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# Protein Crowder Charge and Protein Stability

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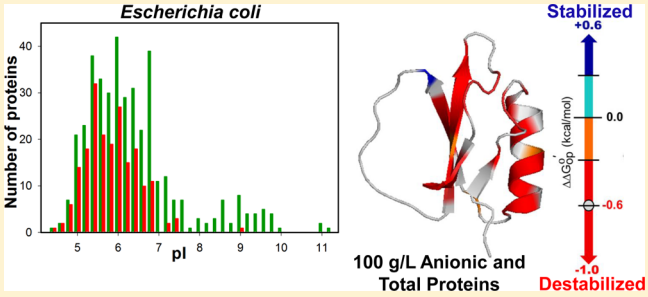
Supporting Information

**ABSTRACT:** Macromolecular crowding effects arise from steric repulsions and weak, nonspecific, chemical interactions. Steric repulsions stabilize globular proteins, but the effect of chemical interactions depends on their nature. Repulsive interactions such as those between similarly charged species should reinforce the effect of steric repulsions, increasing the equilibrium thermodynamic stability of a test protein. Attractive chemical interactions, on the other hand, counteract the effect of hard-core repulsions, decreasing stability. We tested these ideas by using the anionic proteins from *Escherichia coli* as crowding agents and assessing the stability of the anionic test protein chymotrypsin inhibitor 2 at pH 7.0. The anionic protein crowders destabilize the test protein despite the similarity of their net charges. Thus, weak, nonspecific, attractive interactions between proteins can overcome the charge−charge repulsion and counterbalance the stabilizing effect of steric repulsion.




Traditionally, globular proteins were studied in dilute, buffered solutions even though their natural environment is crowded and heterogeneous.[1] With the advent of sensitive biophysical techniques[2] and increased computer power,[3,4] biomolecules are now being studied in more physiologically relevant environments. Here, we focus on how the net charge of crowding molecules affects protein stability.

Globular protein stability is defined as the free energy of the biologically nonfunctional and less compact denatured state minus that of the functional and compact native state.[5] Although crowding effects arise from both steric repulsions and weak nonspecific chemical interactions, only recently have these weak interactions been studied experimentally.[6−11] Steric and chemical interactions are often termed hard and soft, respectively.[12] Scaled particle theory-based explanations of crowding posit that the hard component stabilizes a globular test protein because the decrease in the amount of space available to the test protein by the mere presence of the crowders favors compact species, although these ideas are known to be simplistic.[13] This stabilization is entirely entropic because steric repulsions affect only the arrangement of solute molecules, not the chemical interactions between them.

Chemical interactions can be stabilizing or destabilizing. Soft repulsions reinforce the stabilizing influence of steric repulsions. On the other hand, destabilization of the test protein results when nonspecific attractive interactions dominate, because unfolding exposes the attractive surface.[14,15] Nonspecific attractive interactions between proteins can also facilitate aggregation and misfolding. For example, macromolecular crowding fails to fold a marginally unstable variant of a globular protein.[16] Therefore, it is important to study proteins under several conditions to assess the phenomena that give rise to crowding effects. Gaining an understanding of the nature of crowding will not only provide fundamental knowledge about biology but also help solve practical problems. For instance, such knowledge will facilitate the design of synthetic polymers that increase the stability of industrially useful enzymes and protein-based pharmaceuticals.

Crowding by synthetic polymers is often stabilizing,[17−19] but crowding by individual proteins and by crude *Escherichia coli* lysates can destabilize globular proteins[14,15,20] and impede their diffusion.[21] Both are consistent with the existence of nonspecific attractive interactions,[21,22] although this view is probably too simplistic because it ignores hydration effects.[23] Biological crowders, such as bovine serum albumin (BSA), lysozyme, and *E. coli* lysates, interact nonspecifically with the 7 kDa globular test protein chymotrypsin inhibitor 2 (CI2).[24] The absence of significant CI2 chemical shift changes in unfractionated *E. coli* lysates shows that these interactions are weak, transient, and nonspecific.[15] Synthetic polymers are more inert.[21] Furthermore, results of variable-temperature studies of stability show that crowding effects can be primarily enthalpic,[19,20,25] contrary to predictions based on the assumed primacy of steric repulsions.

The variant of CI2 we use (I29A;I37) has an isoelectric point (pI) of 6.0 and an estimated net charge of −1.0 at pH 7.0. We have studied the stability of CI2 when it is crowded by specific proteins.[14] Having noted that CI2 is more destabilized by lysozyme (pI 11.0), which has a net charge that is the opposite of that of CI2, than by BSA (pI 4.7), which has the same net

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charge, we hypothesized that attractive charge−charge interactions overcome the stabilizing effect of steric interactions. The inside of cells, however, is not crowded by one particular protein. We studied CI2 in crude *E. coli* cell lysates and found that these lysates are also destabilizing.[15] Here, we dissect the lysate to learn about the effects of net protein charge.

In our previous study, we removed metabolites and other small molecules from the crude lysate by dialysis and showed that its protein component reflected that of the *E. coli* proteome. Nevertheless, the lysate still contained nucleic acids and nucleic acid−protein complexes.[15] Here, we focus on the proteins in the lysate. Our hypothesis is that if the net charge of the test protein is same as the net charge of the crowding proteins, then charge−charge repulsions will dominate, enhancing the effects of steric repulsion, shifting the equilibrium toward the native state, and stabilizing CI2.

To focus on the proteins in the lysate, we first removed the nucleic acids and their protein complexes with streptomycin sulfate and polyethylenimine (PEI).[26] Next, we used anion exchange chromatography at pH 7.0 in an attempt to divide the lysate proteins into two fractions, those with a net negative charge and those with a net positive charge. We then used nuclear magnetic resonance-detected amide proton exchange[2] at pH 7.0 to measure the stability of CI2 in buffer and in crowded solutions of the unfractionated and fractionated lysates.

## EXPERIMENTAL PROCEDURES

**Chymotrypsin Inhibitor 2 (CI2).** $^{15}$N-enriched I29A/I37H CI2 was expressed and purified as described previously.[14,15,18,19,27]

***E. coli* Extracts.**[15,27] Typically, six 1 L cultures of *E. coli* were grown to saturation in Luria broth (LB) containing kanamycin (60.0 μg/mL). The cells were pelleted (6500*g* for 30 min at 10 °C) and the pellets stored at −20 °C after the addition of protease inhibitors [Sigma-Aldrich; 0.02 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.14 mM E-64, 1.30 mM bestatin, 0.01 mM leupeptin, 3.0 nM aprotinin, and 0.01 mM sodium EDTA (final concentrations)]. Each pellet was resuspended in 25.0 mL of buffer A [50 mM sodium phosphate (pH 7.0)] and lysed by sonic dismembration on ice for 8 min (Fisher Scientific, model 500 sonic dismembrator, 20% amplitude, 2 s on, 3 s off). The insoluble debris was removed by centrifugation (14000*g* for 30 min at 10 °C). Approximately 180 mL of supernatant was obtained.

**Total Protein Lysate.** Streptomycin sulfate and PEI were used to deplete the lysate of nuclei acids and protein−nucleic acid complexes.[26] Streptomycin sulfate (1.8 g, final concentration of 10.0 mg/mL) was added, and the sample was stirred on ice for 30 min. The solution was centrifuged at 14000*g* and 10 °C for 30 min, and the supernatant was retained. PEI was added to a final concentration of 0.1% (mass per volume) over the course of 1 h while the mixture was being stirred on ice, and the sample was centrifuged as described above. The supernatant was retained. PEI precipitation was repeated until the ratio of the absorbance at 260 nm to the absorbance at 280 nm of the supernatant was <1.2. The supernatant was dialyzed [Thermo Scientific, SnakeSkin, 3K molecular weight cutoff (MWCO)] against 5 L of 10 mM sodium phosphate buffer (pH 7.0) at 4 °C for 72 h. The dialysate was centrifuged at 14000*g* for 1 h at 10 °C. The insoluble nucleic acid and protein−nucleic acid complexes were discarded. The sample was sterile filtered (0.22 μm Durapore polyvinylidene fluoride membrane, Millipore Corp.), and protease inhibitors were added as described above. The sample was lyophilized (Labconco) and stored at −20 °C.

**Anionic Protein Lysate.** Bench-top anion exchange chromatography was performed at room temperature. A 20 cm × 50 mm inner diameter column was packed with a 450 mL slurry of diethylaminoethyl (DEAE) cellulose (GE Healthcare). The final column volume was ∼250 mL. The column was equilibrated at room temperature with 500 mL of buffer A at a flow rate of 1.0 mL/min, and 180 mL of the total protein fraction was loaded. The column was washed (1.0 mL/min) with 1.0 L of buffer A and the eluant retained. Bound proteins were eluted at a rate of 1.0 mL/min with 500 mL of buffer B [buffer A with 1 M NaCl (pH 7.0)]. Both fractions were lyophilized after protease inhibitors had been added. The bound fraction was resuspended in 100 mL of buffer A and dialyzed (Thermo Scientific, SnakeSkin, 3K MWCO) against 5 L of 10 mM sodium phosphate buffer (pH 7.0) for 48 h with a buffer change at 24 h. The sample was then lyophilized to a straw-colored powder and stored at −20 °C.

**Mass Spectrometry.** Proteomic analyses were performed at the University of North Carolina Michael Hooker Proteomics Center. Mass spectrometric data corresponding to peptide fragments from lysates were searched by using MASCOT version 2.3.02 (Matrix Science) via Proteome Discoverer version 1.3.0.339 (Thermo Scientific) against the UniProt *E. coli* database.[28] Nearly all the bound proteins had pI values of <7.0 (Figure 1 and Table S1 of the Supporting Information). We refer to the bound proteins fraction as "anionic" because the experiments were performed at pH 7.0. The unbound fraction had pI values ranging from 4.0 to 11.5 (Table S2 of the Supporting Information). We did not use this fraction.

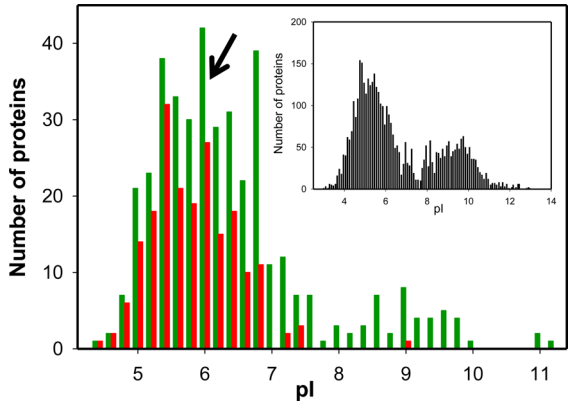


**Figure 1.** Histogram of proteins from the total protein lysate (green) and the anionic protein lysate (red) as a function of isoelectric point (pI). The arrow indicates the pI of CI2. The data are binned in 0.2 pI increments. The inset shows the distribution in the *E. coli* proteome.[34]

**Amide $^1$H Exchange.** Experiments were performed in 100% deuterated solutions [50 mM sodium phosphate (pH 7.0)] at 20 °C unless stated otherwise. Labile protons in the lysates were pre-exchanged with deuterons as described previously.[2,15] Three solutions were prepared. The first solution comprised buffer A. The second solution was made by suspending 600.0 mg of total protein lysate in buffer A to a volume of 6.0 mL. The third solution was prepared by suspending 500.0 mg of the anionic protein lysate in buffer A to a volume of 5.0 mL. The $pH_{read}$ of all solutions was adjusted to


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7.0.[29] Lysate-containing samples were centrifuged at room temperature and 14000*g* for 10 min to remove insoluble material. A modified Lowry assay (Thermo Scientific) was performed on each supernatant after a 100-fold dilution.[15] The 100 g/L total protein solution contained 92 ± 4 g of protein/L of proteins. The 100 g/L anionic protein solution contained 95 ± 2 g of protein/L.

Triplicate NMR-detected amide $^1$H exchange experiments were performed on 1 mM CI2 samples (800 $\mu$L in a 5 mm Norell tube,) in buffer A, 100 g/L total protein lysates, and 100 g/L anionic protein lysate. Each experiment required 24 h. Sample preparation, shimming, and acquisition of serial heteronuclear single-quantum coherence (HSQC) spectra have been described previously.[2,14,15] Briefly, lyophilized $^{15}$N-enriched CI2 was added to 1.0 mL of buffer A. The CI2 concentration was determined by measuring the absorbance at 280 nm ($\varepsilon$ = 7.04 × 10$^3$ M$^{-1}$ cm$^{-1}$).[30] The same mass of CI2 was added to lysate solutions immediately prior to starting acquisition. The shims of the 600 MHz spectrometer (Varian Inova, room temperature, triple resonance, HCN probe) were optimized with a 1 mM CI2 sample in buffer A [10% (v/v) $D_2O$].[15] Twenty-two serial HSQC spectra were acquired with 1024 complex points in the $^1$H dimension and 64 complex increments in the $^{15}$N dimension. Intrinsic rates ($k_{int}$) were calculated with SPHERE[31] at $pH_{read}$ 7.0, 20 °C, and 100% $D_2O$. Data were processed with NMRPipe[32] and NMRView.[33]

## RESULTS

**Lysate Characterization.** Previous analyses show that our dialyzed *E. coli* lysate contains proteins with pI values from 4 to 13,[15] similar to the range predicted from inspecting the *E. coli* proteome,[34] and that a 100 g of dry weight/L lysate solution contained 52 ± 4 g of protein/L, with the remainder comprising mostly nucleic acids.[35] We prepared a refined dialyzed lysate depleted of nucleic acids and nucleic acid binding proteins via streptomycin and PEI precipitations.[26] We call this the total protein lysate. A 100 g of dry weight/L solution of this lysate contained 92 ± 4 g of protein/L, attesting to the effectiveness of the precipitations.

We attempted to divide the total protein lysate into cationic and anionic fractions by using anion exchange chromatography at pH 7.0. Proteins with pI values of <7.0 should be retained, and those with values of >7.0 should be eluted by low-salt buffer. The bound proteins were eluted with high-salt buffer and dialyzed. The two fractions were lyophilized and analyzed by mass spectrometry. Two hundred proteins were identified in the bound fraction, and 193 proteins were identified in the flow through (Tables S1 and S2 of the Supporting Information). Only six of the bound proteins had a pI value of >7.0, affirming the efficiency of chromatography. We define this sample as the anionic protein lysate. A 100 g of dry weight/L solution of this lysate contained 95 ± 2 g of protein/L. In summary, the anionic lysate comprised almost exclusively anionic proteins at pH 7.0.

Ideally, the proteins with pI values of >7.0 should flow through an anion exchange column. Analysis revealed, however, that this fraction comprised proteins with pI values ranging from 4.4 to 11.4. Control experiments showed that the column was not overloaded. Of the 193 proteins identified, only 73 possessed a pI of >7.0. Furthermore, 12 of the 193 proteins were also identified in the bound fraction (highlighted in yellow in Tables S1 and S2 of the Supporting Information). We did not use this fraction because it could not be classified as either anionic or cationic.

The distribution of proteins with respect to pI was assessed (Figure 1). We summed the lists of bound and unbound proteins to give the distribution in the total protein lysate. The inset in Figure 1 shows the distribution from analyzing the *E. coli* genome.[34] Both histograms exhibit similar shapes, indicating that the total protein lysate is a reasonable approximation of the proteome.

**Stability Effects.** We assessed amide $^1$H exchange of 1 mM CI2 in buffer and in 100.0 g of dry weight/L solutions of the total protein lysate and anionic protein lysate at pH 7.0 and 20 °C in triplicate using NMR.[2] The observed rates of exchange ($k_{obs}$, s$^{-1}$) can be converted to free energies of opening ($\Delta G_{op}^{\circ\prime}$) if the intrinsic rates of exchange ($k_{int}$, s$^{-1}$) are rate-determining and unchanged by crowding.[2,36] These conditions are satisfied for lysates at pH 6.5 and 20 °C.[15,27] Global stabilities (Table 1) were calculated by averaging the $\Delta G_{op}^{\circ\prime}$ values for globally exchanging residues.[37] An increase in the pH from 6.5 to 7.0 does not change the stability of CI2 (Table 1).

**Table 1. Global Stabilities (in kilocalories per mole) of CI2**

| | $\Delta G_{op}^{\circ\prime}$ | $\Delta\Delta G_{op}^{\circ\prime}$ |
|---|---|---|
| 50 mM sodium phosphate, pH 6.5 or 7.0, 20 °C[14] | 6.9 ± 0.1 | – |
| 100 g/L bovine serum albumin, pH 6.5, 20 °C[14] | 6.7 ± 0.1 | −0.2 ± 0.1 |
| 100 g of dry weight/L reconstituted lysate, pH 6.5, 20 °C[15] | 6.4 ± 0.1 | −0.5 ± 0.1 |
| 100 g/L anionic proteins, pH 7.0, 20 °C | 6.3 ± 0.1 | −0.5 ± 0.1 |
| 100 g/L total proteins, pH 7.0, 20 °C | 6.3 ± 0.1 | −0.6 ± 0.1 |
| 130 g of dry weight/L reconstituted lysate, pH 6.5, 20 °C[15] | 6.1 ± 0.1 | −0.8 ± 0.1 |
| 50 mM sodium acetate, pH 5.4, 37 °C[18] | 4.9 ± 0.1 | – |
| 100 g/L polyvinylpyrrolidone, pH 5.4, 37 °C[18] | 5.26 ± 0.05 | 0.3 ± 0.1 |
| 100 g/L Ficoll, pH 5.4, 37 °C[19] | 5.4 ± 0.1 | 0.5 ± 0.1 |

$\Delta G_{op}^{\circ\prime}$ values and their uncertainties (Table S5 of the Supporting Information) were used to calculate stability differences, $\Delta\Delta G_{op}^{\circ\prime}$ (crowded − buffer) and the propagated standard error.[38] Both lysates result almost exclusively in negative $\Delta\Delta G_{op}^{\circ\prime}$ values, indicating that the lysates destabilize CI2 (Figure 2).

Destabilization was observed along the entire backbone (Figures 2 and 3), except at Val 34. The unstructured loop of CI2 begins immediately after this residue. Although exchange is too fast to quantify loop stability with the method used here, saturation transfer-based experiments indicate that crowding does not affect exchange rates in this region.[27] The crude lysate also stabilizes Val 34, and the extent of stabilization increases with lysate concentration.[15] Perhaps one or more proteins block exchange by interacting specifically with the loop, or repulsive interactions alter its conformation.

Contrary to our hypothesis, both the total protein lysate and the anionic protein lysate destabilized CI2, and by the same amounts, −0.6 ± 0.1 kcal/mol. We considered the possibility that the destabilization was caused not by the proteins themselves but by their effect on ionic strength. Conductivity measurements show that the proteins in the lysate increase the conductivity by the equivalent of 80 mM NaCl. Our previous work shows that an increase of 150 mM NaCl destabilizes CI2 by only 0.1 kcal/mol.[14]


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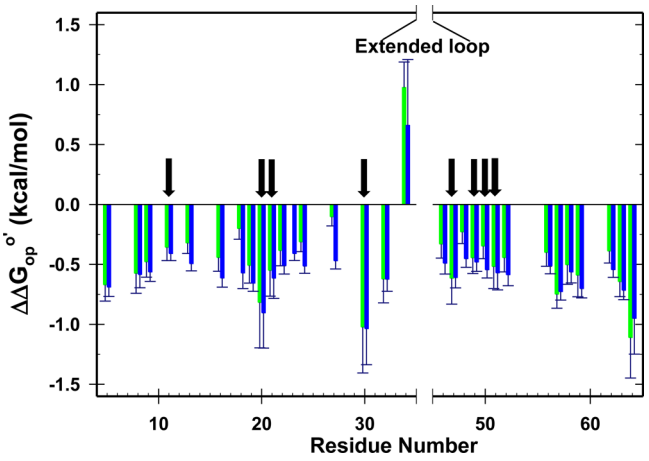


**Figure 2.** Bar graph of $\Delta\Delta G_{op}^{\circ\prime}$ (lysate − buffer) for 100.0 g/L anionic proteins (green) and 100.0 g/L total proteins (blue) vs residue number (20 °C, pH 7.0, 50 mM sodium phosphate). Residues that exchange by global unfolding[37] are denoted with black arrows. The values are the average of three trials under one condition minus the average of three trials under the other condition. The uncertainties are derived from error propagation on the standard deviations of the mean.[38]

## DISCUSSION

**pI Bias of the Proteome.** Many proteomes exhibit a bimodal distribution of isoelectric points.[42] The bias in *E. coli* is toward anionic proteins (Figure 1).[34] The number of acidic proteins is greater, as is their abundance.[4,43] This observation explains why it was easier for us to extract the anionic proteins. Many of the cationic proteins were probably lost during the streptomycin sulfate or PEI precipitation because of their interactions with nucleic acids, which might explain why we were unable to obtain a useful cationic protein lysate. Others have observed this bias against isolating basic proteins from the *E. coli* genome.[43]

**Anionic and Total Protein Lysates Are Both Destabilizing.** It is intuitive that anionic proteins will repulse negatively charged CI2. This repulsion should reinforce crowding-induced steric repulsion, favoring the compact native state over the ensemble of larger unfolded states, and thus increase stability. However, we observed only destabilization. Importantly, the anionic protein lysate is nearly as destabilizing as the total protein lysate (Table 1). This observation is consistent with the idea that nonspecific attractive backbone interactions shift the equilibrium toward the less structured states.

A negatively charged surface should increase the level of repulsion, yet we observe uniform destabilization. Furthermore, we find no correlation between CI2 surface potential and residue level destabilization (Figure 3). We conclude that neither the net charge of CI2 nor the net charges of the crowding proteins can overcome the nonspecific attractive interactions between protein surfaces. This conclusion is consistent with one of our observations about the destabilizing effect of crowding by BSA.[14] Specifically, adding NaCl to screen the charge mitigated but did not eliminate destabilizing interactions.

## SUMMARY AND CONCLUSIONS

We have studied the effects of crowding on the stability of CI2 under several conditions.[14,15,17−19] Synthetic polymers such as PVP and Ficoll are stabilizing,[17−19] but all the physiologically relevant crowders studied so far destabilize CI2,[14,15] Protein L,[16] and ubiquitin near room temperature.[20] We also know that CI2 interacts more strongly with biologically relevant crowders than with these synthetic polymers.[21,24] Even the anionic proteins, which have the same net charge as CI2, interact strongly enough with the backbone to overcome both charge−charge and steric repulsion. We conclude that proteins possess an inherently favorable, and probably ubiquitous, interaction with other proteins. Although weak, these interactions can overcome the stabilizing effect of hard-core repulsions associated with physiologically relevant macromolecular crowding. Nevertheless, the effect of the intracellular environment is not monolithic; both increases and decreases in stability have been observed, strongly suggesting the cells can manipulate protein stability.[44,45] We hope that our work will also motivate the development of more advanced models for explaining the effects of macromolecular crowders.

## ASSOCIATED CONTENT

### Supporting Information

Lists of lysate proteins that did and did not bind the DEAE column (Tables S1 and S2), lists of $\Delta G_{op}^{\circ\prime}$ values for the I29A/

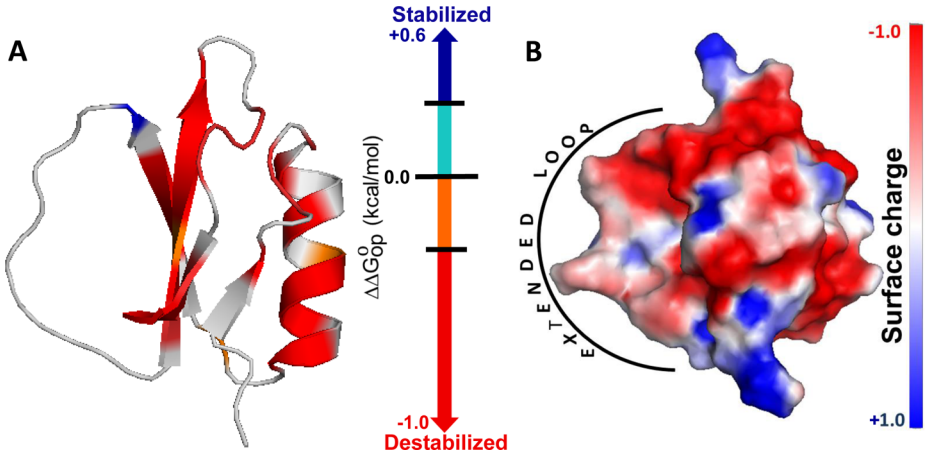


**Figure 3.** Structure of CI2[39] colored by (A) $\Delta\Delta G_{op}^{\circ\prime}$ (lysate − buffer) in a 100.0 g/L sample of anionic protein lysate and (B) electrostatic potential. The color-coded structure for $\Delta\Delta G_{op}^{\circ\prime}$ in 100.0 g/L total protein lysate is essentially identical to that shown. Charge was calculated using the Adaptive Poisson−Boltzmann Solver[40] in PyMOL,[41] which approximates the potential felt by a point charge near the surface.


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I37H variant in buffer, 100.0 g/L total protein lysate, and 100.0 g/L anionic protein lysate (Tables S3−S5), and conductivity measurements of NaCl standards, 50 mM phosphate buffer, and 100.0 g/L total protein lysate (Table S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interests.

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