating the need for perturbants. In these experiments, a ^{15}N-enriched test protein is transferred from a solvent containing H_2O to a crowded solution containing D_2O. Then, serial ^{15}N−^{1}H heteronuclear single quantum correlation (HSQC) experiments are performed to detect the exchange of backbone amide protons for deuterons. The ease of detection arises because the technique is highly sensitive to the low populations of non-native protein.[10]

The rate of exchange, k_{obs}, can be linked to the free energy required to expose each backbone amide to solution, $\Delta G^{0'}_{op}$, if three assumptions hold.[11] First, the test protein must be stable. The protein used here, barley chymotrypsin inhibitor 2 (CI2, 7.4 kDa, pI 6.0) has a stability of greater than 6 kcal/mol in dilute solution at pH 5.4, 37 °C.[7] Second, the rate-determining step is the exchange from the open state. Finally, values from the exchange rate from the open state, k_{int}, must be available. If these assumptions hold

$$\Delta G^{0'}_{op} = -RT \ln\left(\frac{k_{obs}}{k_{int}}\right)$$

The largest $\Delta G^{0'}_{op}$ values occur when all the backbone amide protons are exposed, revealing information about global stability.

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The smaller values assess local stability, which can be as important as global stability.[12]

We have reported increases in the stability of CI2 when crowded by the synthetic polymer, polyvinylpyrrolidone (PVP).[6,7] Synthetic polymers, however, are not physiologically relevant. Here, we examine the effects of two globular proteins as crowding agents, bovine serum albumin (BSA) and hen egg-white lysozyme.

MATERIALS AND METHODS

Vector. The pet28a plasmid (Novagen) containing the gene for an abbreviated version of chymotrypsin inhibitor 2 (CI2) was provided by Andrew Lee (UNC) and altered by site-directed mutagenesis to generate an I29A;I37H variant.[7] Residue 1 of this construct corresponds to residue 20 of the full-length protein. All experiments were performed with the I29A;I37H variant, which we refer to as CI2.

Expression and Purification. To generate ^{15}N-enriched CI2, the plasmid is transformed into BL-21(DE3-Gold) competent *Escherichia coli* cells. All media contain 60 μg/mL kanamycin because the plasmid contains the kanamycin resistance gene. Potential transformants are spread onto Luria Broth (Fisher BioReagents) agar plates and incubated at 37 °C overnight. A single colony is inoculated into 50 mL of Luria Broth and incubated at 37 °C overnight with shaking. The next morning, an 8-mL aliquot is transferred to 100 mL of 2xTY media (1.6 g tryptone, 1.0 g yeast extract, 0.5 g NaCl, 1 mM NaOH in 100 mL H_2O). The culture is incubated at 37 °C with shaking until its optical density at 600 nm reaches 0.8. This culture is spun at 1600*g* for 10 min, and the pellet is resuspended in 1 L of ^{15}N-enriched M9 media (13 g Na_2HPO_4, 4 g dextrose, 3 g KH_2PO_4, 0.5 g NaCl, 1 g $^{15}NH_4Cl$, 2 mM $MgSO_4$, 100 μM $CaCl_2$ in 1 L H_2O). This culture is incubated at 37 °C with shaking until its optical density at 600 nm reaches 0.8, whereupon induction is initiated by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 μM. Protein expression proceeds for 6 h, whereupon the culture is spun at 6500*g*, and the pellet is frozen.

The pellet is resuspended in 25 mL of 25 mM Tris, pH 8.0. Lysis is performed by sonic dismembration for 6 min (500 W dismembrator with a 1/8 in. tip, 20% amplitude, pulse 2 s, rest 2 s). The sample is spun at 14000*g* for 30 min. The supernatant is retained. Streptomycin sulfate (0.250 g) is added with stirring on ice for 30 min, followed by another round of centrifugation. The supernatant is applied to a sterilized 0.22 μm syringe-driven filter. The CI2 is purified by anion exchange chromatography on an AKTA FPLC (GE Healthcare) with a HiLoad Q Sepharose column (GE Healthcare) by using a two-step isocratic approach comprising lysis buffer as the low salt buffer and lysis buffer with 1 M NaCl as the high salt buffer. The pure fractions (as assessed by SDS-PAGE) are pooled and dialyzed against water, followed by purification by size exclusion chromatography. The protein is then lyophilized.

NMR. Amide proton exchange experiments are performed on a 500 MHz Varian Inova spectrometer equipped with an HCN cold probe with a *z*-axis gradient at a ^{1}H sweep width of 8401.6 Hz and a ^{15}N sweep width of 2200 Hz. The ionic strength is kept low to exploit the full potential of the cold probe.[13] Each experiment requires two samples, an optimization sample and an exchange sample. Optimization samples comprise 1 mM CI2 in 50 mM sodium phosphate, pH 6.5, with 15% D_2O, and are used for shim adjustment and pulse width calibration. pH values are direct readings, uncorrected for the isotope effect.[14] Exchange samples are made in 99.9% D_2O, whereupon lyophilized CI2 is added to a final concentration of 1 mM.

Protein crowders are exchanged in D_2O prior to use. One to two grams of protein are suspended in 10 mL of D_2O, pH 10. Exchange is allowed to occur for 2−4 h at room temperature, whereupon the solution is lyophilized overnight and resuspended a second time in 10 mL of D_2O, pH 10. Another round of exchange is performed for 2−4 h, whereupon the pH is readjusted to 7 with dilute HCl. This solution is lyophilized for use in exchange sample preparation.

Twenty to twenty-four consecutive HSQC spectra[15,16] are acquired per exchange sample. Processing is performed with NMRPipe.[17] Assignments have been described.[7] Crosspeak volumes are plotted against time and fit to exponential decays by using NMRViewJ.[18] Experiments using 150 mM NaCl are conducted on a 600 MHz Varian Inova spectrometer equipped with a standard triple resonance HCN probe with three-axis gradients at a ^{1}H sweep of 11990 Hz and a ^{15}N sweep of 2500 Hz. This spectrometer was used because cold probes lose sensitivity at high salt concentrations.[13]

The rate-determining step of exchange is assessed by using HSQC-detected amide proton exchange and quantifying the exchange rate as a function of pH.[11] These experiments are identical to the amide proton exchange experiments described above, except that experiments at pH 5.4 contain 50 mM sodium acetate.

The rate-determining step was also assessed by using nuclear Overhauser spectroscopy-detected amide proton exchange (NOESY-HEX).[11] The experiments are performed in a similar fashion to HSQC-detected amide proton exchange, collecting 50−60 consecutive ^{15}N-filtered NOESY spectra.[19−21] Data are acquired on the 500 MHz spectrometer at a ^{1}H sweep width of 8401.6 Hz. Exchange samples were identical to samples prepared for HSQC-detected experiments. Processing and exponential decay fitting were performed as described for the exchange experiments, but assignments were determined by matching amide−amide crosspeaks to ^{1}H shifts corresponding to pairs of proximal amide protons from the HSQC assignment.

The intrinsic exchange rates, k_{int}, were determined as described[22] for 1 mM CI2 in 50 mM sodium phosphate, pH 6.5, 20 °C containing 0 g/L and 100 g/L BSA or lysozyme. Experiments were performed on the 600 MHz spectrometer at a ^{1}H sweep width of 10000 Hz and a ^{15}N sweep width of 2000 Hz. The water signal was unchanged with mixing times varying from 0 to 53 ms. $R_{1B,app}$ was thus chosen to be 0.01 s^{-1}. As expected,[23] the $R_{1B,app}$ values did not alter the results.

Relaxation experiments were performed on the 600 MHz spectrometer at 20 °C. The R_1R_2 data were acquired and processed as described.[24,25] Briefly, the ^{1}H dimension was acquired with a sweep width of 12000 Hz and comprised 1024 complex points. The ^{15}N dimension was acquired with a sweep width of 2500 Hz and comprised 64 complex increments. For T_1 measurement, the relaxation delays were 0.01, 0.4, 0.6, 0.9, 1.2, and 1.5 s. For T_2 measurement, the delays were 0.01, 0.03, 0.07, 0.09, 0.15, and 0.21 s. Eight transients were acquired per spectrum. The data were processed with NMRPipe[17] and NMRViewJ.[18]

RESULTS

We verified the assumptions required for amide proton exchange determination of protein stability in crowded conditions. First, we determined that exchange from the open state is rate determining both by examining the pH dependence of amide proton exchange and by using the NOESY-HEX experiment.[11]

The pH dependence of exchange was assessed in dilute solution and in both 100 g/L BSA and 100 g/L lysozyme (Table S1 and Figure S1 in Supporting Information [SI]). If exchange from the open state is rate limiting, we expect a pH dependence of exchange. If opening to the exchange-accessible state is rate determining, no such dependence is expected.[10] In dilute solution, the difference in exchange rates at two pH values corresponds to the difference in hydroxide ion concentration (Figure S1, SI).[7] This observation means that exchange from the open state is rate limiting and that CI2 stability is not affected by pH over the range studied. We also observe pH dependence in 100 g/L BSA and lysozyme, suggesting that exchange from the open state is rate determining. When crowded by proteins,

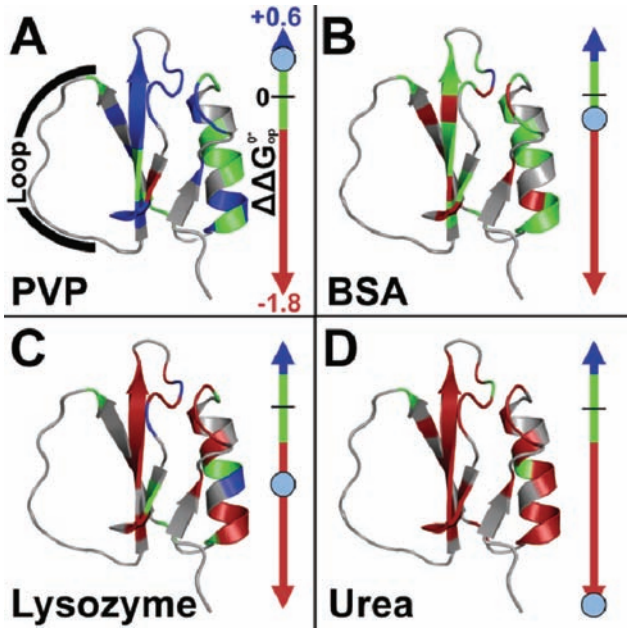

Figure 1. Structure of CI2 (PDB ID: 2CI2) colored by changes in stability compared to dilute solution($\Delta\Delta G^{0*}_{op}$). Stabilization greater than 0.3 kcal/mol is indicated in blue, no effect (between −0.3 and 0.3 kcal/mol) is indicated in green, and destabilization by greater than 0.3 kcal/mol is indicated in red. Exchange for the white residues could not be observed. On the right of each structure is an indicator showing the average $\Delta\Delta G^{0*}_{op}$ value for globally exchanging residues.[28] Results are shown for (A) 100 g/L PVP-40,[6] (B) 100 g/L BSA, (C) 100 g/L lysozyme, (D) 100 g/L urea. The analysis underestimates urea's ability to destabilize CI2 because this compound slows amide proton exchange in unstructured peptides.[29]

however, the change in rate is slightly less than the change in hydroxide ion concentration (Table S1, SI). This result indicates that CI2 stability becomes pH-dependent in protein crowders. We return to this point in the Discussion.

NOESY-detected amide proton exchange experiments were also performed in samples containing 100 g/L BSA (Table S2, SI) to establish the exchange limit. This experiment is preferred for systems exhibiting pH-dependent stability because it provides the exchange limit without requiring a change in conditions.[11] Lysozyme was not studied because interactions between lysozyme and CI2 cause line broadening that prevented analysis.

In NOESY-detected exchange experiments,[11] if exchange from the open state is rate-limiting, then the combined amide−amide crosspeak decay for proximal amide protons equals the sum of the individual decays. Alternatively, if opening is rate determining, then the combined decay equals the individual decays. Table S2 (SI) displays results for two pairs of proximal amide protons. The data confirm the conclusion from our pH dependence experiments that exchange from the open state is rate determining.

It was also necessary to establish whether crowding affects k_{int}. The rate of exchange for residues in the extended loop region of CI2 approximates exchange for an unstructured peptide, because these residues are highly solvent exposed. Unprotected amide protons exchange more quickly than can be assessed with the serial HSQC exchange experiments described above. It is therefore necessary to use phase-modulated clean chemical exchange (CLEANEX-PM) experiments.[22] These results can be analyzed

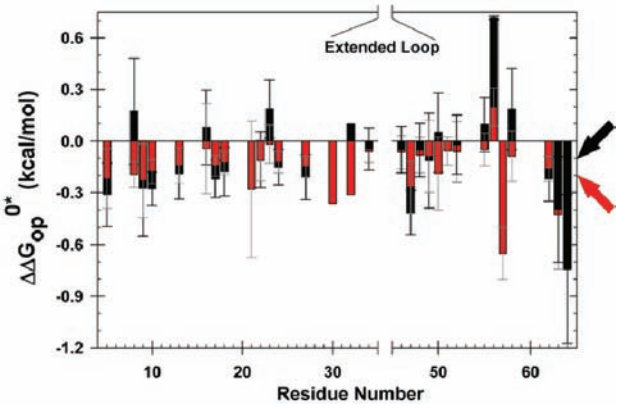

Figure 2. Histogram of $\Delta\Delta G^{0*}_{op}$ versus residue number for CI2 in solution containing 100 g/L BSA (red) and 200 g/L BSA (black). Bars represent the standard error from three trials. Arrows indicate the average $\Delta\Delta G^{0*}_{op}$ values for each set of conditions. Experiments were performed in 50 mM sodium phosphate, pH 6.5, 20 °C.

to determine k_{int}. As shown in Table S3 (SI), k_{int} values are unchanged from dilute solution to 100 g/L of either protein crowder, allowing us to use dilute solution k_{int} values.[26,27]

The results from amide proton exchange (Figure 1 and Tables S4 and S5, SI), performed in triplicate in solutions containing 100 g/L BSA and lysozyme are different from those in solutions with 100 g/L of the synthetic polymer PVP. In PVP, all monitored residues, save one, are stabilized ($\Delta\Delta G^{0*}_{op} > 0$) compared to dilute solution. The average increase for global exchangers[28] is 0.3 ± 0.1 kcal/mol, where the uncertainty is the standard error. For the protein crowders, however, the majority of residues are slightly destabilized, with an average stability decrease for global exchangers of 0.2 ± 0.1 kcal/mol for 100 g/L BSA and 0.6 ± 0.2 kcal/mol for 100 g/L lysozyme. Greater destabilization is observed in 100 g/L urea, which causes an apparent average destabilization for global exchangers of 1.7 ± 0.1 kcal/mol.

Increasing the BSA concentration from 100 g/L to 200 g/L (Figure 2) had minimal effects compared to 100 g/L BSA but resulted in poorer quality spectra, thus increasing the uncertainty in $\Delta\Delta G^{0*}_{op}$. Amide proton exchange experiments could not be performed in 200 g/L lysozyme, because the combination of crosspeak broadening and faster exchange resulted in sparingly few backbone amide proton crosspeaks for analysis. Exchange experiments were also performed in 100 g/L BSA solutions containing 50 mM sodium phosphate in the presence and absence of 150 mM NaCl. Results for these experiments (Figure 3) reveal that adding NaCl mitigates the destabilizing effect of BSA crowding. Ile16, Val47, and Gln59 are stabilized by 0.4−0.5 kcal/mol in NaCl, but several residues remain destabilized (e.g., Val9, Val63). We can find no relationship between the effect of salt and the position or properties of the residues. The results from a control experiment (Figure S2, SI) show that NaCl destabilizes CI2 in dilute solution, indicating that the stabilizing effect of salt shown in Figure 3 arises from attenuating electrostatic interactions involving BSA.

Experiments to determine R_1R_2, the product of longitudinal and transverse relaxation rates respectively,[30] were used to assess the level of weak interactions between CI2 and crowding agents.[24,25] The results are shown in Table 1. Both crowders cause R_1R_2 to exceed the rigid limit value for CI2,[24] indicating the presence of weak interactions.

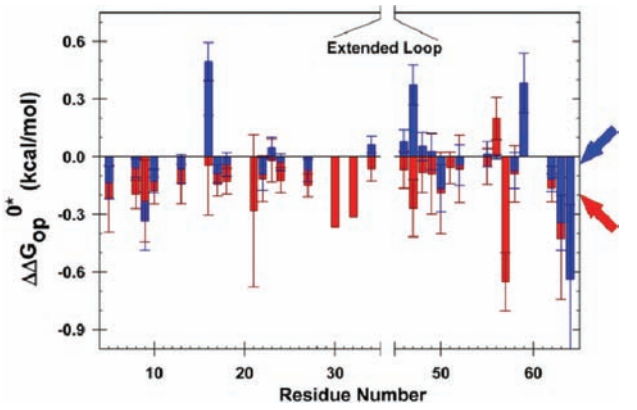

Figure 3. Histogram of $\Delta\Delta G^{0*}_{op}$ versus residue number for CI2 in solution containing 100 g/L BSA without adding NaCl (red) or upon adding 150 mM NaCl (blue). Colored arrows indicate the average $\Delta\Delta G^{0*}_{op}$ values for each set of conditions. Experiments were performed in 50 mM phosphate, pH 6.5, 20 °C.

Table 1. Average Values of R_1R_2 for CI2 in Glycerol and in Protein Crowders[a]

solution	pH	average R_1R_2 (s^{-2})
glycerol	6.5	14.5
100 g/L BSA	6.5	20.7
100 g/L Lysozyme	6.5	31.1

[a] The value of R_1R_2 exceeds the rigid limit value of 19 s^{-2} [24,30] in crowded conditions, indicating the presence of protein−protein interaction. Experiments were performed at 20 °C in 50 mM sodium phosphate, pH 6.5.

DISCUSSION

Protein Stability. The results show that protein crowders can destabilize CI2 (Figure 1). Our stability values using proteins as crowding agents contradict other *in vitro* observations of crowding effects on stability,[6−9] but these previous studies used nonphysiological synthetic polymers as crowding agents. Different results are not surprising, because synthetic polymers and proteins have distinct effects on protein diffusion[25] and enzyme activity.[31] Our results show this trend extends to stability, and are consistent with in-cell results, which indicate either no stability change[3] or destabilization.[4,5] Our data also agree with findings obtained with carboxyamidated RNase T1 in 400 g/L BSA[32] and simulations of protein stability in cellular environments.[33] The lack of correlation between the change in stability and the molecular weight (BSA 66 kDa, lysozyme 15 kDa) and pI (BSA 4.7, lysozyme 11.0) of the crowder suggests that our results are general.

Nonspecific Interactions. As stated in the Introduction, the excluded volume effect is always stabilizing for globular proteins. Paradoxically, we observe that protein-induced crowding can be destabilizing. This apparent contradiction can be reconciled by noting that crowding changes more than the excluded volume. There are also favorable, nonspecific chemical interactions between proteins under crowded conditions.[25,34]

As opposed to excluded volume, these weak nonspecific protein−protein interactions are destabilizing. Such interactions promote unfolding because unfolded species expose more protein surface than does the native state. Urea-induced destabilization (Figure 1) is the classic example of this phenomenon.[29]

NMR spectroscopy is sensitive to weak nonspecific interactions. Typical NMR experiments parameters, however, are also sensitive to viscosity, which complicates comparisons involving viscous crowded solutions. We used a viscosity-independent method[30] to assess the interaction between protein and crowder.[24,25] The fact that both protein crowders result in R_1R_2 values in excess of the rigid limit line (19 s^{-2} for CI2)[24] indicates the presence of weak interactions between the crowding agent and CI2. Even though the R_1R_2 experiment detects only the native state, we suggest these are nonspecific interactions for two reasons. First, it is unlikely that BSA and lysozyme both form specific interactions with CI2. Second, such interactions have been observed in other experiments.[34,35] Consistent with the idea that the strength of the interaction affects stability, lysozyme, which has the larger destabilizing effect (Figure 1), also has stronger interactions (Table 1). In summary, our data indicate that crowding by proteins is a competition between stabilizing volume exclusion effects and destabilizing nonspecific interactions. This conclusion is in complete accord with a recent study that examined both excluded volume and favorable nonspecific intermolecular interactions in a model of the *E. coli* cytoplasm.[33]

Electrostatics and Nonspecific Intermolecular Interactions. The sources of nonspecific interactions include the hydrophobic effect, hydrogen bonding, and electrostatics.[25,34] Spitzer and Poolman suggest that electrostatics play a large role in cells, because the intracellular surface-to-surface distance between proteins is less than the Debye screening length.[36] To test this idea in our *in vitro* system, we performed amide proton exchange experiments in 100 g/L BSA at two NaCl concentrations (NaCl was used to ensure the results do not arise from a Hofmeister effect).

As shown in Figure 3, BSA has less of a destabilizing effect at higher ionic strength. The results from a control experiment (Figure S2, SI) show that NaCl is destabilizing in dilute solution. These results are consistent with the idea that electrostatic interactions between BSA and CI2 are partially responsible for the observed crowder induced decrease in stability. Increasing the NaCl concentration, however, does not remove all the destabilization (e.g., Val9, Val63) indicating that other types of nonspecific interactions are also present.

The electrostatic nature of some of the destabilizing interactions is also suggested by pH-dependent phenomena. The net charge of a protein is affected by solution pH. If the stability of a protein is unaffected by a change in pH, then the exchanges rates are all equally proportional to the difference in H^+ concentration.[10,11] This proportionality is observed for CI2 in dilute solution.[7,28] Adding protein crowders changes this situation (Figure S2, SI), consistent with the presence of electrostatic interactions. In addition, R_1R_2 values for CI2 becomes pH dependent when the solution is crowded with protein.[24] These results are all consistent with the idea that electrostatics play a role in destabilization, in agreement with a simulation-based conclusion.[37] We conclude that adding NaCl mollifies weak but favorable nonspecific interaction between CI2 and the protein crowder, allowing the stabilizing volume exclusion effect to dominate.

Potential Biological Implications. Many protein-mediated biological processes require reversibility. A signaling protein that binds its target irreversibly, an enzyme that fails to release its product, or a transport protein that does not shuttle its cargo is of limited utility, or more probably, toxic.[38] If crowding were to favor strongly one state over another, these equilibria would shift

to such an extent that reversibility would be lost. The ability of the protein crowders to mitigate volume exclusion with nonspecific interactions also might serve to maintain globular protein stability in a range that assures a high concentration of the active form while allowing relatively low expenditure of energy to recycle the components.

CONCLUDING REMARKS

Despite the fact that volume exclusion can only stabilize globular proteins, when proteins act as the crowding agent, stability can be increased, unaffected, or even decreased. The causative nonspecific intermolecular interactions seem to be general and can be modulated by pH and ionic strength, indicating the presence of an electrostatic component. In summary, our results show that under physiologically relevant conditions, protein stability is a competition between stabilizing and destabilizing interactions. This conclusion leads to the idea that protein crowding could tune protein stability in cells.

If valid, this speculation would have several biological implications. First, proteins act as "good neighbors" in crowded conditions by not perturbing each other. Second, the tunability of protein stability could be exploited in biological systems (*e.g.*, mitochondria and the cytoplasm have different macromolecule concentrations). Third, the heterogeneity of the cellular interior can create regions where proteins are stabilized or destabilized, depending on the local degree of volume exclusion and the local extent of nonspecific interactions. Such tunability could result in proteins being stabilized in one region of a cell, but destabilized in another. This work brings us closer to understanding the effects of crowding in the cellular environment.

ASSOCIATED CONTENT

Supporting Information. Figures for pH dependence of exchange and effects of salt on CI2 stability, and tables containing results from NOESY-HEX, CLEANEX-PM, and HSQC-detected amide proton exchange experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Krebs, H. A.; Henseleit, K., *Z. Physiol. Chem.* **1932**, 210, 33−66 [Translation by F. L. Holmes, Hans Krebs; Oxford University Press: New York, NY, USA, 1991; Vol. 1].
(2) Minton, A. P. *Biopolymers* **1981**, *20*, 2093–2120.
(3) Ghaemmaghami, S.; Oas, T. G. *Nat. Struct. Biol.* **2001**, *8*, 879–882.
(4) Ignatova, Z.; Krishnan, B.; Bombardier, J. P.; Marcelino, A. M. C.; Hong, J.; Gierasch, L. M. *Biopolymers* **2007**, *88*, 157–163.
(5) Inomata, K.; Ohno, A.; Tochio, H.; Isogai, S.; Tenno, T.; Nakase, I.; Takeuchi, T.; Futaki, S.; Ito, Y.; Hiroaki, H.; Shirakawa, M. *Nature* **2009**, *458*, 106–109.
(6) Miklos, A. C.; Li, C. G.; Sharaf, N. G.; Pielak, G. J. *Biochemistry* **2010**, *49*, 6984–6991.
(7) Charlton, L. M.; Barnes, C. O.; Li, C.; Orans, J.; Young, G. B.; Pielak, G. J. *J. Am. Chem. Soc.* **2008**, *130*, 6826–6830.
(8) Hong, J. A.; Gierasch, L. M. *J. Am. Chem. Soc.* **2010**, *132*, 10445–10452.
(9) Elcock, A. H. *Curr. Opin. Struct. Biol.* **2010**, *20*, 196–206.
(10) Englander, S. W.; Kallenbach, N. R. *Q. Rev. Biophys.* **1983**, *16*, 521–655.
(11) Miklos, A. C.; Li, C.; Pielak, G. J. *Methods Enzymol.* **2009**, *466*, 1–18.
(12) Matouschek, A. *Curr. Opin. Struct. Biol.* **2003**, *13*, 98–109.
(13) Kelly, A. E.; Ou, H. D.; Withers, R.; Dötsch, V. *J. Am. Chem. Soc.* **2002**, *124*, 12013–12019.
(14) Schowen, B. K.; Schowen, R. L.; Daniel, L. P. *Methods Enzymol.* **1982**, *87*, 551–606.
(15) Bodenhausen, G.; Ruben, D. J. *Chem. Phys. Lett.* **1980**, *69*, 185–189.
(16) Kay, L.; Keifer, P.; Saarinen, T. *J. Am. Chem. Soc.* **1992**, *114*, 10663–10665.
(17) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277–293.
(18) Johnson, B. A.; Blevins, R. A. *J. Biomol. NMR* **1994**, *4*, 603–614.
(19) Kim, S.; Szyperski, T. *J. Am. Chem. Soc.* **2003**, *125*, 1385–1393.
(20) Kupce, E.; Freeman, R. *J. Am. Chem. Soc.* **2003**, *125*, 13958–13959.
(21) Kupce, E.; Freeman, R. *J. Am. Chem. Soc.* **2004**, *126*, 6429–6440.
(22) Hwang, T.-L.; van Zijl, P. C. M.; Mori, S. *J. Biomol. NMR* **1998**, *11*, 221–226.
(23) Bertini, I.; Ghosh, K.; Rosato, A.; Vasos, P. R. *Biochemistry* **2003**, *42*, 3457–3463.
(24) Li, C.; Pielak, G. J. *J. Am. Chem. Soc.* **2009**, *131*, 1368–1369.
(25) Wang, Y.; Li, C.; Pielak, G. J. *J. Am. Chem. Soc.* **2010**, *132*, 9392–9397.
(26) Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W. *Proteins: Struct., Funct., Genet.* **1993**, *17*, 75–86.
(27) Zhang, Y.-Z. *Protein and peptide structure and interactions studied by hydrogen exchange and NMR*. University of Pennsylvania, Philadelphia, PA, USA, 1995.
(28) Neira, J. L.; Itzhaki, L. S.; Otzen, D. E.; Davis, B.; Fersht, A. R. *J. Mol. Biol.* **1997**, *270*, 99–110.
(29) Lim, W. K.; Rösgenc, J.; Englander, S. W. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 2595–2600.
(30) Kneller, J. M.; Lu, M.; Bracken, C. *J. Am. Chem. Soc.* **2002**, *124*, 1852–1853.
(31) Derham, B. K.; Harding, J. J. *Biochim. Biophys. Acta* **2006**, *1764*, 1000–1006.
(32) Qu, Y. X.; Bolen, D. W. *Biophys. Chem.* **2002**, *101*, 155–165.
(33) McGuffee, S. R.; Elcock, A. H. *PLoS Comput. Biol.* **2010**, *6*, e1000694.
(34) Stradner, A.; Sedgwick, H.; Cardinaux, F.; Poon, W. C. K.; Egelhaaf, S. U.; Schurtenberger, P. *Nature* **2004**, *432*, 492–495.
(35) Martorell, G.; Adrover, M.; Kelly, G.; Temussi, P. A.; Pastore, A., *Proteins: Struct., Funct., Bioinf.* **2011**, *79*, 1408−1415.
(36) Spitzer, J. J.; Poolman, B. *Trends Biochem. Sci.* **2005**, *30*, 536–541.
(37) Tanizaki, S.; Clifford, J.; Connelly, B. D.; Feig, M. *Biophys. J.* **2008**, *94*, 747–759.
(38) Rodkey, F. L.; Oneal, J. D.; Collison, H. A.; Uddin, D. E. *Clin. Chem.* **1974**, *20*, 83–84.