

Review of: "Impact of crowded environments on binding between protein and single-stranded DNA"

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Background

Cells retain large amounts of macromolecules within their plasma membranes, up to 400 g/L depending on their physiologic state.^[1] Mutual exclusion is a fundamental property of polymers in solution.^{[2][3]} Classically, this excluded volume effect is treated as hard-core repulsion: an entropic depletion force that drives folding and interactions towards more compact states.^{[4][5]} More recent studies show that depletion forces are supplemented by lower-potential “soft” interactions which may even be attractive.^{[6][7][8][9][10]} While the impact of crowders on protein stability and dynamics in solution has been recognized since the early works of Ogston^[11] and Laurent^[12], their theoretical treatment continues to be debated.^{[13][14][15][16][17][18]} Experimental measurements remain a mainstay of studying biological interactions in a crowded environment.

In their study, Köhn *et al.* characterize the impact of macromolecular crowding on the binding of single-stranded DNA, specifically oligothymidines (dTn, n = 5 to 7 nucleotides) by cold shock protein B from *Bacillus subtilis* (*BsCspB*, 67 residues), using polyethylene glycol (PEG1 and PEG8, 1k and 8k), ethylene glycol (EG), dextran (Dex20, 20k), and glucose as cosolutes. Monomer-polymer pairs enable a comparison of both chemical composition and size. On the one hand, PEG and EG act more like an organic solvent, which displays hydrophobic tendencies. On the other hand, Dex and glucose are more polar. The authors hypothesize that weak chemical interactions dominate excluded volume effects in crowding protein/DNA binding.

Technical review

***BsCspB* structure is conserved under crowding.** High concentrations of any cosolute always raises a concern of specific interactions that would obscure the intended interpretation of the data. To assess this possibility, the authors used heteronuclear NMR spectroscopy to probe the equilibrium structures of free *BsCspB* and dTn-bound complexes. An extensive series of 1D (¹H and ³¹P) and 2D (¹H-¹⁵N HSQC and ¹H-¹³C HSQC) experiments showed substantially similar chemical shift perturbations (CSP) or intensity in both the protein and DNA upon binding whether in dilute solution or up to 300 g/L of crowding agent. While the CSP data is presented as overlays of the free and bound states under identical

conditions in the main text (e.g., Figure 1), overlays of free or variously bound spectra under dilute and crowded conditions may also be informative. Regardless, NMR evidence under neutral buffered conditions suggests that crowding agents do not drastically alter the average structure of *BsCspB* upon binding at the residue level.

Crowding decreases *BsCspB*/ssDNA affinity and kinetics in a crowder-dependent manner.

The authors used steady-state fluorescence spectroscopy (based on intrinsic tryptophan fluorescence quench upon binding) to determine *BsCspB*/dTn binding affinity at equilibrium, identifying differences between solutions treated with the different treatments. The presence of PEG or EG significantly reduced dTn binding affinity for *BsCspB*. Limited data for PEG1 and PEG8 in dT7 binding showed little incremental effect by the larger crowder. In contrast, equivalent % w/v glucose was much less perturbative than EG, and crowding by Dex20 produced the opposite effect, enhancing dTn affinity with increasing concentration. While the MW of glucose is about three times that of EG, it is difficult to discern whether the decrease in affinity by EG and glucose (each measured at a single %w/v, representing two different activities) represented cosolute interactions or colligative hydration effects. The divergent effects of glucose and Dex20 on binding affinity demonstrate the impact of cosolute size on binding, which oppose and overcome the negative contributions by the monomer.

To tease out the subtle binding affinity differences, the authors used stopped-flow techniques to quantify the kinetics of *BsCspB*/dTn binding. Remarkably, the association rate constant (k_{on}) of the complex decreases linearly with the %w/v of the crowding agent essentially independently of cosolute identity and size (Table 3G). In contrast, the dissociation rate constant k_{off} differed widely depending on the cosolute (Table 3H). While the polymers (PEG1 and Dex20) showed relatively little effect on k_{off} out to 300 g/L, their monomers produced qualitatively opposite differences: EG increased k_{off} monotonically with concentration, whereas glucose sharply decreased k_{off} at 100 g/L before showing no additional effect (Table 3H). The non-monotonic effect of glucose seems to imply complex effects of glucose on the rate-limiting step in the dissociation of the *BsCspB*/dTn complex. Taken together, the authors conclude that chemical interactions dominate in crowding. Interestingly, the presented kinetic parameters generally underestimate the equilibrium dissociation constants ($K_D = k_{off}/k_{on}$) by a factor of two or more, suggesting the potential of steps in *BsCspB*/dTn binding that are not fully captured by the 1:1 model used to fit the equilibrium data.

Cosolute effects on solvent exchange by *BsCspB*. Given the apparent dissociation kinetics, the authors returned to NMR to interrogate (unbound) *BsCspB* by amide hydrogen exchange (Figure 4). At equivalent %w/v (240 g/mL), PEG1 and Dex20 were generally less perturbative on the solvent exchange rate constant (k_{ex}) than their monomers, whose effects were most pronounced at residues with rapid solvent exchange under dilute conditions. Noting a rough correspondence in the trends in k_{ex} and the dissociation rate constant, the authors proposed that cosolute effects in dTn binding are mediated through the “hydration shell” around *BsCspB*.

Amide hydrogen exchange is most directly interpreted in terms local solvent accessibility and

reactivity of proton exchange. In previous work, the authors have correlated solvent accessibility with the conformational stability of free *BsCspB*.^[19] In the present study, the authors acknowledge that the solvent exchange profiles only partially explain the dissociation kinetics data. Namely, there was no obvious difference in the exchange profiles for EG and glucose that would track their quantitative effects on dTn binding affinity. Nor was there any pattern in the corresponding profiles for PEG1 and Dex20 to explain the qualitatively opposite effects of PEG and Dex20 on binding affinity. Uncertainty may be partly due to a lack of data on the dTn-bound complex itself, or at least unbound dTn. Another confounding factor may be uncontrolled cosolute effects on conformation and binding on the one hand, and the intrinsic reactivity of proton exchange with solvent on the other, for which resolution requires measurement over a range of pH.

Perspective

To place the crowding effects from this study in perspective, it is instructive to consider the relative sizes (e.g., radii of gyration, R_g) of the macromolecules involved. Using their crystal structures, the R_g of *BsCspB* (PDB: [1CSP](#)) and a *BsCspB*/dT6 complex ([2ES2](#)) may be computed to be 1.1 and 1.3 nm, respectively, which would somewhat underestimate their true values as fluctuating folded states in solution. For free dTn, we may estimate an R_g up to 0.7 nm for dT7 based on SAXS measurements of poly-dT.^[20] Solution dimensions of PEG and Dex, which are commercially important excipients, are extensively reported in the literature:^{[21][22][23]} $R_g \sim 1.0$ nm for PEG1, ~ 2.0 nm for PEG8, and ~ 2.2 nm for Dex20. These crowding agents are therefore the same order in size as *BsCspB* and dTn. One wonders how the apparent balance of “soft” and “hard” effects of crowding might depend on crowder size and whether excluded-volume effects might increasingly contribute if much larger crowders were studied.

Overall, the authors make a case for soft interactions on ligand binding of proteins, following a “bottom-up” approach that prioritizes defined chemical characteristics. As a result, the authors gained insight into physicochemical properties driving crowding of *BsCspB*/dTn binding, justifying their choice of synthetic crowders over naturally derived crowders such as reconstituted cell lysate. That said, the use of one model system and two pairs of cosolutes necessarily limits the generalizability of the results from a biological relevance perspective.

Paper in context

Research into the binding properties of transcription factors under native-like conditions bears implications for our understanding of gene expression, as the authors stress in their paper. Biophysical data on crowded protein/DNA interactions remains limited relative to protein folding and interactions, DNA stability, and diffusion.^{[24][25][26][27][28][29][30]} Interest has recently arisen on how crowding modifies target site search via DNA-mediated diffusion.^{[31][32][33]} Nevertheless, there has been limited progress in relating the stability and interactions of nucleoprotein complexes in dilute solution, on which a large body of literature exists, to their behavior under crowded conditions. The welcome study by Köhn *et al.* shows that crowding

with PEG and Dex20 can significantly impact, in an identity-dependent manner, single-stranded DNA binding by BsCspB, without exerting major structural changes on the protein.

In the literature, attribution to soft interactions in macromolecular crowding remains somewhat secondary to excluded-volume effects. Binding studies that report similar observations as Köhn *et al.*, namely a lack of major structural change upon crowding and the volume of proteins affecting binding, frequently favor hard interactions as the primary contributor. It may be the case that binding (wherein at least the receptor or ligand is well folded) is more sensitive to soft interactions, whereas conformational transitions involving highly unfolded structures are more sensitive to excluded-volume effects. The relative effectiveness of low-MW cosolutes to exert osmotic pressure is generally well recognized. On the flip side, while all cosolutes make exclusion contributions in solution^[34], excluded volumes scale with the effective radius cubed. Low-MW cosolutes, therefore, do not yield the crowding effects of polymers at experimentally relevant % w/v. Parallel evaluation of constituent monomers of polymeric crowders thus represents a well-reasoned control for helping resolve the two classes of contributions.

In closing, we regret the omission of numerous colleagues who contribute to our understanding of macromolecular crowding in our attempt for concision.

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