FEBS Letters 587 (2013) 394–397

journal homepage: www.FEBSLetters.org

Hypothesis

# Polymer crowders and protein crowders act similarly on protein folding stability

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ARTICLE INFO

*Article history:*
Received 1 January 2013
Revised 11 January 2013
Accepted 13 January 2013
Available online 23 January 2013

Edited by Peter Brzezinski

*Keywords:*
Macromolecular crowding
Protein folding
Crossover temperature

ABSTRACT

**Recently a polymer crowder and two protein crowders were found to have opposite effects on the folding stability of chymotrypsin inhibitor 2 (CI2), suggesting that they interact differently with CI2. Here we propose that all the macromolecular crowders act similarly, with an entropic component favoring the folded state and an enthalpic component favoring the unfolded state. The net effect is destabilizing below a crossover temperature but stabilizing above it. This general trend is indeed observed in recent experiments and hints experimental temperature as a reason for the opposite crowding effects of the polymer and protein crowders.**


## 1. Introduction

There is now growing recognition that the crowded conditions found in cellular environments can significantly impact the equilibria of biochemical processes such as protein folding [1]. A number of studies [2–6] reported increases in protein folding stability by polymer crowders such as Ficoll and dextran, although the magnitudes are modest, of the order of 1–2 $k_BT$ ($k_B$: Boltzmann constant; $T$: absolute temperature) around room temperature. Extending these studies, Pielak and co-workers [7–9] investigated the effects of both a polymer crowder, poly(vinylpyrrolidone) (PVP), and two protein crowders, lysozyme and bovine serum albumin (BSA), on the folding stability of a small protein chymotrypsin inhibitor 2 (CI2), a known reversible two-state folder [10]. In line with the other studies, the polymer crowder PVP was found to have a moderate stabilizing effect on CI2, but the two protein crowders were found to be destabilizing, leading to the suggestion that polymers and proteins behave differently as crowding agents. Here we propose that both polymer and protein crowders act similarly on the test protein, with an entropic component that favors the folded state and an enthalpic component that favors the unfolded state. This unifying mechanism explains the apparently opposite effects of the polymer and protein crowders as well as the very recent temperature-dependent crowding effects of Pielak and co-workers [11,12].

## 2. Temperature-dependent change of crowding effects on folding stability: existence of a crossover temperature

The temperature dependence of the unfolding free energy, $\Delta G$, can be derived based on the generally accepted assumption that $\Delta C_p$, the change in heat capacity upon unfolding, is temperature-independent. Then the unfolding enthalpy and unfolding entropy depend on temperature as [13]:

$$\Delta H(T) = \Delta H(T_{ref}) + \Delta C_p(T - T_{ref}) \quad (1)$$

$$\Delta S(T) = \Delta S(T_{ref}) + \Delta C_p \ln(T/T_{ref}) \quad (2)$$

where $T_{ref}$ is an arbitrary reference temperature. From these two components, one obtains

$$\Delta G(T) = \Delta H(T) - T\Delta S(T) \quad (3a)$$

Another standard thermodynamic relation is

$$\Delta S(T) = -\frac{\partial \Delta G(T)}{\partial T} \quad (3b)$$

We choose $T_{ref}$ to be the temperature, denoted as $T_s$, where $\Delta G(T)$ is maximum. The maximum of any function corresponds to a zero slope, therefore it follows from Eq. (3b) that, at $T = T_s$, $\Delta S$ is zero and hence the value of $\Delta H$, denoted as $\Delta H_s$, is the same as the $\Delta G$ maximum. Consequently

*Abbreviations:* BSA, bovine serum albumin; CI2, chymotrypsin inhibitor 2; PVP, poly(vinylpyrrolidone)

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0014-5793/$36.00 
http://dx.doi.org/10.1016/j.febslet.2013.01.030

$$\Delta H(T) = \Delta H_s + \Delta C_p(T - T_s) \quad (4)$$

$$\Delta S(T) = \Delta C_p \ln(T/T_s) \quad (5)$$

Now consider the folding equilibrium in a crowded solution. The changes in $\Delta H$, $\Delta S$, and $\Delta G$ by crowding will be denoted as $\delta\Delta H$, $\delta\Delta S$, and $\delta\Delta G$, respectively. We first make the simplifying assumption that crowding does not affect $\Delta C_p$. This amounts to neglecting the temperature dependence of $\delta\Delta H$. Given that the effects of crowding on folding stability are found to be small in all cases and the "observable" temperature range is usually narrow, this zeroth-order assumption seems justified. Then crowding can only affect the other two parameters, $T_s$ and $\Delta H_s$. Let the temperature at which the unfolding free energy in the crowded solution achieves maximum be $T_s + \delta T_s$, and the latter maximum be $\Delta H_s + \delta\Delta H_s$. We have

$$\delta\Delta H = \delta\Delta H_s - \Delta C_p \delta T_s \quad (6)$$

$$\delta\Delta S = -\Delta C_p \ln(1 + \delta T_s/T_s) \quad (7)$$

Note that $\delta\Delta S$ is only affected by $\delta T_s$ but $\delta\Delta H$ can be affected by both $\delta T_s$ and $\delta\Delta H_s$, and both $\delta\Delta H$ and $\delta\Delta S$ are independent of temperature.

Based on the preceding observation, we define three kinds of crowding behaviors (Fig. 1). A purely "entropic" crowder is one that results in a non-zero $\delta\Delta S$ but a zero $\delta\Delta H$, and is obtained when a change in the maximum-stability temperature is accompanied by a compensating change in the maximum stability such that $\delta\Delta H_s = \Delta C_p \delta T_s$. Assuming $\delta\Delta S < 0$ (corresponding to a positive $\delta T_s$), an entropic crowder results in an increase in the unfolding free energy at every temperature, and the increase grows with increasing $T$ (since the entropic component of $\Delta G$ is weighted by $T$). An "enthalpic" crowder, obtained when only $\Delta H_s$, not $T_s$, is changed, results in a uniform decrease in $\Delta G$ (assuming $\delta\Delta H_s < 0$) at all temperatures. Finally a "compound" crowder has both $\delta\Delta H$ and $\delta\Delta S$ non-zero. For $\delta T_s > 0$ and $\delta\Delta H < 0$, the compound crowder results in a $\Delta G$-vs.-$T$ curve that crosses that for the dilute solution. The existence of a crossover temperature, $T_x$, is a signature of the compound crowding behavior. At the crossover temperature, $\delta\Delta G = 0$, by which we find

$$T_x = \frac{\delta\Delta H}{\delta\Delta S} = \frac{-\delta\Delta H}{\Delta C_p \ln(1 + \delta T_s/T_s)} \approx \frac{-\delta\Delta H}{\Delta C_p \delta T_s} T_s \quad (8)$$

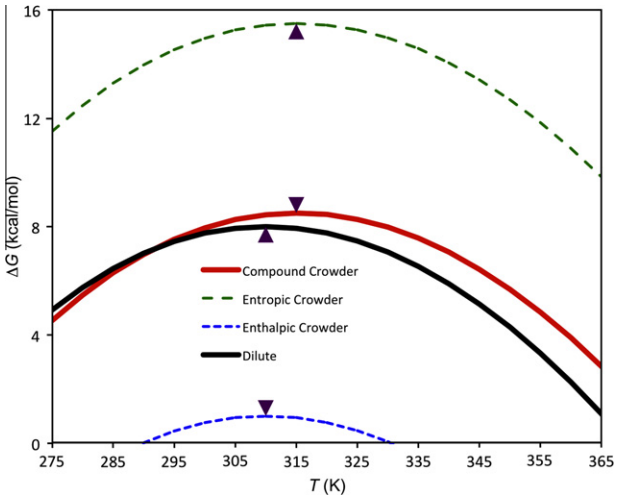


**Fig. 1.** Three kinds of crowding behaviors. The curve for the dilute solution is calculated using typical experimental values ($\Delta C_p$ = 1.5 kcal/mol/K; $T_s$ = 310 K (indicated by arrow); and maximum stability $\Delta H_s$ = 8 kcal/mol). The enthalpic, entropic, and compound crowders have $\delta T_s$ = 0, 5, and 5 K; and $\delta\Delta H$ = −7, 0, and −7 kcal/mol, respectively.

When the temperature goes from below $T_x$ to above it, the crowder changes from being a destabilizer to a stabilizer.

All intermolecular interactions have hard-core repulsion. This repulsion between the test protein molecule and the surrounding crowder molecules leads to an entropic component favoring the folded state of the test protein (i.e., $\delta\Delta S < 0$), because the folded state is more compact and hence experiences less repulsion by the crowder molecules (Fig. 2A and B). This was predicted initially by theoretical models based on representing test protein and crowders as hard particles [14] and more recently by molecular simulations based on atomistic or coarse-grained representations [15–17]. (The hard-core repulsion could result in compaction of unfolded proteins, as observed in some experiments [18–20].) Potentially macromolecular crowders could behave like an entropic crowder. However, intermolecular interactions do not die out beyond the hard core; the soft part of the interactions is generally attractive. This soft part of interactions (also referred to as chemical interactions [12,21]) between a test protein molecule and crowder molecules is modeled in recent simulations [22–24]. As the unfolded state is more open and hence the residues are more accessible to crowder molecules, the soft part of interactions will lead to an enthalpic component that favors the unfolded state (i.e., $\delta\Delta H < 0$) (Fig. 2A and B). Therefore all macromolecular crowders are expected to exhibit the compound crowding behavior, with a crossover temperature at which, $\delta\Delta G$, the effect of crowding on the unfolding free energy, changes sign (Fig. 2C). Note that, according to Eq. (8), $T_x$ is increased by increasing the magnitude of $\delta\Delta H$ and decreased by increasing $\delta T_s$. A crossover temperature has also been predicted for the effects of crowding on protein binding stability [25].

## 3. Experimental evidence for crossover temperature

Recently Pielak and co-workers studied the temperature-dependent crowding effects of two polymers (PVP and Ficoll) and two proteins (lysozyme and BSA), all at 100 g/l, on the folding stability of ubiquitin [12]. Their results for all the four crowders, re-analyzed here (Fig. 3 and Supplementary Fig. SI), conform to the compound crowding behavior, with upshift in the maximum-stability temperature (signifying $\delta\Delta S < 0$) and crossing of the $\Delta G$-vs.-$T$ curves for dilute and crowded solutions (signifying $\delta\Delta H < 0$). For each crowder, the net effect is destabilizing below a crossover temperature but stabilizing above it. The crossover temperatures for PVP, Ficoll, lysozyme, and BSA are 48 °C, 28 °C, 24 °C, and 37 °C, respectively.

This brings us to a simple explanation for the opposite effects of the polymer and protein crowders observed by Pielak and co-workers on the CI2 stability [7–9]. We notice that the experiments with the polymer crowder (PVP) were done at a higher temperature, 37 °C, whereas the experiments with the lysozyme and BSA crowders were done at a lower temperature, 20 °C. As any crowder exhibiting the compound crowding behavior would have a stabilizing effect at some higher temperature and a destabilizing effect at some lower temperature, the difference in the experimental temperatures seems to be a significant contributing factor for the opposite crowding effects observed.

This conclusion is reinforced by a re-analysis (Fig. 4) of the limited temperature-dependent data of 100 g/l Ficoll crowding on CI2 published very recently [11]. The data is consistent with a 5 °C upshift in the maximum-stability temperature and a 3.8 kcal/mol decrease in $\Delta H$. These parameter values result in a crossover temperature of 12 °C, below which Ficoll is expected to become destabilizing.

The destabilization of CI2 by 100 g/l lysozyme at 20 °C was 0.6 kcal/mol [9]. No temperature dependent data are available for this crowder. As illustration, the 0.6 kcal/mol destabilization at 20 °C can be produced by a 10 °C upshift in the maximum-stability