Macromolecular crowding: an important but neglected aspect of the intracellular environment

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Biological macromolecules have evolved over billions of years to function inside cells, so it is not surprising that researchers studying the properties of such molecules, either in extracts or in purified form, take care to control factors that reflect the intracellular environment, such as pH, ionic strength and composition, redox potential and the concentrations of relevant metabolites and effector molecules. There is one universal aspect of the cellular interior, however, that is largely neglected – the fact that it is highly crowded with macromolecules. It is proposed that the addition of crowding agents should become as routine as controlling pH and ionic strength if we are to meet the objective of studying biological molecules under more physiologically relevant conditions.

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Current Opinion in Structural Biology 2001, 11:114–119

0959-440X/01/$ – see front matter

Abbreviations
BSA bovine serum albumin
FITC fluorescein isothiocyanate
GFP green fluorescent protein

Introduction

Macromolecular crowding presents an unusual combination of features:

1. It is ubiquitous, occurring in the interiors of all types of cell.

2. It is predicted by theory to have large effects on the interactions between all types of macromolecule, with consequent effects on the reaction rates and equilibria of many macromolecular reactions.

3. Despite the foregoing, it is a property that, with certain exceptions, is ignored by most biochemists.

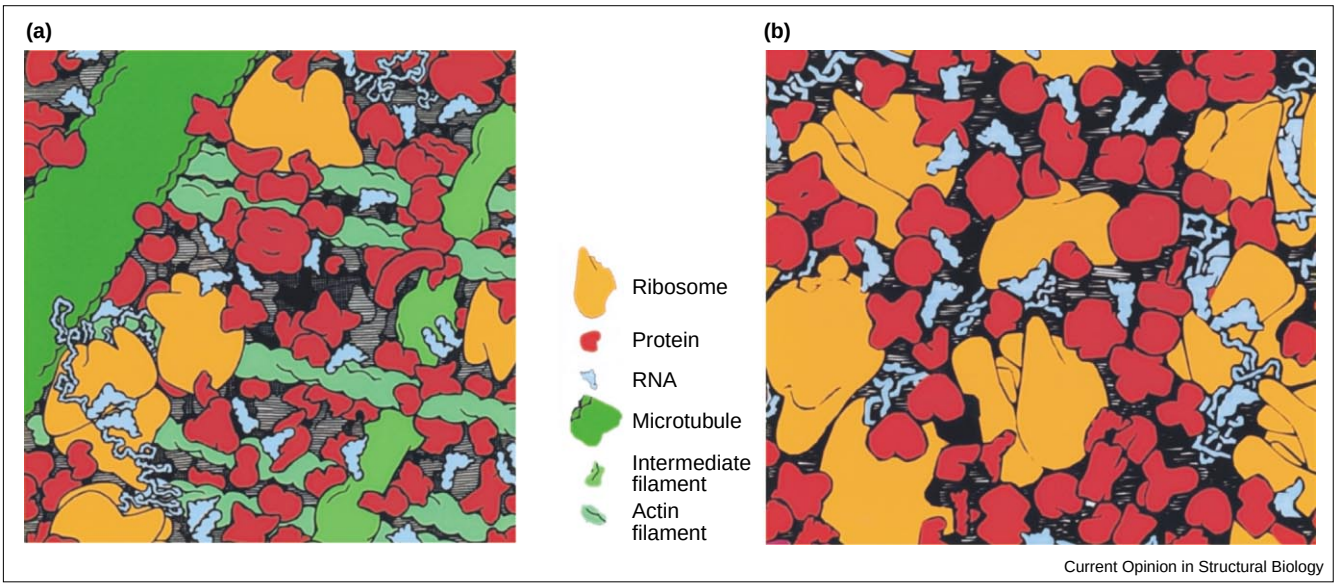
The last point was made 11 years ago by GB Ralston [1], who concluded that "while the phenomenon of macromolecular nonideality has been known for many years, and the biological relevance of macromolecular crowding demonstrated unequivocally, there is a resounding silence in this area from the standard biochemistry texts". A glance at current biochemistry textbooks reveals that this neglect continues, leading to a more recent comment by one of the founders of this field, AP Minton, that, while "an increasing number of investigators are adding substantial concentrations of water-soluble polymers to their reaction mixtures in order to simulate aspects of the highly volume-occupied intracellular environment, ...such investigators still comprise a very small fraction of the number who claim physiological relevance for their *in vitro* results" [2]. Biological macromolecules have evolved to function in the crowded conditions characteristic of intracellular milieux, so it is remarkable, not to say remiss, that most investigations of the properties of such macromolecules are still carried out in uncrowded buffers. The principal exceptions are studies of DNA transcription and replication, for which it has been found to be necessary to add crowding agents in order to relate *in vitro* properties to *in vivo* counterparts [3].

The purpose of this review is to complement the discussion of the principles and implications of crowding theory presented in this issue last year [4••] by summarising the predicted quantitative effects of crowding and describing several specific examples of the effects of crowding on biologically relevant properties of proteins. The hope is that the remarkable effects of adding crowding agents will persuade more researchers to regard such agents as factors to control in their *in vitro* experiments as important as variables such as pH.

Basics

The term macromolecular crowding, as applied to biological systems, describes the fact that the total concentration of macromolecules inside cells is so high that a significant proportion of the volume is physically occupied and, hence, unavailable to other molecules. Crowding is more precisely termed the excluded volume effect, which emphasises the fact that it is a purely physical nonspecific effect originating from steric repulsion. The effective concentration, or thermodynamic activity, of each macromolecular species inside cells is thus greater than its actual concentration and this difference has kinetic and thermodynamic consequences for the properties of that macromolecule. The concentration of total protein inside cells is in the range 200–300 g/l, whereas that of RNA is in the range 75–150 g/l. Thus, the total concentration of protein and RNA inside *Escherichia coli* is in the range 300–400 g/l, depending on the growth phase [5], whereas red blood cells contain about 350 g/l of haemoglobin alone. Polysaccharides also contribute to crowding, especially in the extracellular matrix of tissues such as collagen. Thus, crowding occurs outside, as well as inside, the cell; even blood contains about 80 g/l of protein. In general, cellular interiors are 20–30% volume-occupied by macromolecules, so this range defines the relevant concentration of crowding agents to use when trying to simulate biological crowding conditions *in vitro*. Figure 1 illustrates this degree of crowding for the cytoplasm of a eukaryote and a prokaryote.

Figure 1



The crowded state of the cytoplasm in (a) eukaryotic and (b) *E. coli* cells. Each square illustrates the face of a cube of cytoplasm with an edge 100 nm in length. The sizes, shapes and numbers of macromolecules are approximately correct. Small molecules are not shown. Adapted with permission from [21].

Predictions

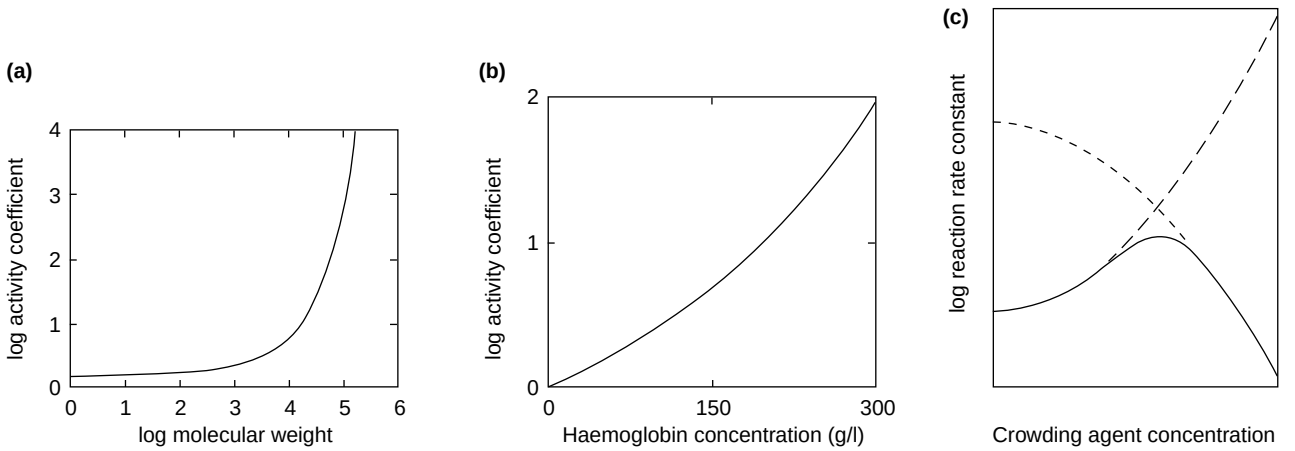
Thermodynamic approaches [1–3,4••,5–8] to the quantification of the excluded volume effect make two major predictions of relevance to biological systems.

Diffusion coefficients (D) will be reduced by factors up to 10-fold. The average time a molecule takes to move a certain distance by diffusion varies as D^{-2}, so if D is reduced 10-fold, it will take 100 times as long for a molecule to move a certain distance. This reduction applies to both small and large molecules, so the rate of any process that is diffusion-limited will be reduced, whether the process involves small molecules, large molecules or both. The diffusion of large molecules will, however, be impeded more than that of small molecules.

Equilibrium constants for macromolecular associations may be increased by two to three orders of magnitude, depending upon the relative sizes and shapes of macromolecular reactants and products, and of background macromolecules. This effect on the thermodynamic activity of macromolecules arises from the reduction in volume obtained when such molecules bind to one another. The more solute molecules present in a solution, and the larger they are, the less randomly they can be distributed. Thus, the configurational entropy of each macromolecular solute species becomes smaller and its contribution to the total free energy of the solution increases as the total concentration of macromolecules rises. The reduction in excluded volume when molecules bind to one another thus favours the binding event, as it leads to a decrease in the total free energy of the solution. In other words, the most favoured state excludes the least volume to the other macromolecules present. This conclusion applies not just to associating macromolecules, but to all processes in which a change in excluded volume occurs, for example, the collapse of newly synthesised polypeptide chains and the unfolding of proteins induced by environmental stresses such as heat shock.

The crowding effect on activity is largely restricted to macromolecules. Figure 2a illustrates how the activity coefficient of a molecule (defined as the ratio of its thermodynamic concentration to its actual concentration) in a background crowded by 300 g/l haemoglobin varies with its molecular weight; the activity coefficient increases significantly only for molecules larger than 10^3 molecular weight (note that Figure 2a is a log/log plot). Thus, crowding does not greatly affect the activity of metabolites and small ions. For many macromolecules in the size range found in cells, however, the effect on activity is very large — note the sharp increase as the molecular weight becomes larger than that of the predominant crowding species (haemoglobin in this case). A simple example taken from [5] will make the point: the equilibrium constant in dilute solution for a spherical homodimer and a monomer of molecular weight 40,000 will shift towards dimerisation by a factor in the range 8–40-fold (depending on the partial specific volume of the protein) if the protein is expressed inside *E. coli*. For a tetramer, the shift in equilibrium towards tetramerisation would be in the range 10^3–10^5. Thus, this aspect of the crowding effect is exerted by large molecules on large molecules and can be surprisingly large.

Figure 2



Characteristics of macromolecular crowding. (a) The dependence of activity coefficient on molecular weight in a solution crowded by 300 g/l haemoglobin. (b) The dependence of activity coefficient on concentration of crowding agent. In this example, the molecular species whose activity is measured is identical to the crowding agent – both are haemoglobin. (c) The dependence of reaction rate on concentration of crowding agent, in cases in which the reaction is either diffusion-limited (short-dashed curve) or transition-state-limited (long-dashed curve). The overall reaction rate is transition-state-limited at low concentrations of crowding agent and diffusion-limited at high concentrations (continuous curve). Adapted with permission from [6,7,22], respectively.

A misunderstanding sometimes encountered is that crowding makes all macromolecules bind to one another. This is clearly not the case; crowding enhances the inherent tendency of macromolecules to bind to one another, but it does not create this tendency *de novo* — if it did, the cytoplasm would be solid! It is the case, however, that the occurrence of cytoskeletal structures in eukaryotic cells is predicted from crowding theory to increase association constants over and above those found in free solution by confining macromolecules to restricted volumes [9]. The nature of the cytoplasm is still under debate, but crowding considerations suggest that it might be considered to be like a gel, in the sense that most macromolecules may exist as components of large complexes, rather than as independent entities (see Update).

Nonlinearity

The effect of crowding on the thermodynamic activity of macromolecules is highly nonlinear with respect to the concentration of the crowding agent. Figure 2b shows experimentally determined values for the activity coefficient of haemoglobin with respect to the concentration of haemoglobin, that is, in this case, the crowding agent and the interacting species are identical. From this graph, it can be calculated that the activity coefficient at 340 g/l is about 27-fold larger than that at 100 g/l (note that Figure 2b is a semi-log plot). This nonlinearity is important to bear in mind when choosing concentrations of crowding agents to use *in vitro*; 100 g/l sounds a high concentration to a biochemist, but it is too low to adequately simulate the effect of crowding inside, for example, a cell of *E. coli* (see below).

Opposing effects

The effects of crowding on diffusion and activity oppose each other with respect to reaction rates and are illustrated in Figure 2c. Consider a bimolecular association of the form:

$$A + B \leftrightarrow AB^* \leftrightarrow AB$$

where A and B may be, for example, interacting proteins producing amyloid fibrils or a repressor binding to DNA, and AB* is the transition state. If the overall rate-limiting step is the encounter rate of A with B, the reaction is diffusion-limited. But crowding reduces diffusion, so the rate will fall as the concentration of crowding agent increases (short-dashed curve in Figure 2c). If, on the other hand, the rate-limiting step is the conversion of the transition state to the AB dimer, the conversion of A and B to AB* can be treated as being at equilibrium. But crowding increases association via its effect on activity, so this equilibrium is displaced to the right and the overall reaction rate will increase as the concentration of crowding agent rises (long-dashed curve in Figure 2c). However, the maximal reaction rate possible for any bimolecular reaction must be set ultimately by the encounter rate of the two components, so that even for transition-state-limited reactions, the reaction rate will eventually fall when the concentration of crowding agent becomes high enough (continuous curve in Figure 2c). The effect of crowding on reaction rate is thus complex and depends crucially on the nature of the reaction and on the concentration of crowding agent. It is for this reason that there is an urgent need for more researchers to study comprehensively the effects of crowding agents on macromolecular interactions.

Examples of crowding effects

In vivo diffusion rates

Table 1 lists measurements of diffusion coefficients for both large and small molecules inside a range of cells, relative to their diffusion in water. In the cytoplasm of eukaryotic cells, diffusion of both large and small molecules is slowed three to fourfold, whereas in *E. coli* cytoplasm, the diffusion of green fluorescent protein (GFP) is slowed 11-fold. These measurements are broadly consistent with the degree of crowding of the cytoplasm illustrated in Figure 1, but it is important to note that mobility inside cells is likely to be affected not just by crowding, but by other factors, such as binding to other molecules, including relatively immobile structures such as cytoskeletal components ([9,10•]; see also Update).

Table 1

Diffusion of large and small molecules inside cells.

System	Molecular species	D/D_0*	References
Water	GFP	1	
CHO cell cytoplasm	GFP	0.31	[23]
CHO cell mitochondria	GFP	0.23–0.34	[24]
E. coli cytoplasm	GFP	0.088	[10•]
BSA (200 g/l)	FITC BSA	0.25	[25]
3T3 fibroblast cytoplasm	Carboxyfluorescein	0.27	[26]
3T3 fibroblast cytoplasm	FITC dextrans	0.27	[27]
Erythrocyte cytoplasm	Lactate	0.32	[28]

*Ratio of translational diffusion coefficient to that in water.

Direct measurement of crowding-induced enhancement of protein association

Early studies detected the self-association of proteins such as myoglobin, aldolase and ovalbumin in concentrated solutions of these proteins, but it is only recently that a method has been developed to measure the self-association of proteins in dilute solution induced by the crowding effects of high concentrations of a second macromolecule to which the dilute proteins do not bind [11••]. This method, called tracer sedimentation equilibrium, was used to measure the self-association of dilute solutions of two proteins: fibrinogen at 0.25–1.0 g/l, labelled with either ^{125}I or fluorescein isothiocyanate (FITC), in the presence of bovine serum albumin (BSA) as the crowding agent at 0–100 g/l; and tubulin at 0.2–0.6 g/l, labelled with rhodamine, in the presence of dextran as the crowding agent at 0–100 g/l.

It was observed that, at BSA concentrations exceeding 40 g/l, fibrinogen forms homodimers; at 80 g/l BSA, the activity coefficient of fibrinogen is calculated to be 10. As the latter concentration of BSA is close to the total protein concentration in blood plasma, this observation suggests that the activity of fibrinogen in its natural environment is an order of magnitude larger than that exhibited in the uncrowded solutions in which its properties are usually studied. The average molar mass of tubulin was found to increase monotonically with increasing dextran concentration to produce soluble tubulin oligomers; this observation suggests that, inside the cell, such soluble oligomers may be intermediates in the assembly of insoluble microtubules. Recent work using the tracer sedimentation equilibrium method has shown that the self-association of monomers of the bacterial cell division protein FstZ is enhanced by the addition of crowding agents in a manner quantitatively described by a hard sphere model for excluded volume effects (G Rivas *et al.*, personal communication; see Now in press).

Sedimentation equilibrium is one of the best methods for measuring the thermodynamic activity of macromolecules in solution, so the extension of this method for use with crowded solutions presents a new opportunity for the quantitative properties of such molecules to be measured under more physiological conditions than hitherto.

Enhancement of protein folding and aggregation

Theory predicts that crowding should enhance two aspects of protein folding: the initial collapse of polypeptide chains, whether newly synthesised inside the cell or refolding on dilution from denaturant in the test tube, and the association of partly folded chains into nonfunctional aggregates. The first reports testing these predictions utilise the fact that oxidised hen lysozyme refolds from denaturant very rapidly with high efficiency, whereas reduced lysozyme folds much more slowly and suffers from extensive aggregation [12••,13••]. The addition of crowding agents, both synthetic (Ficoll 70 and dextran 70) and natural (ovalbumin and BSA), at concentrations up to 200 g/l, to refolding oxidised lysozyme has no effect, but addition to reduced lysozyme abolishes refolding by causing all the chains to aggregate. This aggregation is prevented by the addition of protein disulfide isomerase, which acts both as a catalyst to speed the rate of disulfide bond formation and as a molecular chaperone to shield hydrophobic surfaces [12••]. At concentrations of crowding agent low enough to permit some reduced chains to escape aggregation, the rate of correct refolding of these chains can be increased up to fivefold compared with the rate in uncrowded buffers; this effect is interpreted in terms of the stabilisation of compact refolding intermediates induced by the excluded volume effect [13••].

These observations need both more detailed study to test this interpretation and extension to a variety of other proteins to determine their universality, but they have two general implications: first, some polypeptide chains may fold more rapidly *in vivo* than they refold *in vitro* and second, aggregation of partly folded polypeptide chains is an even bigger problem inside the cell than it appears to be from observations of protein refolding inside the test tube. This implication could explain why cells contain molecular chaperones, even though most denatured proteins will refold correctly *in vitro* in the absence of other macromolecules [14,15].

Enhancement of chaperonin action

The chaperonin family of molecular chaperones present in prokaryotes consists of two interacting protein components, GroEL and GroES. There is abundant *in vitro* and *in vivo* evidence that this system functions to assist protein folding by preventing the aggregation of similar or identical partly folded polypeptide chains, according to the Anfinsen cage model [15]. In this model, each partly folded chain is sequestered from other such chains by binding to the apical domains of a GroEL cage. Subsequent binding of GroES to the open end of this cage triggers the release of the bound chain into the centre of the cage, where it can continue to fold, and simultaneously prevents the folding chain from leaving the cage for a time set by the ATP hydrolysis cycle — around 15 s at 23°C. For some chains, it has been observed *in vitro* that this time is insufficient for the chains to fold to a point at which binding to GroEL no longer occurs; instead, the partly folded chain is released into the buffer and rebinds to another GroEL molecule. But if this release occurs *in vivo*, aggregation-sensitive chains would appear in the cytosolic medium, which would appear to render the system pointless. Thus, the problem is how to reconcile the release and rebinding observed for some slow folding proteins *in vitro* with the *in vivo* evidence that the chaperonin system functions to prevent aggregation [16].

The ability of crowding to enhance the association of interacting macromolecules suggests that the solution to this problem may be that, *in vivo*, the crowded environment prevents the release of the partly folded polypeptide chain from the open GroEL cage long enough to allow it to rebind to the same GroEL oligomer and thus avoid exposure to the cytosolic medium. This possibility was tested by repeating the *in vitro* experiments in the presence of either Ficoll 70 or dextran 70 at concentrations up to 300 g/l. Such additions prevent the release of partly folded chains of rhodanese into the buffer; instead, the chains are retained and continue to fold inside the same GroEL oligomer to which they initially bound [17]. Similar effects are given by the addition of *Xenopus oocyte* extracts, provided that the latter are used at a total protein concentration of 200 g/l; lowering the concentration by only half allows released chains to appear in the buffer, confirming the importance, illustrated in Figure 2b, of using the appropriate concentration of crowding agent.

Studies with intact yeast [18] and mammalian [19] cells confirm that partly folded proteins capable of binding to GroEL do not appear in the cytosol *in vivo* except under stress conditions. Thus, crowding is not all bad — it favours aggregation, but also enhances the operation of chaperones that prevent aggregation [20].

Conclusions

These examples reinforce earlier conclusions [1,2] that the effects of crowding are so large and diverse that the addition of crowding agents at biologically relevant concentrations to solutions containing macromolecules should become a routine variable to study. This is unlikely to happen until enough editors of learned journals reject manuscripts on the grounds that this important variable has not been controlled.

Update

An excellent review has appeared recently that discusses the considerable body of evidence that the interior of cells is poorly described by the dilute solution paradigm assumed by most biochemists [29•].

Acknowledgements

Thanks are due to Allen Minton and Ulrich Hartl for commenting on the manuscript, and to Howard Dalton for provision of office facilities.

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Now in press

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