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Effects of solution crowding on actin polymerization reveal the energetic basis for nucleotide-dependent filament stability

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Abstract

Actin polymerization is a fundamental cellular process involved in cell structure maintenance, force generation, and motility. Phosphate release from filament subunits following ATP hydrolysis destabilizes the filament lattice and increases the critical concentration (C_c) for assembly. The structural differences between ATP- and ADP-actin are still debated, as well as the energetic factors that underlie nucleotide-dependent filament stability, particularly under crowded intracellular conditions. Here, we investigate the effect of crowding agents on ATP- and ADP-actin polymerization, and find that ATP-actin polymerization is largely unaffected by solution crowding, while crowding agents lower the C_c of ADP-actin in a concentration-dependent manner. The stabilities of ATP- and ADP-actin filaments are comparable in the presence of physiological amounts (~30% w/v) and types (sorbitol) of low molecular weight crowding agents. Crowding agents act to stabilize ADP-F-actin by slowing subunit dissociation. These observations suggest that nucleotide hydrolysis and phosphate release *per se* do not introduce intrinsic differences in the *in vivo* filament stability. Rather, the preferential disassembly of ADP-actin filaments in cells is driven through interactions with regulatory proteins. Interpretation of the experimental data according to osmotic stress theory implicates water as an allosteric regulator of actin activity and hydration as the molecular basis for nucleotide-dependent filament stability.

INTRODUCTION

Most of biochemistry is performed in dilute solution ($< 1 \text{ mg ml}^{-1}$ protein) so that common physical and chemical formalisms that assume ideality can be applied to experimental analysis, yet eukaryotic cells contain high concentrations (on the order of $50\text{--}400 \text{ mg ml}^{-1}$) of various macromolecules in solution and in structured complexes and arrays. The effect of a crowded intracellular environment on biochemical reactions can be significant.^{1,2} To examine the effects of volume exclusion, researchers have used the aid of various “crowding agents”, also referred to as osmolytes, cosolutes, and cosolvents. The nature of these inert crowding agents varies from small molecules such as glycerol and betaine, to simple sugars such as sucrose and trehalose, to polymers such as dextrans and polyethylene glycols.

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The dynamic process of actin polymerization and depolymerization underlies diverse cellular processes including motility, contractility, intracellular transport, and the maintenance of cell shape.³ ATP hydrolysis and release of inorganic phosphate (P_i) drives actin filament dynamics by modulating filament stability and interaction with regulatory proteins. Actin monomers (G-actin) bound to either ATP or ADP can polymerize to form filamentous actin (F-actin), however ATP-actin has a >10-fold lower affinity (K_d) for filament barbed ends than does ADP-actin ($0.1 \mu\text{M}$ for Mg^{2+} -ATP-actin vs. $1.5 \mu\text{M}$ for Mg^{2+} -ADP-actin) under physiological ionic conditions⁴, and the monomeric pool of actin in cells is predominantly in the ATP state to allow for rapid filament assembly at the leading edge of cell protrusions⁵. Filament destabilization⁶ upon nucleotide hydrolysis and P_i release permits disassembly of filaments in cells and recycling of actin monomers⁷ and is the result of a conformational change in the constituent actin subunits.

The effect of crowding agents on the extent of Mg^{2+} -ATP-actin polymerization is negligible. Sucrose, glycerol, ethylene glycol, or dextran T-40 (MW = 40,000) do not affect the C_c (Ref 8). Glycerol, TMAO, glucose, sorbitol, glucopyranoside, or sucrose also have no detectable effects on the C_c of Mg^{2+} -ATP-actin⁹. The effects of solution crowding on Mg^{2+} -ADP-actin polymerization has not been evaluated.

There remains no consensus regarding the structural differences between ATP- and ADP-actin and how these contribute to filament energetics and assembly dynamics. All ATP- and ADP-actin structures have closed nucleotide binding clefts, except a profilin-ATP-actin structure,¹⁰ and disordered DNase I-binding loops, except TMR-ADP-actin.¹¹ However, given the differential binding of accessory proteins to ATP- and ADP-actin, conformational rearrangement of actin upon nucleotide hydrolysis and P_i release is anticipated. Molecular dynamics simulations^{12,13} and a crystal structure of chemically modified actin¹⁴ favor a nucleotide-dependent conformation of actin subdomain 2, but structural studies of non-polymerizable actin mutants¹⁵ challenge this prediction.

In this study, we investigate the effect of crowding agents on the C_c of ATP- and ADP-actin and show that crowding has significant impact on the difference in stability between the two nucleotide states of F-actin. We model our results according to several crowding theories, and in doing so, gain insight into the structural and functional differences between ADP- and ATP-actin, as well as the energetic basis for their different stabilities.

RESULTS

Effects of crowding agents on the C_c of actin

The concentration of Mg^{2+} -ADP-actin or Mg^{2+} -ATP-actin* monomers required to form filaments (i.e. the critical concentration for polymerization, C_c) was determined fluorometrically using pyrenyl-actin.¹⁶ Under our experimental conditions (50 mM KCl, 1 mM MgCl_2 , 1 mM EGTA, and 10 mM imidazole pH 7.0, 25°C), the C_c of ATP-actin is $0.13 \pm 0.08 \mu\text{M}$, and that of ADP-actin is $1.40 \pm 0.17 \mu\text{M}$ (Fig. 1 and Table 1; uncertainties represent standard deviation of the mean of >10 independent measurements), in agreement with previous measurements made under comparable conditions.^{4,17,18}

The effects of 15 crowding agents on the C_c of ADP-actin and ATP-actin were investigated. Glucose, galactose, sorbitol, sucrose, raffinose, stachyose, and trehalose are simple sugars; glycerol, TMAO, glycine, and betaine are small molecules known to regulate the osmotic balance of animal cells¹⁹; dextran 1500, PEG-MME 550, PEG 8000, and Ficoll 70 are

*From this point, we shall no longer specify the divalent metal associated with actin, as all our experiments were done with Mg^{2+} -actin.

polymers. Sorbitol and betaine are two of the predominant mammalian cell osmolytes examined in this study¹⁹.

None of the crowding agents examined affect the C_c for ATP-actin polymerization (Fig. 1) in accordance with previous studies^{8,9,20}. In contrast, all 15 agents lower the C_c of ADP-actin in a concentration-dependent manner (Fig. 1 & Supplementary Information). The C_c of ADP-actin is comparable to that of ATP-actin at the highest concentration (~1–2 molal, comparable to the small molecule solute concentration in vertebrate cells) of most crowding agents investigated, including sorbitol and betaine. This observation suggests that the stability of ADP-actin in crowded environments such as those found in animal cells does not largely differ from that of ATP-actin as implicated in models of *in vivo* actin dynamics derived from studies done in dilute solutions.

Theoretical interpretations of crowding-dependence of C_c

Excluded volume theories—A variety of theories have been formulated to explain the effects of crowding agents on biochemical equilibria. A subset of these theories (depletion attraction theory, covolume theory, and scaled particle theory) conceptualize crowding purely in terms of excluded volume effects; that is, they are based upon the thermodynamic consequences of having a reduced volume available to the reaction components due to the presence of the numerous crowding molecules. In general, these theories predict that crowding will favor states with a reduced surface area-to-volume ratio²¹; namely, it will induce more compact structures (folding) or self-association (dimerization, aggregation). These theories are independent of the chemical nature of species and treat all solution components as hard particles, usually spheres. Because the dimensions of ATP- and ADP-actin are comparable, excluded volume theories such as depletion attraction, covolume, and scaled particles (see Theory section) will predict identical effects for these two nucleotide states of actin and thus cannot account for the observed differential effect of crowding on the extent of ATP- vs. ADP-actin polymerization. In fact, the lack of a crowding effect on the C_c of ATP-actin is unanticipated by these hard particle theories (see Theory section). Therefore, theories other than those based on excluded volume (*i.e.* binding-based) are needed to explain the differential effects of crowding on ADP- and ATP-actin.

Binding-based theories—Crowding theories that consider the chemical nature of solute, solvent, and protein species account for the observed effects of crowding on the ADP-actin C_c , as well as the weak effect on the C_c for ATP-actin assembly. Osmotic stress theory²² (OST) is a binding-based theory that treats water as a participant in the reaction and, accordingly, crowding agents as dilutants of water. By modulating the chemical activity of water, as measured by changes in osmotic pressure, the linkage of water binding or release to macromolecular reactions can be assayed using Wyman's or Tanford's relations²³. OST therefore states that the variance of K with solution osmolality, Π , can be related to the number of excess water molecules, ΔN_{ew} , released or bound from the protein vicinity upon going from reactants to products:

$$\frac{\partial \ln K}{\partial \text{osmolality}} = - \frac{\Delta N_{ew}}{55.6} \quad (1)$$

We interpret the osmolyte-dependence of the ATP- and ADP-actin C_c 's according to OST (Fig. 2a). In our system, the “reactants” are an actin monomer and the end of an actin filament, and the “product” is an elongated actin filament, so ΔN_{ew} represents the loss or gain in hydration of an actin subunit upon going from monomer to polymer. We only analyzed the effects of crowding agents for which measured osmotic pressure data is available. We did not extrapolate beyond the range of available data because solution non-ideality can cause large discrepancies

between calculated ($\Pi = RTc$, where c is the molal solute concentration) and actual osmotic pressures at higher crowding agent concentrations (upwards of 2 molal).

The slope of $-\ln(K/K_0)$ vs. Π yields the change in the effective number of water molecules bound (ΔN_{ew}) to an actin subunit upon polymerization. The value of ΔN_{ew} approximates zero for ATP-actin, indicating no significant change in hydration of an ATP-actin monomer upon incorporation onto a filament end. In marked contrast, ΔN_{ew} is negative for ADP-actin independent of the crowding agent used (Fig. 2a), indicating that water is released upon ADP-actin filament elongation. The value of ΔN_{ew} for ADP-actin polymerization ranges from ~ 13 with betaine to >200 with stachyose. Actin filament bundling by even modest concentrations ($\sim 5\%$) of large PEGs and dextrans²⁴ precludes investigation of these high molecular weight polymers.

The effect of glycerol is not well-explained by OST, as indicated by a non-linear relationship between $-\ln(K/K_0)$ and Π (Fig. 2a). Non-linear behavior may arise from (i) two interconverting states of the macromolecular reactant or product, (ii) deformation of the macromolecule by high osmotic pressure, and/or (iii) inability to exclude solute at high osmotic pressure. Glycerol is known to bind some proteins²⁵ and may also interact directly with actin leading to deviation from the behavior predicted by OST.

The value of ΔN_{ew} associated with ADP-actin polymerization correlates with the molecular weight of all crowding agents examined (except for glycerol, Fig. 2b). This behavior is not predicted *a priori* by OST, but may arise because a larger number of actin-bound waters becomes inaccessible to the crowding agent as the size of the crowding agent increases, consistent with steric exclusion of the agents. In invoking this explanation, the crowding agent size-dependence of the number of ΔN_{ew} is predicted to plateau. However, this behavior was not observed, possibly because the size of crowding agent needed to attain such a plateau is above the range investigated here

Preferential interaction (PI) theory is a binding-based alternative to osmotic stress theory²⁶. The PI of a protein is a measure of its relative affinity for water and crowding agent (also referred to as cosolvent). PIs between a solvent component and a protein determine the effect of that cosolvent on processes & reactions involving that protein. This cosolvent effect can be expressed thermodynamically as the transfer free energy (TFE) of the protein from pure water to a cosolvent-containing medium²⁷ (see Methods). The effect of cosolvent on the free energy change ($\Delta\Delta G^\circ$) of a self-association equilibrium is therefore the difference between the TFE of a monomer in the context of a polymer and that of a monomer in solution:

$$\Delta\Delta G^\circ = \Delta\mu_{A,ir}^{filament} - \Delta\mu_{A,ir}^{monomer} \quad (2)$$

The TFE for a protein molecule can be calculated by summing the TFE's of its constituent amino acid side chains and backbone moieties, taking into consideration the solvent accessibility of each residue. Amino acid TFE data have been tabulated for 0.5 M sucrose, and 1.0 M sorbitol, TMAO, betaine, and sucrose.^{28,29} We have, therefore, limited our analysis to these cosolvent conditions. In addition, because the change in $\Delta\Delta G^\circ$ is approximately linear with m_C (supplementary information), we extrapolated our data to these cosolvent concentrations. The sources of the ADP-G-actin, ATP-G-actin, and F-actin structures used to generate the TFE data are given in Table 3. As the only atomic models available for actin filaments are for ADP-actin³⁰, we used the same model for both ADP- and ATP-actin filament calculations.

Comparison of the TFE calculations and the extrapolated $\Delta\Delta G^\circ$ values (Table 3) reveals that PI analysis does not predict nucleotide-dependent crowding effects on actin filament stability. It should be noted that the accuracy of these calculations is dependent on the correctness of the

structures chosen to represent reactant and product. We examined another filament³¹ model (data not shown), but it also could not account for the experimental data.

Molecular dynamics simulations

Molecular dynamics simulations of ATP- and ADP-actin monomers (see Methods) indicate equal hydration independent of the bound nucleotide throughout the 50-ns simulation (Fig. 3). No residues had a statistically significant difference in local solvent density between the two structures. This result indicates that changes in hydration must occur at the polymer level (see Fig. 5).

Effects of crowding agents on the kinetics of actin polymerization

To investigate the kinetic basis of ADP-actin stabilization by crowding agents, we measured by spinning-disc confocal microscopy the elongation rates of ATP- and ADP-actin in the presence and absence of crowding agents (Fig. 4). The actin subunit association (k_+) and dissociation (k_-) rate constants were obtained from the slope and y-intercept, respectively, of the best linear fits of the actin concentration-dependence of the observed barbed end elongation rate (k_{obs} in subunits sec^{-1} ; Fig. 4a). Observed elongation rates at the pointed end were also measured, but the large relative error³² prevented accurate analysis of crowding-induced changes. The biologically relevant crowding agent, sorbitol, slows association and dissociation of ATP- and ADP-actin subunits. Association rates for ATP- and ADP-actin are slowed to the same extent (~2 fold) by 16% w/v sorbitol presumably because of the increase in viscosity and corresponding reduction in translational diffusion coefficients⁸. The values of k_+ and k_- are affected to the same extent for ATP-actin, and so there is no net effect on the C_c , in accordance with previous measurements using similar crowding agents⁸. However, the k_- of ADP-actin is reduced to a greater extent than k_+ , and this accounts for the overall reduction of the C_c in the presence of sorbitol (Fig. 4b).

DISCUSSION

Implications for in vivo actin filament assembly and polymerization dynamics

Current models for actin polymerization dynamics are based on equilibrium and kinetic measurements made in dilute solution. Our results indicate that solution crowding significantly influences actin assembly and disassembly. Although the C_c , and therefore energetics, of ATP-actin are unaffected by crowding, ADP-actin is far more stable under crowded conditions than in dilute solutions. A conservative estimate of crowding in cells is 25% volume occupancy of non-solvent components². The C_c of ADP-actin is considerably lower under these conditions than in dilute solutions (Fig. 1).

Sorbitol, TMAO, and betaine are the crowding agents present in eukaryotic and mammalian cells, and they appear to be functionally interchangeable¹⁹, consistent with colligative properties of inert molecules. To estimate the stability of ADP-actin under physiological crowding conditions, we therefore examine results at ~25% volume occupancy of sorbitol (equivalent to 30% w/v). Under these conditions, ADP- and ATP-actin have comparable stabilities as evidenced by their nearly identical C_c (~0.1 μM for both ADP-actin and ATP-actin). This observation suggests that *in vivo*, the conformational change(s) that accompanies nucleotide hydrolysis and inorganic phosphate (P_i) release does not destabilize the actin filament lattice and promote disassembly of ADP-actin subunits as implicated by models of actin-based cell motility. Therefore, hydrolysis and P_i release serve a different role than to destabilize actin filaments.

The affinity of regulatory proteins for actin depends on the actin-bound nucleotide. ATP hydrolysis and P_i release would therefore demarcate aged filament so that they interact

differentially with regulatory proteins such as ADF/cofilin, Arp2/3, and profilin. That is, the functional differences between ATP- and ADP-F-actin would depend largely on nucleotide-dependent interactions with regulatory proteins. This prediction would imply that the binding of regulatory proteins is insensitive to or enhanced by crowding agents, which has been shown for cofilin³³, profilin³⁴, and thymosin β 4 (Ref 35).

Actin polymerization is a non-equilibrium system in the cell, and so rates of addition and dissociation of monomer subunits from filaments end are important in determining actin dynamics *in vivo*. The kinetic mechanism of stabilization of ADP-F-actin by crowding agents appears to be suppression of the dissociation of ADP-actin subunits (Fig. 4). This suggests an even greater role for co-factor proteins in the disassembly of actin filaments *in vivo*, as turnover of actin subunits from existing filaments is essential for maintaining a pool of actin monomers for rapid filament assembly at the leading edge of cells³.

The crowded nature of the cell is not due to the overabundance of any one molecule but rather the interplay of numerous species of diverse size and chemical nature. The experiments in this work are done with individual crowding agents and therefore mixtures of crowding agents may have different effects. However, the observation that ADP-actin is stabilized by crowding agents of diverse size and chemical nature (small charged molecules, polar sugars, and large aliphatic polymers) and none appear to destabilize ADP-actin (i.e. increase C_c) suggests that mixtures will have a similar impact on actin filament stability.

Consideration of crowding theories

The effect of crowding is generally thought of as non-specific volume exclusion or confinement, resulting in aggregation, folding, and adsorption³⁶. This view considers the impact of crowding from the standpoint of the chemical activity of the macromolecular species of interest. One can also take into account the influence of volume occupancy on the chemical activity of water, which can be thought of as specific effects dependent on the chemical nature of both the crowding species and the macromolecule of interest. It has been suggested that in terms of water behavior, the cell behaves more like a protein crystal than dilute solution³⁷. While both non-specific excluded-volume and specific water activity-based crowding effects undoubtedly play a role *in vivo*, studies tend to consider only one when drawing conclusions on how crowding impacts the biological process under investigation. Here, we consider both excluded volume and binding-based theories in interpreting our results.

Excluded volume-based theories such as depletion attraction, covolume, and scaled particle theories, cannot explain the nucleotide-dependent effect of crowding agents on actin polymerization because the protein-dependent parameter in these theories – macromolecule size (see Theory section) – is essentially identical for ATP- and ADP-actin. Binding-based theories, such as OST and preferential interaction, are capable of accounting for the different response to crowding agents observed for ATP- and ADP-actin. These theories are sensitive to alterations in the chemical nature of reacting species, in particular smaller conformational changes that result in differences in hydration energies.

Preferential interaction effects are calculated from the change in solvent-exposure of protein side chain residues, which requires detailed structural knowledge of both reactants and products. As the impact of nucleotide and assembly state on actin conformation is still debated, application of preferential interaction to the process of actin polymerization is limited in its utility. It is therefore not surprising that PI is unable to predict the differential effects of crowding on ATP- vs. ADP-actin. Moreover, this result suggests that the conformation of actin subunits differs between ATP- and ADP-actin filaments.

Nucleotide-linked differences in hydration

Interpretation of the data according to OST implies that water is released upon elongation of an ADP-actin filament, while no water is released upon ATP-actin assembly (Fig. 2a). The larger entropic contributions to the energetics of ADP-actin filament assembly than for ATP-actin filament assembly ($T\Delta S^\circ$ for Mg^{2+} -ADP-actin is $13.1 \text{ kcal mol}^{-1}$ and that of Mg^{2+} -ATP-actin is $10.9 \text{ kcal mol}^{-1}$)³⁸ supports the notion of a greater change in the number solvent interactions in ADP- vs. ATP-actin assembly. The observation that dissociation of terminal ADP-actin subunits is slowed by crowding implicates assembly-linked conformational rearrangements of the ADP-actin subunit, which are likely be accompanied by changes in hydration.

Calculation of hydration differences between ATP- and ADP-actin monomers from MD simulations (Fig. 3) suggests that these species interact with solvent to a similar degree. Taking this result together with OST analysis, we can construct a thermodynamic cycle of actin's nucleotide-dependent water associations which suggests that ADP-actin filaments are less hydrated than ATP-actin filaments (Fig. 5). Differences in hydration energies between the two nucleotide states of actin filaments can account for their different stabilities in dilute solution and their discrepant responses to crowding. Water therefore acts as an allosteric regulator of actin activity.

Implications for actin monomer structure

A structural difference between ATP- and ADP-actin is proposed to be located in the D-loop of subdomain 2, which is thought to be unstructured in ATP-actin and a helix in ADP-actin¹⁴. It is unlikely that this region is responsible for the differential effects of crowding agents on ATP- and ADP-actin polymerization because surface area considerations would predict this region to be more hydrated in ATP-actin, as an unstructured loop exposes more surface area than a structured one. If this were the case, ATP-actin would release more water than ADP-actin upon polymerization, assuming burial of the D-loop in both filament structures³⁰. However, this is not observed and therefore, some other region or regions must be the source of the nucleotide-dependent differences in hydration.

Another structural difference thought to exist between ADP- and ATP-actin is the conformation (open vs. closed) of the nucleotide binding cleft. Given actin's homology to other ATP-binding proteins (hexokinase, Arp2/3), nucleotide cleft would be closed in the ATP state and open in the ADP-state. This has been observed in one crystal structure of actin, and there is limited biochemical data to support it. Dehydration of the nucleotide cleft could result in the release of ~60 waters based on changes in surface area (data not shown), which is within the range of ΔN_{ew} values observed for ADP-actin assembly (Table 2). The ability of covolume theory, which models actin as a hard sphere, to predict the effects of crowding on ADP-actin (manuscript in preparation) does not support the idea that ADP-actin undergoes a large conformational change upon polymerization. It is possible that the magnitude of change in shape and size in actin accompanying cleft closure is not great enough to impact packing considerations, thereby implicating other regions of the actin molecule³⁹.

THEORY

Depletion attraction

Depletion attraction arises when two large particles amongst a background of numerous smaller particles come within a distance equal to the diameter of the smaller particle to each other, such that the volume excluded to the smaller particles by each of the large particles overlaps. This results in an overall increase in the volume available to the smaller particles, and thereby an

increase the⁴² entropy of the system and an overall reduction in the free energy⁴⁰. Asakura and Oosawa⁴¹ formulated a theory that permits estimation of the attractive depletion force:

$$\frac{\Delta G^{0'}}{k_B T} = \left(1 + \frac{3r_A}{2r_C}\right) \varphi \quad (3)$$

where $\Delta G^{0'}$ is the free energy gained when two large particles associate, r_A and r_C are the radii of the large and small spheres, respectively, and φ is the volume fraction occupied by the smaller spheres. Depletion attraction theory predicts that crowding agents will lower the C_c of ATP- and ADP-actin as polymerization results in a decrease in the volume excluded to the crowding agents.

Covolume theory

Covolume analysis relates the combined volume of protein and crowding agent (i.e. the covolume) to the second virial coefficient of the protein, which allows for the prediction of the extent to which equilibrium will be perturbed by the crowding agent. The effect on the equilibrium constant of a protein association reaction is a function of the covolume increment ($\Delta V_{\text{covolume}}$) and of the molar volume of the crowding agent ($M_C \bar{v}_C$) according to:

$$\frac{\partial \ln K}{\partial m_C} = \Delta V_{\text{covolume}} - M_C \bar{v}_C \quad (4)$$

The covolume increment, defined as the difference in covolume between product and reactant states, for the end-to-end association of a spherical monomer and a spherocylindrical polymer is:⁴³

$$\Delta V_{\text{covolume}} = -\frac{2\pi N_A}{3} r_C^2 (3r_A + 3r_C) \quad (5)$$

where r_A is the radius of actin, r_C is the radius of the solute, and N_A is Avogadro's number. Thus, as there is a decrease in the covolume ($\Delta V_{\text{covolume}}$) of an actin subunit upon addition to a filament end, covolume theory predicts that the affinity (K) of ATP- and ADP-actin monomers for filament ends will increase in the presence of crowding agents.

Scaled particle theory

Scaled particle theory⁴⁴ (SPT) computes the work required to insert a test particle into a hard-sphere fluid. The activity coefficient of the test particle (γ) is related to the sizes, shapes, and densities of the species present in the fluid. Modeling all species as spheres and treating water as a continuum solvent,⁴⁵ the activity coefficient for an actin monomer in a solution of crowding agent can be expressed as:

$$\ln \gamma_A = -\ln(1 - S_3) + r_A \left(\frac{6S_2}{1-S_3} \right) + r_A^2 \left(\frac{12S_1}{1-S_3} + \frac{18S_2^2}{(1-S_3)^2} \right) + r_A^3 \left(\frac{8S_0}{1-S_3} + \frac{24S_1S_2}{(1-S_3)^2} + \frac{24S_2^3}{(1-S_3)^3} \right) \quad (6)$$

Each term of the form S_j ($0 \leq j \leq 3$) is given by:

$$S_j = \frac{\pi}{6} \rho_C (2r_C)^j \quad (7)$$

where ρ_C is the number density of crowding agent, which is equal to $N_A m_C$. We assume that the activity of an actin filament end is unchanged upon elongation and therefore that the effect of the crowding agent on the standard free energy change of the actin elongation reaction is:

$$\Delta \Delta G^{0'} = -RT \ln \gamma_A \quad (8)$$

Therefore, SPT predicts that the activity coefficient for an ATP- or ADP-actin monomer will decrease in the presence of crowding agent, and consequently, ATP- and ADP-actin filament formation will be favored.

Osmotic Stress

The Gibbs-Duhem equation allows us to relate the change in chemical potential of the macromolecule, M , to changes in the chemical potentials of water and cosolvent (crowding agent):

$$d\mu_M = -n_s d\mu_s - n_w d\mu_w \quad (9)$$

where n_s and n_w are the number of solute and water molecules in the system. If we consider that such a relation exists both in the local domain of the protein and in bulk solution, a change in μ_M with addition of solute s can be expressed as:

$$d\mu_M = - \left[N_w - N_s \left(\frac{n_w}{n_s} \right) \right] d\mu_w = - N_w \left[1 - \left(\frac{N_s}{N_w} \right) \left(\frac{n_w}{n_s} \right) \right] d\mu_w \quad (10)$$

where n_s and n_w are now the number of solute and water molecules in bulk solution, and N_s and N_w are the number of solute and water molecules in the local domain of the protein. We can define the number of *excess* waters, N_{ew} :

$$N_{ew} = - N_w \left[1 - \left(\frac{N_s}{N_w} \right) \left(\frac{n_w}{n_s} \right) \right] \quad (11)$$

and the change in macromolecular chemical potential ($d\mu_M$) is thus:

$$d\mu_M = N_{ew} d\mu_w \quad (12)$$

Water activity is related to osmotic pressure, Π , by the molecular volume of water: $\bar{V}_w d\Pi = d\mu_w$, and $\Pi = (kT/\bar{v}_w)(\text{osmolality}/55.6)$.

It is important to note that the dependence of the equilibrium constant ($-kT \ln K = \Delta\mu_M$) on osmotic pressure gives us the change in the number of *excess* waters in the local domain of the protein, not the *absolute* number. Also, the magnitude of N_{ew} will depend on the extent to which the ratio N_s/N_w differs from that in bulk solution, n_s/n_w – i.e. the degree of exclusion of cosolvent from protein. Thus, N_{ew} for solutes that are *not* completely excluded represents a lower bound on the change in hydration. Nevertheless, this formalism can still be used to reliably assess changes in protein hydration and surface area⁴⁶.

Preferential interaction

Preferential interactions between a solution component and protein determine that cosolvent's effect on protein stability, solubility, and reactivity. This effect is expressed thermodynamically as the transfer free energy of the protein ($\Delta\mu_{A,tr}$) from pure water to a cosolvent-containing medium,²⁷ according to:

$$\Delta\mu_{A,tr} = \int_0^{m_C} \left(\frac{\partial \mu_A}{\partial m_C} \right) dm_C = \mu_A^{m_C} - \mu_A^w \quad (13)$$

where the superscript m_C refers to a cosolvent solution of molality m_C , the superscript w refers to pure water, and the integral is performed under constant temperature, pressure, and protein concentration. Therefore, the effect of cosolvent on the free energy change ($\Delta\Delta G^{o'}$) of the actin self-association equilibrium is the difference between the transfer free energy of a monomer in the context of a filament and that of a monomer in solution:

$$\Delta\Delta G^{o'} = \Delta\mu_{A,tr}^{filament} - \Delta\mu_{A,tr}^{monomer} \quad (14)$$

The TFE for a protein molecule is calculated by summing the TFE's of its constituent amino acid side chains and backbone moieties:²⁸

$$\Delta\mu_{A,ir} = \sum_{aa=Ala}^{aa=Val} \sum_i^{n_{aa}} \alpha_i^{sc} \Delta g_{ir,aa}^{sc} + \Delta g_{ir}^{bb} \sum_{aa=Ala}^{aa=Val} \sum_i^{n_{aa}} \alpha_i^{bb} \quad (15)$$

where n_{aa} is the number of amino acids of type aa , α_i is the fractional solvent accessibility of residue i , $\Delta g_{ir,aa}$ is the TFE of an amino acid of type aa , and the superscripts sc and bb refer to side chain and backbone, respectively.

METHODS

Materials

All chemicals and reagents were of the highest purity commercially available. ATP and ADP were purchased from Sigma (St. Louis, MO.) Crowding agents (galactose, glucose, raffinose, sucrose, stachyose, Dextran1500, and Ficoll70) were purchased from Sigma or JT Baker (Phillipsburg, NJ) and dissolved in water. 0.1 volumes of 10X buffer was added to yield a final solution of 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 10 mM imidazole pH 7.0.

Purification and Labeling of Actin

Actin was purified from rabbit skeletal muscle,⁴⁷ labeled with pyrenyl-iodoacetamide⁴⁸ or Alexa Fluor 488 carboxylic acid succinimidyl ester (Molecular Probes, Eugene, OR)³², and gel-filtered at 4°C over Sephacryl S-300HR equilibrated with G-buffer (0.2 mM ATP, 0.1 mM CaCl₂, 0.5mM dithiothreitol, 1 mM NaN₃, 2 mM Tris-HCl, pH 8.0). Actin concentrations were determined by absorbance at 290 nm and corrected for labeling.

Equilibrium sample preparation

Ca²⁺-ATP-actin was converted to Mg²⁺-ADP-actin by incubation on ice with 80 μM MgCl₂ and 200 μM EGTA for 3 min to exchange divalent cation,¹⁷ followed by addition of 20 U mL⁻¹ of hexokinase, 200 μM ADP, and 1 mM glucose and incubation at 4 °C for 2–3 hours.³⁵ Polymerization was initiated by addition of 10X buffer to yield a final solution of 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 10 mM imidazole (pH 7.0) in Buffer A. A concentrated (25 μM) stock of Mg²⁺-ADP-actin (35% pyrene labeled) was allowed to polymerize for 1 hour at room temperature before being diluted to a series of desired final concentrations (0.5–5 μM). These diluted samples were briefly sonicated in a water bath and allowed to equilibrate for ~ 4 hours. Attainment of equilibrium was assessed by verifying that the critical concentration did not change after over-night incubation. Results were independent of the percent of pyrene-actin (data not shown).

Fluorimetry and data analysis

The fluorescence intensities (FI) of equilibrated F-actin samples were measured at 22°C with a Molecular Devices Spectra Max Gemini XPS (Sunnyvale, CA) plate reader at an excitation wavelength of 366 nm and an emission wavelength of 407 nm. FIs were corrected by subtracting the intensity value of buffer. The critical concentration (C_c) for assembly was determined from the x -intercept of the best fit of the highly fluorescent samples (i.e. those containing F-actin) to a linear function. This assumes an infinitely sharp transition between the regime of monomer (low pyrene fluorescence) and the regime of polymer (high pyrene fluorescence), which is the case for highly cooperative helical polymers such as actin.⁴⁹

Calculation of Solvent-Accessible Surface Area (ASA)

ASA calculations of the monomeric ADP-actin crystal structure (accession code 1J6Z) and of the Holmes model of the actin filament³⁰ were performed using the GETAREA server (http://pauli.utmb.edu/cgi-bin/get_a_form.tcl),⁵⁰ with a user-defined atomic library for ADP and HMS (methylated histidine). Coordinates for the actin filament were downloaded from the FTP server 149.217.48.3. A subunit from the interior of the filament was used for the filament transfer free energy calculations, as it has all relevant contacts with neighboring subunits. For the free monomer structure, the rhodamine molecule was removed; the “A” conformations of Met132, Ser265, and Ser348 were used; and the ASA data for residues 1-4 and 373-375, which are unstructured or truncated in 1J6Z, were taken from calculations performed on a filament subunit in isolation, which has these residues built back in.

Molecular dynamics calculations

Molecular dynamics simulations were carried out for actin monomers with bound ADP or ATP.¹³ Since these simulations were performed in explicit solvent, the nucleotide-dependent interaction of water with actin could be evaluated. To assess the differences in hydration between ADP- and ATP-actin, we quantitated the solvent density within the first solvation shell surrounding each amino acid residue. For the solvent model used (TIP3P),⁵¹ the first minimum in the radial distribution function occurs at 3.4 Å. We used all non-hydrogen atoms for each residue in the protein and calculated the total number of water molecules within this 3.4 Å cutoff. These values were averaged over the 50 ns time course of the simulations for ADP- and ATP-actin, yielding a mean number of water molecules and standard deviation for each amino acid in actin. We applied a t-test to determine significant differences between the two nucleotide states and identified 16 amino acid residues with a p-value < 5×10⁻⁵.

Analysis of actin polymerization kinetics

Mg²⁺-ADP-actin was prepared as described above, except it was spun at ~250,000 g for 55 min at 4°C prior to use in microscopy experiments to remove pre-formed nuclei. This was done purely to improve the quality of images and did not impact the observed rate of growth (data not shown). Ca²⁺-ATP-actin was converted to Mg²⁺-ATP-actin by incubation on ice with 100 μM MgCl₂, 200 μM EGTA for 1 min. Samples contained 30% Alexa actin. Experiments were carried out in flow chambers⁵² treated with 10–30 nM NEM-myosin II and washed with 1% BSA prior to use. Actin polymerization was initiated by mixing with polymerization buffer in the presence or absence of crowding agent at room temperature, to yield a final solution of 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 0.2 mM ADP or ATP, 10mM imidazole (pH 7.0), 100 mM DTT, 0.1 mg mL⁻¹ glucose oxidase, 0.02 mg mL⁻¹ catalase, 15 mM glucose, and 0.5% w/v methylcellulose. The polymerization reaction was immediately passed into the flow chamber; nucleation occurred spontaneously within 10 min for all samples.

The presence of crowding agents altered the index of refraction of solutions and precluded the use of evanescent wave illumination, which is typically employed to observe single actin filaments. Instead, the growth of single actin filaments was observed on an inverted IX-71 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a spinning-disc confocal unit (UltraView RS, Perkin Elmer) and a 100X/1.4 NA objective (Plan-Apo). Excitation was from a 488-nm argon-ion laser, and images were acquired for 100 ms on a charge-cooled CCD camera (ORCA-ER, Hamamatsu) at a resolution of 7.4 pixels per μm. Analysis of filament dynamics was performed in Image J using plug-ins kindly provided by Dr. T.D. Pollard⁵².

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ASA	accessible surface area
F-actin	filamentous actin
G-actin	monomeric actin
TFE	transfer free energy

References

1. Ellis RJ. Macromolecular crowding: Obvious but underappreciated. *Trends Biochem Sci* 2001;26:597–604. [PubMed: 11590012]
2. Ellis RJ, Minton AP. Cell biology: Join the crowd. *Nature* 2003;425:27–28. [PubMed: 12955122]
3. Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 2003;112:453–465. [PubMed: 12600310]
4. Pollard TD. Rate constants for the reactions of atp- and adp-actin with the ends of actin filaments. *J Cell Biol* 1986;103:2747–2754. [PubMed: 3793756]
5. Welch MD, Mallavarapu A, Rosenblatt J, Mitchison TJ. Actin dynamics in vivo. *Curr Opin Cell Biol* 1997;9:54–61. [PubMed: 9013669]
6. Orlova A, Egelman EH. Structural basis for the destabilization of f-actin by phosphate release following atp hydrolysis. *J Mol Biol* 1992;227:1043–1053. [PubMed: 1433285]
7. Theriot JA. The polymerization motor. *Traffic* 2000;1:19–28. [PubMed: 11208055]
8. Drenckhahn D, Pollard TD. Elongation of actin filaments is a diffusion-limited reaction at the barbed end and is accelerated by inert macromolecules. *J Biol Chem* 1986;261:12754–12758. [PubMed: 3745211]
9. Fuller N, Rand RP. Water in actin polymerization. *Biophys J* 1999;76:3261–3266. [PubMed: 10354451]
10. Chik JK, Lindberg U, Schutt CE. The structure of an open state of [beta]-actin at 2.65 Å resolution. *J Mol Biol* 1996;263:607–623. [PubMed: 8918942]
11. Dominguez R, Graceffa P. Solution properties of tmr-actin: When biochemical and crystal data agree. *Biophys J* 2003;85:2073–2074. [PubMed: 14507675]
12. Chu JW, Voth GA. Allostery of actin filaments: Molecular dynamics simulations and coarse-grained analysis. *Proc Natl Acad Sci USA* 2005;102:13111–13116. [PubMed: 16135566]
13. Zheng X, Diraviyam K, Sept D. Nucleotide effects on the structure and dynamics of actin. *Biophys J* 2007;93:1277–1283. [PubMed: 17526584]
14. Otterbein LR, Graceffa P, Dominguez R. The crystal structure of uncomplexed actin in the adp state. *Science* 2001;293:708–711. [PubMed: 11474115]
15. Rould MA, Wan Q, Joel PB, Lowey S, Trybus KM. Crystal structures of expressed non-polymerizable monomeric actin in the adp and atp states. *J Biol Chem* 2006;281:31909–31919. [PubMed: 16920713]

16. Cooper JA, Walker SB, Pollard TD. Pyrene actin: Documentation of the validity of a sensitive assay for actin polymerization. *J Muscle Res Cell Motil* 1983;4:253–262. [PubMed: 6863518]
17. Gershman LC, Selden LA, Kinoshian HJ, Estes JE. Preparation and polymerization properties of monomeric adp-actin. *Biochim Biophys Acta* 1989;995:109–115. [PubMed: 2930792]
18. Lal AA, Brenner SL, Korn ED. Preparation and polymerization of skeletal muscle adp-actin. *J Biol Chem* 1984;259:13061–13065. [PubMed: 6490645]
19. Yancey PH. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology* 2005;208:2819–2830. [PubMed: 16043587]
20. De La Cruz EM, Mandinova A, Steinmetz MO, Stoffler D, Aebi U, Pollard TD. Polymerization and structure of nucleotide-free actin filaments. *J Mol Biol* 2000;295:517–526. [PubMed: 10623543]
21. Minton AP. Influence of macromolecular crowding upon the stability and state of association of proteins: Predictions and observations. *J Pharm Sci* 2005;94:1668–1675. [PubMed: 15986476]
22. Parsegian VA, Rand RP, Fuller NL, Rau DC. Osmotic stress for the direct measurement of intermolecular forces. *Method Enzymol* 1986;127:400–416.
23. Tanford C. Extension of theory of linked functions to incorporate effects of protein hydration. *J Mol Biol* 1969;39:539. [PubMed: 5357211]
24. Suzuki A, Yamazaki M, Ito T. Osmoelastic coupling in biological structures: Formation of parallel bundles of actin filaments in a crystalline-like structure caused by osmotic stress. *Biochemistry* 1989;28:6513–6518. [PubMed: 2790010]
25. Na GC, Timasheff SN. Interaction of calf brain tubulin with glycerol. *J Mol Biol* 1981;151:165–178. [PubMed: 7328652]
26. Timasheff SN. In disperse solution, “Osmotic stress” Is a restricted case of preferential interactions. *Proc Natl Acad Sci USA* 1998;95:7363–7367. [PubMed: 9636154]
27. Timasheff SN. The control of protein stability and association by weak interactions with water: How do solvents affect these processes? *Annu Rev Biophys Biomol Struct* 1993;22:67–97. [PubMed: 8347999]
28. Liu Y, Bolen DW. The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes. *Biochemistry* 1995;34:12884–12891. [PubMed: 7548045]
29. Auton M, Bolen DW. Predicting the energetics of osmolyte-induced protein folding/unfolding. *Proc Natl Acad Sci USA* 2005;102:15065–15068. [PubMed: 16214887]
30. Lorenz M, Popp D, Holmes KC. Refinement of the f-actin model against x-ray fiber diffraction data by the use of a directed mutation algorithm. *J Mol Biol* 1993;234:826–836. [PubMed: 8254675]
31. Holmes KC, Angert I, Kull FJ, Jahn W, Schroder RR. Electron cryo-microscopy shows how strong binding of myosin to actin releases nucleotide. *Nature* 2003;425:423–427. [PubMed: 14508495]
32. Fujiwara I, Vavylonis D, Pollard TD. Polymerization kinetics of adp- and adp-pi-actin determined by fluorescence microscopy. *Proc Natl Acad Sci USA* 2007;104:8827–8832. [PubMed: 17517656]
33. Cao W, Goodarzi JP, De La Cruz EM. Energetics and kinetics of cooperative cofilin-actin filament interactions. *J Mol Biol* 2006;361:257–267. [PubMed: 16843490]
34. Vinson VK, De La Cruz EM, Higgs HN, Pollard TD. Interactions of *acanthamoeba* profilin with actin and nucleotides bound to actin. *Biochemistry* 1998;37:10871–10880. [PubMed: 9692980]
35. De La Cruz EM, Ostap EM, Brundage RA, Reddy KS, Sweeney HL, Safer D. Thymosin-beta(4) changes the conformation and dynamics of actin monomers. *Biophys J* 2000;78:2516–2527. [PubMed: 10777749]
36. Minton AP. Molecular crowding: Analysis of effects of high concentrations of inert cosolutes on biochemical equilibria and rates in terms of volume exclusion. *Method Enzymol* 1998;295:127–149.
37. Fulton AB. How crowded is the cytoplasm? *Cell* 1982;30:345–347. [PubMed: 6754085]
38. Kinoshian HJ, Selden LA, Estes JE, Gershman LC. Thermodynamics of actin polymerization; influence of the tightly bound divalent cation and nucleotide. *BBA - Protein Struct M* 1991;1077:151–158.
39. Guan JQ, Takamoto K, Almo SC, Reisler E, Chance MR. Structure and dynamics of the actin filament. *Biochemistry* 2005;44:3166–3175. [PubMed: 15736927]
40. Marenduzzo D, Finan K, Cook PR. The depletion attraction: An underappreciated force driving cellular organization. *J Cell Biol* 2006;175:681–686. [PubMed: 17145959]

41. Asakura S, Oosawa F. Interaction between particles suspended in solutions of macromolecules. *J Polym Sci* 1958;33:183–192.
42. Shearwin KE, Winzor DJ. Effect of sucrose on the dimerization of alpha-chymotrypsin. Allowance for thermodynamic nonideality arising from the presence of a small inert solute. *Biophys Chem* 1988;31:287–294. [PubMed: 3233302]
43. Tellam RL, Sculley MJ, Nichol LW, Wills PR. The influence of poly(ethylene glycol) 6000 on the properties of skeletal-muscle actin. *Biochem J* 1983;213:651–659. [PubMed: 6412688]
44. Lebowitz JL, Helfand E, Praestgaard E. Scaled particle theory of fluid mixtures. *J Chem Phys* 1965;43:774–779.
45. Minton AP. Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers* 1981;20:2093–2120.
46. Courtenay ES, Capp MW, Anderson CF, Record MT. Vapor pressure osmometry studies of osmolyte-protein interactions: Implications for the action of osmoprotectants in vivo and for the interpretation of “Osmotic stress” Experiments in vitro. *Biochemistry* 2000;39:4455–4471. [PubMed: 10757995]
47. Spudich JA, Watt S. The regulation of rabbit skeletal muscle contraction. I Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J Biol Chem* 1971;246:4866–4871. [PubMed: 4254541]
48. De La Cruz EM. Cofilin binding to muscle and non-muscle actin filaments: Isoform-dependent cooperative interactions. *J Mol Biol* 2005;346:557–564. [PubMed: 15670604]
49. Oosawa, F.; Asakura, S. *Molecular biology, an international series of monographs and textbooks.* Academic Press; New York: 1975. *Thermodynamics of the polymerization of protein.*
50. Fraczkiewicz R, Braun W. Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J Comput Chem* 1998;19:319–333.
51. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J Chem Phys* 1983;79:926–935.
52. Kuhn JR, Pollard TD. Real-time measurements of actin filament polymerization by total internal reflection fluorescence microscopy. *Biophys J* 2005;88:1387–1402. [PubMed: 15556992]

Figure 1. Effect of crowding agents on the critical concentration of ADP-actin and ATP-actin
 Black squares represent actin in the absence of crowding agent. Solid symbols and lines represent ADP-actin data and fits, respectively; open symbols and dotted lines represent ATP-actin data and fits, respectively. Concentrations of crowding agent are given in % w/v.

Figure 2. Osmotic stress theory

The slope of the change in equilibrium constant in the presence of crowding agent ($-\ln(K/K_0)$) plotted versus solution osmotic pressure (Π) yields information on the change in number of waters (ΔN_{ew}) bound or released. (a) ADP-actin data analyzed according to OST. (b) ATP-actin data analyzed according to OST. (c) N_{ew} as calculated from OST for ADP-actin (black circles) and ATP-actin (red circles) plotted as a function of crowding agent molecular weight. Line display the inear trend of the data.

Figure 3. Molecular Dynamics Simulations

Number of waters molecules(N_w) associated with ATP-actin (red) and ADP-actin (black).

Figure 4. Effect of crowding agents on actin polymerization kinetics

Top, effect of 16% w/v sorbitol (open symbols) on the concentration-dependence of observed elongation rate (k_{obs}) at the barbed end of ADP-actin (black) and ATP-actin (red) filaments. Behavior in dilute buffer solution (closed symbols) is shown for comparison. The slope of such a graph is k_+ and the y-intercept is k_- . *Bottom*, comparison of the relative viscosity of the sorbitol solutions (η/η_0), the reciprocals of the relative rates of association (k_+^0/k_+) and dissociation (k_-^0/k_-) for ATP- and ADP-actin.

Figure 5. Thermodynamic cycle of ΔN_{ew} in the presence of sorbitol (16% w/v)

Depicted is the thermodynamic cycle of coupled nucleotide hydrolysis and actin polymerization. D, ADP; T, ATP; G, G-actin (monomer); F, F-actin (filament). ΔN_{ew} , the difference in number of effective bound waters between two neighboring states, is shown for the outer arrow of the reaction cycle. The values of ΔN_{ew} for D-G → D-F and T-G → T-F were obtained from OST analysis. The value of ΔN_{ew} for D-G → T-G was obtained from MD simulations. The value of ΔN_{ew} for T-F → D-F, in red, was calculated from the thermodynamic cycle.

Table 1Effect of crowding agents on the C_c of ATP- and ADP-actin.

Solute ^a	C_c (μ M)	
	ADP	ATP
Buffer	1.40 $\pm$ 0.17	0.13 $\pm$ 0.08
Betaine		
11	0.98 $\pm$ 0.08	
21	0.77 $\pm$ 0.07	
31	0.48 $\pm$ 0.07	
42	0.45 $\pm$ 0.03	0.26 $\pm$ 0.03
Sorbitol		
7.5	0.753 $\pm$ 0.06	
15	0.474 $\pm$ 0.04	0.13 $\pm$ 0.01
23	0.236 $\pm$ 0.03	0.10 $\pm$ 0.03
30	0.134 $\pm$ 0.02	
Glucose		
11	1.07 $\pm$ 0.04	
22	0.64 $\pm$ 0.03	
34	0.32 $\pm$ 0.07	
45	0.20 $\pm$ 0.02	0.24 $\pm$ 0.09
Sucrose		
15	1.06 $\pm$ 0.06	
25	0.87 $\pm$ 0.13	
35	0.61 $\pm$ 0.11	
45	0.35 $\pm$ 0.05	0.047 $\pm$ 0.03
Ficoll 70		
4	1.39	
8	0.94	
12	0.71	
16	0.48	
PEG 8000		
2	0.57 $\pm$ 0.05	
6	0.32 $\pm$ 0.10	
8	0.21 $\pm$ 0.07	
11	0.20 $\pm$ 0.14	

^a Solute concentrations are given in % w/v.

Table 2

Results of OST analysis

C.A. ^a	MW(g)	ADP	ΔN_{EW}	ATP
Glycine	75.07	-74.5 ± 1.9		-11.4 ± 0.2
glycerol	92.1	-14.6 ± 2.5		-0.80 ± 0.56
Betaine	117.5	-13.1 ± 1.0		
glucose	180	-34.6 ± 1.7		-2.4 ± 10
galactose	180	-38.3 ± 1.3		
sorbitol	182	-52.1 ± 2.2		10.4 ± 6.4
trehalose	342.3	n/a		-2.9 ± 1.8
sucrose	343	-46.3 ± 6.3		
raffinose	505	-95.7 ± 12.2		
PEG-MME	550	-116 ± 9.5		
stachyose	667	-222 ± 18		

^a C.A., crowding agent.

Table 3

Results of preferential interaction analysis.

		0.5 M sucrose		1.0 M sucrose		$\Delta \Delta G^{\circ}$ (kcal mol ⁻¹) 1.0 M sorbitol		1.0 M TMAO		1.0 M betaine	
		<i>calc</i>	<i>obsd</i>	<i>calc</i>	<i>obsd</i>	<i>calc</i>	<i>obsd</i>	<i>calc</i>	<i>obsd</i>	<i>calc</i>	<i>obsd</i>
ADP	<i>calc</i>	-0.26		-0.43		-0.20		-0.37		0.28	
	<i>obsd</i>	-0.42		-0.87		-1.12		-1.55		-0.25	
ATP	<i>calc</i>	-0.30		-0.49		-0.27		0.38		2.05	
	<i>obsd</i>	0.0		0.0		0.0		0.0		0.0	