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Macromolecular Crowding by Polyethylene Glycol Reduces Protein Breathing

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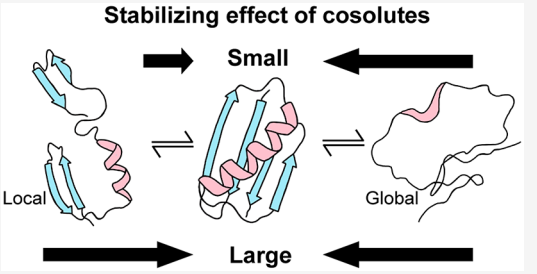
ABSTRACT: Most efforts to understand macromolecular crowding focus on global (i.e., complete) unfolding, but smaller excursions, often called breathing, promote aggregation, which is associated with several diseases and the bane of pharmaceutical and commercial protein production. We used NMR to assess the effects of ethylene glycol (EG) and polyethylene glycols (PEGs) on the structure and stability of the B1 domain of protein G (GB1). Our data show that EG and PEGs stabilize GB1 differently. EG interacts with GB1 more strongly than PEGs, but neither affects the structure of the folded state. EG and 12000 g/mol PEG stabilize GB1 more than PEGs of intermediate size, but EG and smaller PEGs stabilize GB1 enthalpically while the largest PEG acts entropically. Our key finding is that PEGs turn local unfolding into global unfolding, and meta-analysis of published data supports this conclusion. These efforts provide knowledge that can be applied to improve biological drugs and commercial enzymes.



Many small single-domain globular proteins unfold in a two-state manner[1] when unfolding is detected using methods such as calorimetry, fluorimetry, or circular dichroism spectropolarimetry (CD). The biologically active folded state is both a structural and a thermodynamic state, comprising a narrow ensemble of conformations. The inactive globally unfolded state is well-defined thermodynamically but comprises a much larger ensemble of non-native structures.[2]

For this class of proteins, methods such as those listed above sense only two states because they detect ensemble averages. In fact, the unfolded ensemble is large even under non-denaturing conditions, but its members are sparsely populated: for a typical globular protein with a stability of 7 kcal/mol at room temperature, only about one in 10^5 molecules are not in the folded state. There are many members of the non-native ensemble where only a part of the protein is unfolded. Englander and Kallenbach called these locally unfolded states and associated them with protein breathing.[3] Although exceedingly rare under physiologically relevant conditions, these locally unfolded states are important because a change in the ensemble, for instance by changing solution conditions or substituting one amino acid with another, can lead to aggregation—which is associated with several protein-related diseases[4] and the scourge of companies working to produce, purify, store, and ship biological drugs and industrial enzymes.[5] We set out to determine how adding high concentrations of macromolecules alters local unfolding.

Although undetectable by many techniques, these rare but important locally unfolded states are made visible at equilibrium by NMR-detected hydrogen−deuterium exchange (HDX). HDX allows quantification of test protein stability in solution at the residue level with or without cosolute.[3,6,7] A buffered D_2O solution is added to a dried ^{15}N-enriched test protein. Serial $^{15}N-^{1}H$ heteronuclear single-quantum coherence (HSQC) spectra are acquired to detect the exchange of backbone amide protons for deuterons as described by the reaction

$$\mathrm{N{-}H(closed)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \mathrm{N{-}H(open)} \xrightarrow{k_{int}} \mathrm{N{-}D(open)} \underset{k_{op}}{\overset{k_{cl}}{\rightleftharpoons}} \mathrm{N{-}D(closed)} \quad (1)$$

where k_{op} and k_{cl} are the first-order opening and closing rate constants, and k_{int} is the intrinsic rate constant of amide proton exchange[8] from the open state. The second step is irreversible because the reaction is carried out in D_2O.

If the test protein is stable and k_{int} is rate-determining ($k_{cl} > k_{int}$),[6] then exchange occurs by the EX2 mechanism[6] and the observed exchange rate (k_{obs}) of a backbone amide proton can be linked to the equilibrium constant for opening, K_{op}

$$k_{obs} = K_{op}k_{int} \quad (2)$$

which can be converted to the free energy of opening[7]

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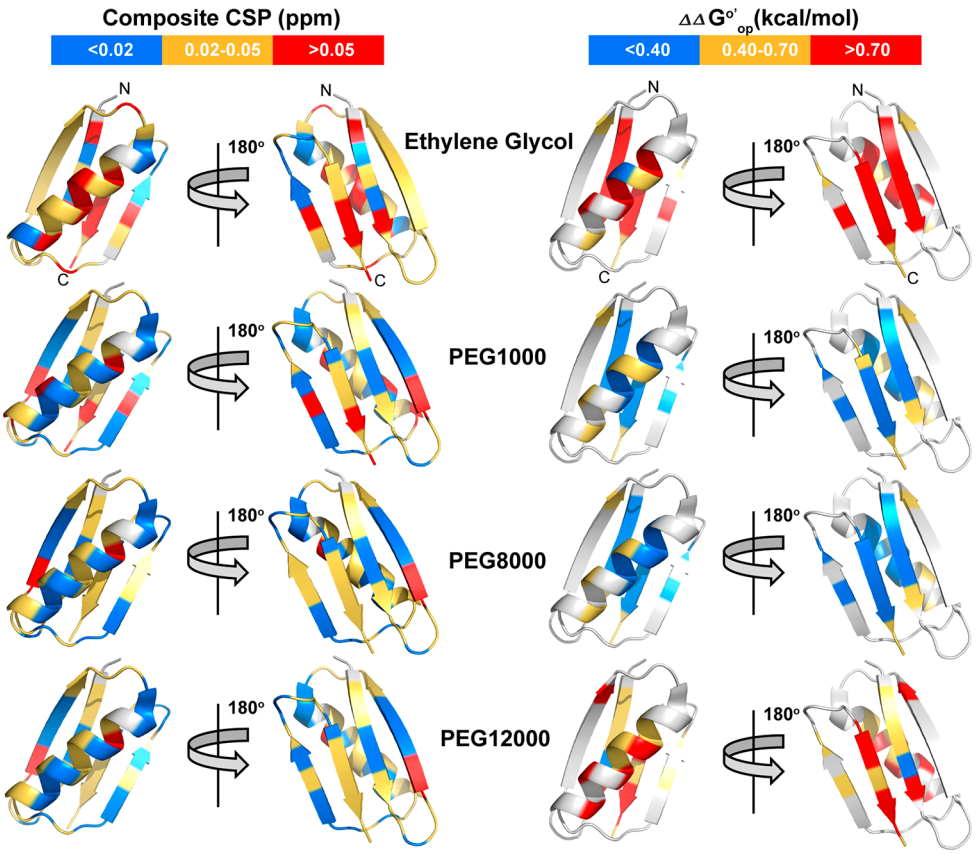

Figure 1. Structures of GB1 (PDB ID: 2QMT) colored by composite chemical shift perturbation (CSP) and change in stability compared to buffer at 310 K, pH 7.5 ($\Delta\Delta G^{o'}_{op} = \Delta G^{o'}_{op,cosolute} - \Delta G^{o'}_{op,buffer}$). Results are shown for 200 g/L ethylene glycol, PEG1000, PEG8000, and PEG12000. Left panel: Colors associated with categories of composite CSP are shown at the top. Gray indicates lack of data. Right panel: Colors associated with categories of $\Delta\Delta G^{o'}_{op}$ are shown at the top. Gray residues exchange too quickly to estimate rates. N- and C-termini are labeled in the top structures.

$$\Delta G^{o'}_{op} = -RT\ln K_{op} = -RT\ln\frac{k_{obs}}{k_{int}} \quad (3)$$

where R is the gas constant and T is the absolute temperature. The largest $\Delta G^{o'}_{op}$ values are observed for residues that are only exposed to the solution upon complete unfolding, providing the global stability. Smaller values are observed for residues that exchange by breathing.[9]

Importantly, if cosolutes are not enriched in ^{15}N, they are invisible to NMR-detected HDX and other ^{15}N-directed NMR experiments making NMR useful for crowding studies. In addition, HDX can be performed as a function of temperature to provide the enthalpic and entropic components of $\Delta G^{o'}_{op}$.[10] Furthermore, chemical shift changes can be used to correlate the thermodynamic data with changes in protein structure and protein−cosolute interactions.[11]

Crowding effects on protein and protein complex stability arise from hard repulsions and chemical interactions.[12] Hard repulsion, which means that two molecules do not interact until they touch, at which point repulsion rises steeply, is entropic and always stabilizing because unfolded states are larger than the folded state.[12] Attractive cosolute−protein chemical interactions (e.g., hydrogen bonding) weaken, and repulsive chemical interactions (i.e., like charges) reinforce hard repulsions, resulting in destabilization if attractive or stabilization if repulsive.

The cytoplasm is crowded with macromolecular cosolutes whose concentration can exceed 300 g/L.[13] Quantitative efforts in physiologically relevant environments show the importance of chemical interactions. For example, the stability of the side-by-side dimer made from the B1 domain of protein G (GB1)[14] increases in both prokaryotic and eukaryotic cells compared to buffer, probably because of repulsive chemical interactions between the GB1 monomer and intracellular macromolecules.[15] On the other hand, the reaction of the GB1 domain-swapped dimer[16,17] changes from an equilibrium between a folded dimer and a molten globule monomer in buffer to one between the dimer and unfolded monomers in *Escherichia coli* cells, suggesting that attractive chemical interactions in cells unfold the protein.[18]

Although not directly physiologically relevant, the crowding effects of synthetic polymers are essential to the pharmaceutical and chemical industries where they are used as excipients to protect biological drugs, vaccines, and commercial enzymes.[19−21] Polyethylene glycols (PEGs), which are used to formulate pharmaceutically and industrially important proteins and vaccines,[21] are the most commonly employed synthetic polymers for crowding experiments because of their high solubility, low polydispersity, and availability in a range of molecular weights. Understanding how PEGs protect proteins against aggregation will help in the search for new, more effective excipients.

Here, we use the 6.2 kDa GB1 monomer[22] as our test protein. Under all the conditions used here, GB1 exchanges by the EX2 mechanism, and 11 of its 56 residues exchange via global unfolding in buffer (K4, L5, and I6 in the β1-strand; A26, E27, and F30 in the α-helix; T44 in the β3-strand; and T51, F52, T53, and V54 in the β4-strand) as shown by the

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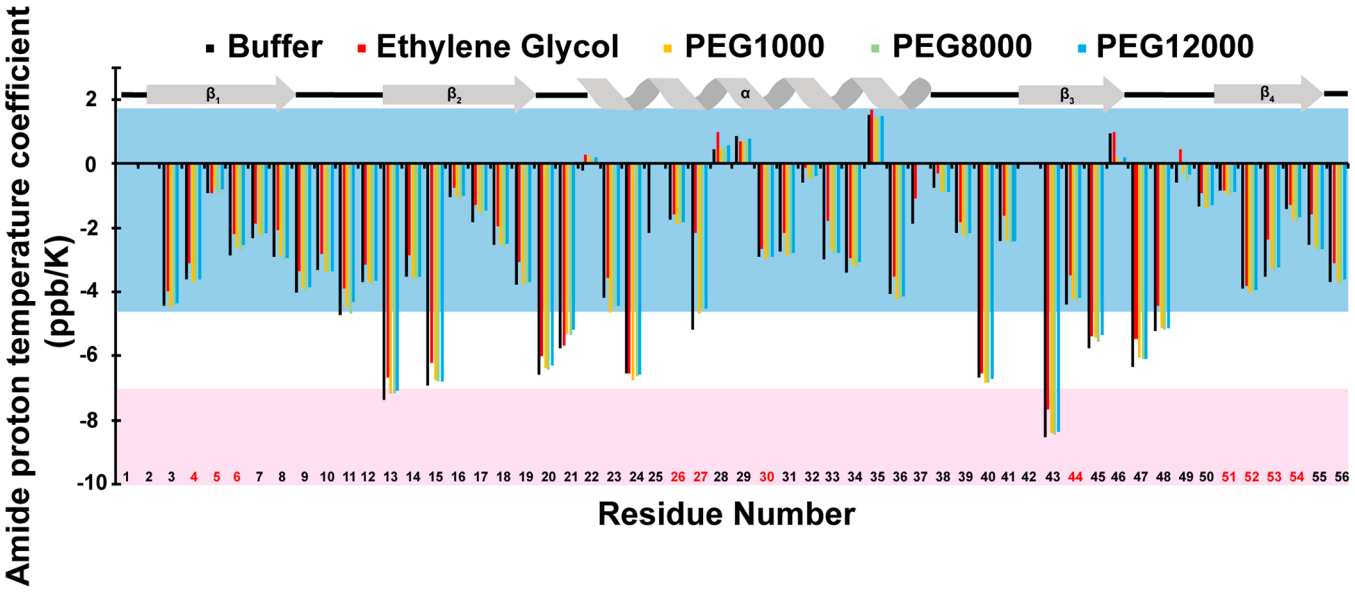

Figure 2. Amide proton temperature coefficients in buffer, 200 g/L ethylene glycol, PEG1000, PEG8000, and PEG12000. Bars ending in the blue box have a ≥85% probability of participating in an intramolecular hydrogen bond. Bars ending in a pink box have a ≤20% probability of participating in an intramolecular hydrogen bond. Red residue numbers are global unfolders. Coefficients are the slope of a plot of amide proton chemical shifts against temperature from 298 to 313 K in 5 K increments at pH 7.5.

correspondence between the hydrogen−deuterium exchange and differential scanning calorimetry data.[23] The other residues are either local unfolders or prolines. We used HDX to assess stability effects of 200 g/L solutions of ethylene glycol, 1000 g/mol PEG (PEG1000), 8000 g/mol PEG (PEG8000), and 12000 g/mol PEG (PEG12000) on GB1 and chemical shift perturbation (CSP) measurements to assess interaction of the cosolutes on the protein. When dealing with synthetic polymers it is important to consider the overlap concentration, c^*, the concentration where polymers[24] in solution cease to act as individual molecules and begin to form a mesh where the space between the strands becomes important. These concentrations are 190 g/L for PEG 1000, 38 g/L for PEG8000, and 28 g/L for PEG12000. Therefore, all our PEG data were acquired at or above c^*.

We used CD to test whether the cosolutes affect the secondary structure of the folded state (Figure S1). Spectra in all the cosolutes are identical to the spectrum in buffer alone, suggesting that none of the cosolutes have a significant effect on secondary structure.

CD data are useful, but NMR chemical shift data are much more sensitive. CSP is a simple and convenient metric for assessing cosolute−protein interactions (Figure S2).[25]

$$\text{Composite CSP} = \sqrt{(\delta^{HN}_{cosolute} - \delta^{HN}_{buffer})^2 + ((0.154)(\delta^{N}_{cosolute} - \delta^{N}_{buffer}))^2} \quad (4)$$

The CSP patterns for PEG1000, PEG8000, and PEG12000 are similar but different from the pattern for ethylene glycol (Figure 1), and a majority of the CSP values in ethylene glycol are greater than those in PEGs.

Given the lack of change in CD spectra, the CSP data could reflect changes in tertiary structure. However, this scenario seems unlikely because the interiors of native globular proteins are already almost perfectly packed,[26] which means that tertiary structure changes should usually be destabilizing, but as described next, all the cosolutes increase stability. We suggest that the chemical shift changes reflect the enhanced interaction of ethylene glycol with folded GB1, consistent with a report suggesting that a strong ethylene glycol−folded protein interaction explains the stabilizing effect of ethylene glycol.[27]

To gain information about how the cosolutes might change intraprotein hydrogen bond strength we measured amide proton temperature coefficients (the slope of a plot of amide proton chemical shifts against increasing temperature). Cierpicki and Otlewski showed that 85% of amide protons involved in an intraprotein hydrogen bond have coefficients more positive than −4.6 ppb/K.[28] Coefficients more negative than −7.0 ppb/K suggest a hydrogen bond probability <20%.[28]

Amide proton temperature coefficients for the native state were determined in buffer and in 200 g/L solutions of the cosolutes (Figure 2). About 80% of all hydrogen bonded amides (as defined in PDB ID: 2QMT) have coefficients greater than −4.6 ppb/K and with average values of −3 ± 2 ppb/K for buffer, ethylene glycol, and PEGs. For all conditions, none of the coefficients changed from the 85% to the 20% category (Figure 2). For PEGs, between 24 and 35 of the 52 coefficients are more positive compared to buffer, while for ethylene glycol, 50 of 52 have more positive values. We conclude that ethylene glycol has a stronger effect on hydrogen bonds than do the PEGs, but the effect is not large. In summary, the CD, CSP, and temperature coefficient data suggest that none of the cosolutes affect its folded structure, but ethylene glycol interacts slightly more strongly with GB1 than do the macromolecular cosolutes.

Initial HDX experiments were performed in triplicate in cosolute solutions (pH 7.5, 310 K) containing 200 g/L ethylene glycol, PEG1000, PEG8000, and PEG12000 (Tables S1−S5). The k_{int} values in buffer at 310 K were obtained using the online Server Program for Hydrogen Exchange Rate Estimation.[8,29] We used poly-DL-alanine and one-dimensional ^{1}H saturation-transfer[30,31] to quantify the effect of ethylene glycol and PEGs on k_{int} (Table S6). The value of k_{int} in buffer

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is 1.0 ± 0.1 s^{-1}. Values in ethylene glycol and PEGs vary from 0.6 to 0.7 s^{-1} with the same uncertainty. Considering that $\Delta G^{o'}_{op}$ is proportional to the logarithm of k_{int}, these small changes do not affect our conclusions. We then calculated $\Delta G^{o'}_{op}$ for each assigned backbone residue of GB1 to assess the effects of PEG on stability. All sites for which data are available show increased stability ($\Delta\Delta G^{o'}_{op} > 0$) in all cosolutes (Figure 1).

To obtain more information, we estimated the van't Hoff enthalpies of opening ($\Delta H^{o'}_{op}$) by plotting $-R \ln K_{op}$ for each residue against $1/T$. We allocated the $\Delta\Delta H^{o'}_{op}$ ($\Delta\Delta H^{o'}_{op} = \Delta H^{o'}_{op,cosolute} - \Delta H^{o'}_{op,buffer}$) data into three tranches and superimposed them on the GB1 structure (Figure 3). Positive

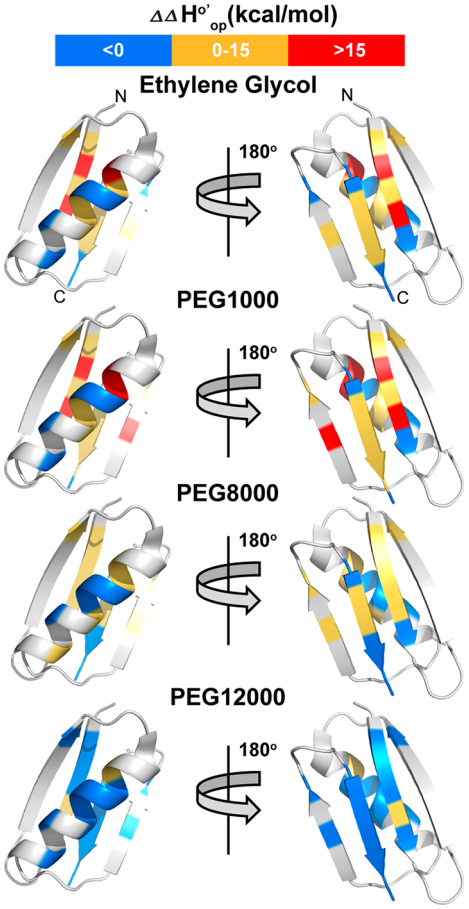

Figure 3. Structures of GB1 (PDB ID: 2QMT) colored by the change in enthalpy compared to buffer. Results are shown for 200 g/L ethylene glycol, PEG1000, PEG8000, and PEG12000. Colors associated with categories of $\Delta\Delta H^{o'}_{op}$ are shown at the top. Gray residues exchange too quickly to estimate rates. N- and C-termini are labeled in the top structures.

values of $\Delta\Delta H^{o'}_{op}$ for over half of the residues and for all the global unfolding residues are observed for ethylene glycol, PEG1000, and PEG8000. The result is strikingly different for PEG12000: $\Delta\Delta H^{o'}_{op}$ is negative for almost all residues, local and global. (This result was so surprising we repeated the PEG12000 experiments and obtained the same outcome.) Since all the cosolutes increase $\Delta\Delta G^{o'}_{op}$, this result means that below a molecular weight of 12000 g/mol PEG stabilization is enthalpically driven but switches to entropically driven for the largest PEG.

Stability does not correlate in a simple way with cosolute size. The increases at the extremes of molecular weight—ethylene glycol and PEG12000—are greater than those in PEGs 1000 and 8000. Furthermore, the patterns for stability differ from the patterns for CSPs. For CSPs, the patterns for PEGs 1000, 8000, and 12000 are similar but different from the pattern for ethylene glycol. These results suggest that ethylene glycol and PEG12000 stabilize GB1 in fundamentally different ways. The temperature dependence of the amide chemical shifts suggests that ethylene glycol interacts more strongly with the folded state than do the PEGs. These data suggest that preferential interaction of ethylene glycol with the folded state caused by the larger number of hydroxyl groups per gram compared to PEGs explains both the stronger interaction and the larger stabilizing effect of ethylene glycol, as has been suggested by others.[27,32]

The PEGs are stabilizing but have only a small effect on structure and hydrogen bonding. $\Delta\Delta H^{o'}_{op}$ shows a general decrease with PEG size and becomes negative for PEG12000. These data indicate that stabilization becomes more entropic with increasing PEG molecular weight and becomes entirely entropic at PEG 12000. This increase in entropic stabilization is consistent with simple theory, but there is a persistent enthalpic effect, which is not explained by simple ideas about crowding.[12]

To further understand why ethylene glycol and PEG12000 behave differently, we classified individual $\Delta\Delta G^{o'}_{op}$ values for GB1 as global unfolders and local unfolders.[23] In ethylene glycol, global unfolders contribute more than local unfolders to GB1 stability (Figure 4A). However, in PEG1000, 8000, and 12000, global unfolders contribute no more stabilization than do local unfolders. We hypothesized that macromolecular crowding by PEG tends to turn breathing into global unfolding.

We analyzed the results from 12 other NMR-detected HDX studies of several globular proteins (ribonuclease A, hisactophilin, chymotrypsin inhibitor 2, and GB1) in stabilizing (trehalose, glycine betaine, polyvinylpyrrolidones, Ficoll-70, sucrose) and destabilizing (urea) cosolutes (Figure 4B and Table S7).[10,31,33−37] All the polymer data were acquired at above their overlap concentrations.[38] To compare data from different cosolutes, we normalized $\Delta\Delta G^{o'}_{op}$ values. Specifically, we divided $\Delta\Delta G^{o'}_{op}$ for each residue of a particular cosolute−protein combination by the average of the three largest $\Delta\Delta G^{o'}_{op}$ values for each data set. For urea experiments, we used the absolute value of $\Delta\Delta G^{o'}_{op}$. Consistent with our hypothesis, small cosolutes affect global unfolding residues more than local unfolding residues, but the difference disappears for macromolecular cosolutes (Figure 4B).

The data presented here and those from the literature indicate that both stabilizing and destabilizing small cosolutes affect global stability more than local stability, but larger stabilizing cosolutes increase the similarity of these two classes of residues. In their classic study, Bai et al. used the denaturant guanidinium chloride (GdmCl) and HDX to show that the opening free energies of global unfolding residues in cytochrome c have a higher dependence on GdmCl concentration than do locally unfolding residues and interpreted this observation as showing that families of locally unfolding residues represent intermediates.[9] This conclusion, taken together with our analysis, suggests that crowding by synthetic polymer cosolutes tends to make local unfolders

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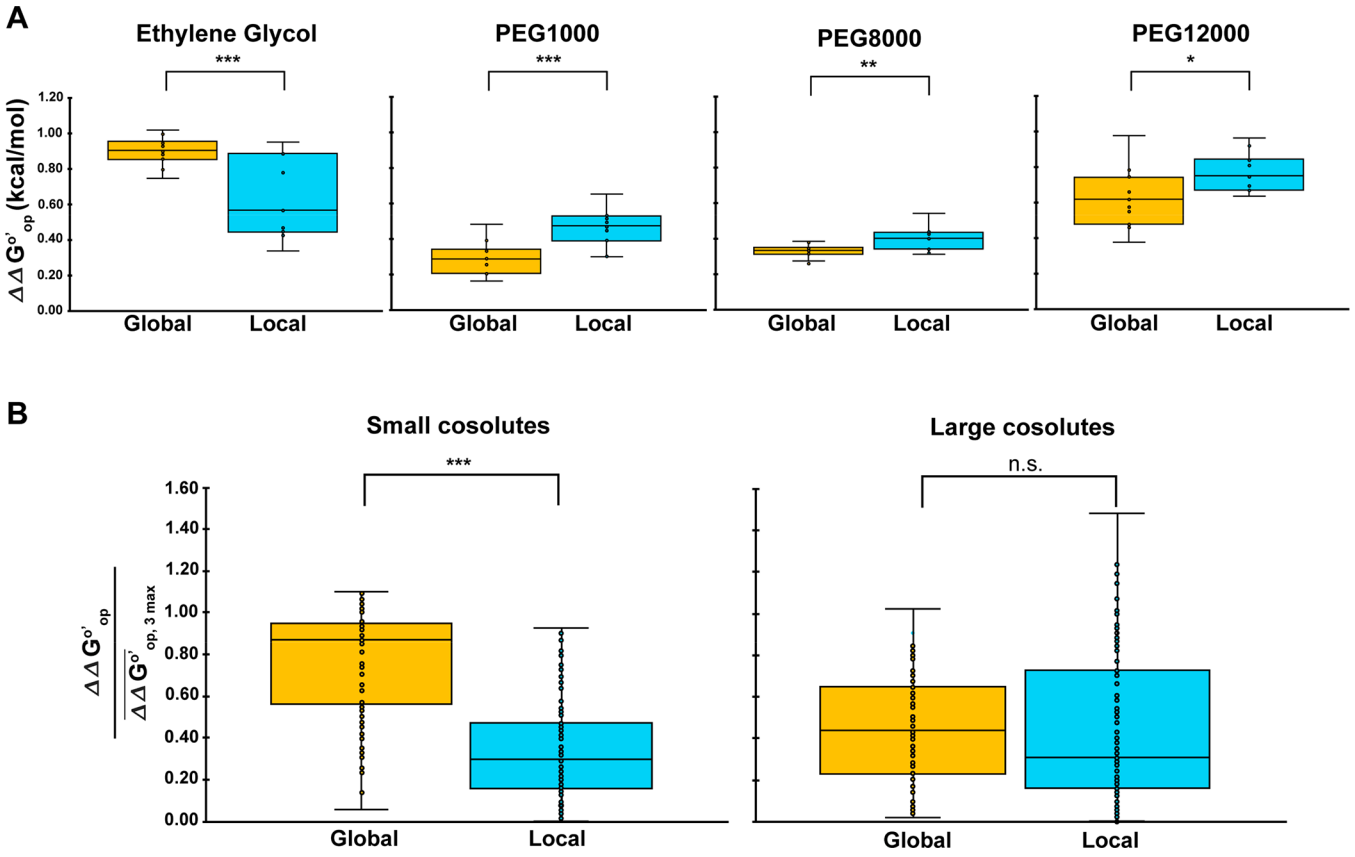

Figure 4. Box plots of (A) GB1 opening free energies for ethylene glycol, PEG1000, PEG8000, and PEG12000 and (B) normalized opening free energies for small and large cosolutes. $\Delta\Delta G^{o'}_{op}$ values used to produce the plots for small and large cosolutes include the four cosolute-protein combinations from this study and 12 additional combinations from the literature. To facilitate comparison of $\Delta\Delta G^{o'}_{op}$ across protein−cosolute pairs, the absolute value of $\Delta\Delta G^{o'}_{op}$ for every residue in each protein−cosolute combination is divided by the mean $\Delta\Delta G^{o'}_{op}$ of the three largest $\Delta\Delta G^{o'}_{op}$ values. In the box plot, the center line indicates the median and horizontal box edges represent upper and lower quantiles. Student's *t* test $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., not significant.

more like global unfolders, indicating a potential loss of intermediates and signaling a change in the mode of unfolding.

This suggestion may have practical applications. Intermediates in protein folding expose hydrophobic surface, which can lead to aggregation. Our results suggest that formulation with PEG and other synthetic polymers may increase the performance of biological drugs not only by increasing stability but also by lowering the concentration of aggregation-prone intermediates. This effect, however, is not universal; synthetic polymers can accelerate aggregation of some globular proteins[39] but probably via surface interactions between the crowders and the native state.[40] Thus, the need remains to assess synthetic polymers with different surface properties.

In summary, we used NMR-detected HDX and chemical shift data to study how ethylene glycol and different PEGs affect GB1 stability at the residue level. Ethylene glycol and PEGs both stabilize GB1 but do so in different ways. Ethylene glycol affects global unfolding residues more than local unfolding residues. The largest PEG affects global and local unfolders nearly equally. The temperature dependence of the HDX data shows that stabilization by the largest PEG is entropic, consistent with simple ideas about crowding, but there is an enthalpic effect, which is not predicted by simple theories. We further analyzed HDX studies from the literature, and the results support our PEG-GB1 data. We conclude that small cosolutes affect global stability more than local stability, suggesting that potential new excipients should be assessed for their effect on both global and local stability.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.3c00271.

Materials and methods; CD spectra; bar plots of chemical shift perturbations; tables of backbone amide exchange rates in buffer, ethylene glycol, PEG1000, PEG8000, and PEG12000; table of T1 and k_{int}; table of hydrogen−deuterium exchange studies (PDF)

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Notes

The authors declare no competing financial interest.

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