Macromolecular crowding: obvious but underappreciated

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Biological macromolecules evolve and function within intracellular environments that are crowded with other macromolecules. Crowding results in surprisingly large quantitative effects on both the rates and the equilibria of interactions involving macromolecules, but such interactions are commonly studied outside the cell in uncrowded buffers. The addition of high concentrations of natural and synthetic macromolecules to such buffers enables crowding to be mimicked *in vitro*, and should be encouraged as a routine variable to study. The stimulation of protein aggregation by crowding might account for the existence of molecular chaperones that combat this effect. Positive results of crowding include enhancing the collapse of polypeptide chains into functional proteins, the assembly of oligomeric structures and the efficiency of action of some molecular chaperones and metabolic pathways.

A characteristic of the interiors of all cells is the high total concentration of macromolecules they contain. Such media are termed 'crowded' rather than 'concentrated' because, in general, no single macromolecular species occurs at high concentration but, taken together, the macromolecules occupy a significant fraction (typically 20–30%) of the total volume. This fraction is thus physically unavailable to other molecules. This steric exclusion generates considerable energetic consequences that are not generally appreciated and that are the subject of this review. Biological macromolecules have evolved to function inside such crowded environments. For example, the total concentration of protein and RNA inside a cell of *Escherichia coli* is in the range of 300–400 g l^{-1} (Ref. 1). An artist's impression of this degree of crowding is shown in Fig. 1. Similar pictures are available for the major compartments of eukaryotic cells[2].

Despite this physiological feature of the intracellular environment, biochemists commonly study the properties of macromolecules in solutions with a total macromolecular concentration of 1–10 g l^{-1} or less, in which crowding is negligible. The few exceptions include reticulocyte lysates and *Xenopus* oocyte extracts, which can be made without significant dilution, and *in vitro* studies of DNA replication and transcription, in which it has been found necessary to add crowding agents to extracts to obtain properties akin to those seen *in vivo*[3]. Overlooking this difference matters because crowding has both thermodynamic and kinetic effects on the properties of macromolecules, a fact that has been known for at least four decades since systematic studies by Ogston and by Laurent[4]. These effects are so large that it can be stated with some confidence that many estimates of reaction rates and equilibria made with uncrowded solutions in the test tube differ by orders of magnitude from those of the same reactions operating under crowded conditions within cells[5,6].

A sceptic might observe that this difference does not matter because molecular biology continues to be remarkably successful despite its lack of quantitation[7]. It is also true that editors of learned journals do not routinely reject manuscripts on the grounds that the lack of experiments to determine the effects of crowding raises doubts about the relevance of the conclusions to the *in vivo* situation. For this reason, crowding is not part of the biochemical *zeitgeist*, and it is rarely discussed in standard textbooks of biochemistry and molecular biology. However, the ultimate aim of these disciplines is to understand how intact organisms work, and the current determination of total genome sequences is triggering a renaissance in protein biochemistry as genes for many proteins with unknown functions are being discovered. The increasing availability of total genome databases is also stimulating the development of methodologies to measure changes in transcriptomes and proteomes, with the aim of modelling the molecular determinants of cellular behaviour in a more comprehensive manner[8]. The time is thus ripe to review the principles and experimental studies of macromolecular crowding. The aim of this article, and of related articles[9,10], is to encourage more researchers to include this factor both in their studies of isolated macromolecules in the test tube and in their models for how these molecules function inside living cells.

Principles

Molecular crowding is more accurately termed the excluded volume effect, because the mutual impenetrability of all solute molecules is its most basic characteristic. This nonspecific steric repulsion is always present, regardless of any other attractive or repulsive interactions that might occur between the solute molecules. Thus, crowding is similar to gravity – it cannot be avoided and organisms have to cope with its consequences. How much of the intracellular volume is unavailable to other macromolecules depends upon the numbers, sizes and shapes of all the molecules present in each compartment. This conclusion applies both to the bulk soluble phases in each compartment and to the smaller volumes confined by cytoskeletal elements in

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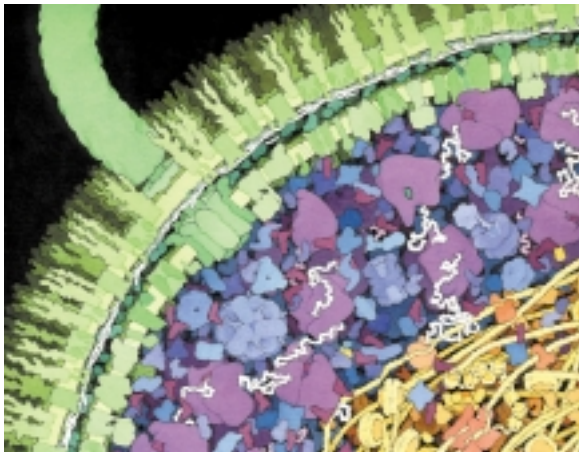

Fig. 1. Representation of the approximate numbers, shapes and density of packing of macromolecules inside a cell of *Escherichia coli*. Small molecules are not shown. Reproduced, with permission, from Ref. 47.

eukaryotic cells. The cytoskeleton constitutes a dense lattice in which the fluid part of the cytoplasm is dispersed in small elements or pores, with dimensions comparable to the size of large macromolecular complexes. Volume exclusion by the pore boundaries to the macromolecules within is a type of crowding called macromolecular confinement[9,11,12].

In general, cellular interiors are 20–30% volume-occupied by macromolecules of specific volume close to 1 ml g^{-1}, so these values define an approximate range of 200–300 g l^{-1} to be considered when using physical theory to calculate the consequences of crowding inside cells[11–15]. Crowding is not confined to cellular interiors, but also occurs in the extracellular matrix of tissues such as cartilage; even blood plasma contains ~80 g l^{-1} of protein, a concentration sufficient to cause significant crowding effects (see Practicalities section).

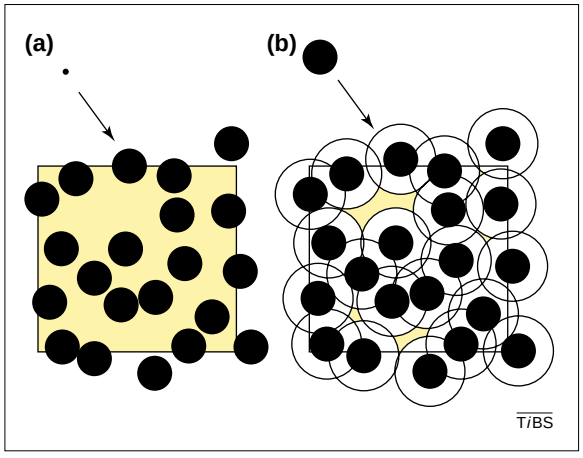

Fig. 2. The importance of size in volume exclusion. The squares define volumes containing spherical macromolecules occupying ~30% of the available space. (a) The centre of an introduced small molecule has access to virtually all of the remaining 70% of the space, indicated in yellow. (b) The centre of an introduced molecule similar in size to the macromolecules is excluded from most of this 70% as it cannot approach these macromolecules to a distance less than that indicated by the open circles. Reproduced, with permission, from Ref. 9.

Solute size and excluded volume

The importance of the size of a molecule in determining the magnitude of the intracellular volume that is excluded to that molecule is so striking as to be counterintuitive, but can be grasped from Fig. 2. The squares outline elements of volume containing spherical macromolecules (black) that occupy ~30% of the total volume, a value typical of intracellular compartments. The volume available to another molecule is defined as the fraction that can be occupied by the centre of that molecule. Obviously, if the introduced molecule is small relative to the macromolecules already present (Fig. 2a), it can access virtually all the space between these macromolecules – the volume available is depicted in yellow. However, if the introduced molecule is similar in size to that of the macromolecules (Fig. 2b), the available volume is much less than might be expected because the centre of that molecule can approach the centre of the other macromolecules to no less than the distance at which the surfaces of the two molecules meet; this distance is indicated by the open circle around each macromolecule. It follows that the centre of the added macromolecule can occupy only that part of the total volume that is exterior to any open circle. The volume available to the large molecule (Fig. 2b) is much less than that available to a small molecule (Fig. 2a), as can be seen by comparing the yellow areas. The available volume per macromolecule thus defines an effective concentration that can be much higher than the actual concentration in a crowded medium. The consequence of this is that effects of crowding on reaction equilibria and reaction rates are highly non-linear with respect to the sizes and concentrations of the interacting molecules.

Effects of crowding on reaction equilibria

Equilibrium constants are properly expressed in terms of effective concentrations (or thermodynamic activities) rather than in terms of actual concentrations. The ratio of effective concentration to actual concentration is termed the activity coefficient (Box 1). The activity coefficient of haemoglobin has been determined by measuring how the osmotic pressure varies with the actual concentration of haemoglobin[16]. Figure 3a illustrates the striking non-linearity of the activity coefficient with the actual concentration of haemoglobin; the effective concentration of haemoglobin exceeds its actual concentration by tenfold at 200 g l^{-1} and by 100-fold at 300 g l^{-1}. This experimentally determined non-linearity is accounted for quantitatively by a model in which each haemoglobin molecule is represented as a rigid spherical particle of diameter 59 Å; X-ray crystallography indicates that haemoglobin molecules are spheroids of dimension $50 \times 55 \times 65$ Å.

Quantitative studies with cell extracts show that the high concentrations of macromolecules within *E. coli* cells produce large increases in activity coefficient values[1,3]. Figure 3b illustrates how the

Box 1. Volume exclusion and thermodynamic activity

Physical chemists simplify reality by making calculations for molecules under 'ideal' conditions (i.e. those in which the finite size of the molecules has no effect), and extrapolate experimental measurements to zero concentration. However, real molecules are not ideal and their total concentration in biological systems is often very high. The effect of volume exclusion for a solute molecule can be handled mathematically as follows.

The chemical potential of solute species i, or the differential change in solution free energy associated with the introduction of an infinitesimal amount of this solute species into the solution, can be partitioned into ideal and nonideal contributions (Eqn 1):

$$\mu_i = \mu_i^{ideal} + \mu_i^{nonideal} \quad [1]$$

The ideal contribution is the free energy change expected in the absence of solute–solute interactions (Eqn 2):

$$\mu_i^{ideal} = \mu_i^0 + kT\ln c_i \quad [2]$$

where μ_i^0 is the standard state chemical potential of species i , k is Boltzmann's constant, T the absolute temperature, and c_i the concentration in units proportional to density of molecules (e.g. molar or w/v units). The nonideal contribution is the free energy change associated with the equilibrium free energy of interaction between a molecule of solute species i and all other solute molecules in the solution $\langle f_i \rangle$ (Eqn 3):

$$\mu_i^{nonideal} = kT\ln\gamma_i \quad [3]$$

where γ_i, called the activity coefficient, is defined as $\gamma_i = \exp(\langle f_i \rangle / kT)$. Eqns 1–3 can be combined to yield Eqn 4:

$$\mu_i = \mu_i^0 + RT\ln a_i \quad [4]$$

where a_i ($= \gamma_i c_i$) is an effective concentration called the thermodynamic activity.

It is essential to keep in mind that the theory of chemical equilibrium is based upon the use of activities rather than concentrations. All equilibrium relations should, strictly speaking, be written as functions of species activities rather than of concentrations. As solutions become progressively more dilute, solute–solute interaction, and hence the nonideal contribution to the chemical potential, diminishes to the point where it can safely be neglected, and $a_i \sim c_i$ for all solute species. Under these conditions, the solution can be said to behave in an ideal fashion. However, when solute concentrations are large, it is inappropriate to neglect solute–solute interactions: one cannot safely assume that a_i is approximately equivalent to c_i. In particular, in a crowded biological medium, the volume excluded by macromolecules to other macromolecules can cause the activity of some species to exceed their respective concentrations by one or more orders of magnitude.

activity coefficient of a macromolecule inside such a cell increases dramatically with its molecular weight. The activity coefficient triples as the molecular weight passes 3000 but exceeds two orders of magnitude as the molecular weight passes 50 000. Thus, the effect of crowding on thermodynamic activity is exerted *by* large molecules *on* other large molecules – hence the term 'macromolecular crowding'[17].

The main effect of crowding on biochemical equilibria is to favour the association of macromolecules. Equilibrium constants for such reactions can be increased by as much as two to three orders of magnitude, depending on the relative sizes and shapes of the reactants and products, and on those of the background macromolecules. For example, the equilibrium constant for the association of a spherical monomer of molecular weight 40 000 into a homodimer is 8–40-fold larger (depending on the specific volume of the protein) if the protein is expressed in *E. coli*, compared to its value in an uncrowded solution[1]. For a tetramer, the shift in equilibrium towards tetramerization is in the range 10^3–10^5.

This striking effect arises from the reduction in volume obtained when macromolecules bind to one another. As the number and size of molecules in a solution increase, the less randomly they can be distributed. The configurational entropy of each macromolecular species becomes smaller, and its contribution to the total free energy of the solution increases, as the total concentration of macromolecules rises; this effect can be envisaged by considering the situation in Fig. 2b. The binding event is favoured because the reduction of excluded volume, and the concomitant increase in available volume, decreases the total free energy of the solution. Thus, the most favoured state is that which excludes the least volume to all the other macromolecules present.

This is a general conclusion, and means that crowding not only favours association reactions, but also affects all those biochemical processes in which a change of excluded volume occurs (e.g. the collapse of newly synthesized polypeptide chains into compact functional proteins, the unfolding of proteins induced by stress, the formation of oligomeric structures such as fibrin, collagen and multienzyme complexes in metabolic pathways, and the association of proteins into nonfunctional aggregates such as bacterial inclusion bodies and the plaques in human amyloid diseases). The effects of crowding on the reactivity of macromolecules is also implicated as one of the ways in which cells sense and respond to changes in their overall volume, induced by osmotic alterations caused by transport and metabolism[17–19].

Effects of crowding on reaction rates

One result of crowding that is easy to grasp is the reduction in molecular diffusion rates[13,20]. This reduction applies to both large and small molecules, but is greater for large molecules. Imagine an airport lounge that is crowded with people. The time it takes for an adult to move from the entrance to the ticket office is clearly increased by the need to avoid other people, compared with the time required to cross an empty lounge. The same is true for a child, but a child can more easily avoid other people than the adult, and therefore move faster, because it is smaller. Diffusion coefficients (D) for both large molecules (e.g. green

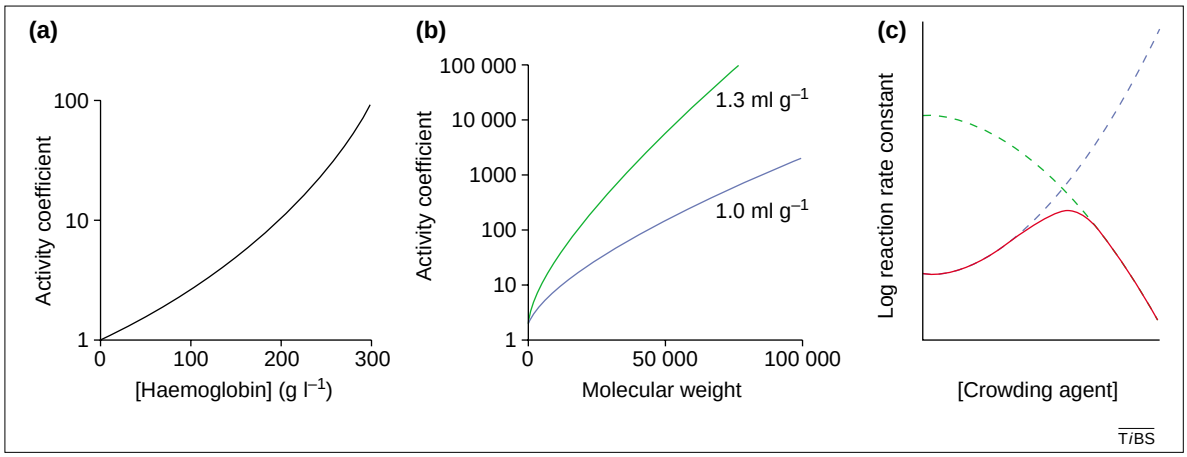

Fig. 3. The highly nonlinear characteristics of macromolecular crowding. (a) The dependency of activity coefficient on concentration of crowding agent. (b) Estimated activity coefficients for spherical molecules of increasing molecular weight in the cytoplasm of *Escherichia coli*. The curves are drawn for two specific volumes. (c) Dependence of reaction rate constant on the degree of crowding in cases where the reaction is either diffusion-limited (green curve) or transition state-limited (blue curve). The reaction rate is transition-state limited at low degrees of crowding and diffusion-limited at high degrees of crowding (red curve). Note that the reaction rate constant in (c) is represented on a log scale. Reproduced, with permission, from Refs 15, 3 and 13, respectively.

fluorescent protein) and small molecules (e.g. carboxyfluorescein) have been measured inside a range of cells, and are reduced from three- to tenfold compared to their values in water[10,20,21]. The average time a molecule takes to diffuse a certain distance varies with 1/D so, if D is reduced tenfold, the molecule will take ten times longer to move this distance. Thus, the rate of any biochemical process that is diffusion-limited will be reduced by crowding, whether this process involves large molecules, small molecules or both types of molecule.

The effects of crowding on biochemical reaction rates are complex because although crowding reduces diffusion, it increases thermodynamic activities. The net result of these opposing effects depends upon the precise nature of each reaction. Figure 3c illustrates this complexity for a simple reaction of form $A + B \leftrightarrow AB^* \leftrightarrow AB$, where AB* is the transition complex. If the overall rate of this reaction is limited by the encounter rate of A with B, crowding will reduce the overall rate by reducing diffusion. But, if the reaction rate is limited by the activity of AB*, crowding will increase the rate because crowding increases activity. The maximum rate possible for any multimolecular reaction is ultimately set by the encounter rate of the components, so even for transition-state-limited reactions, the rate will eventually fall as crowding increases. The effect of crowding on reaction rate thus depends upon the nature of the reaction and on the degree of crowding and, for this reason, it is necessary to measure the quantitative effects of crowding in all studies of macromolecular interactions if these studies are to be regarded as physiologically relevant[6].

Practicalities

It is possible, in principle, to measure the effects of crowding on the interactions of extracted macromolecules by adding to the solution high concentrations of macromolecules that mimic those found within cells. There are problems with this approach (reviewed in Ref. 1). The ideal crowding agent should have a molecular weight in the range 50 000–200 000, be highly water-soluble and not be prone to self-aggregation; a globular, rather than an extended, molecular shape prevents solutions becoming too viscous to handle. The agent should be easily available in highly purified form so that the use of high concentrations does not introduce problems associated with contaminants. The most important criterion, however, is that the agent should not interact with the system under test, except via steric repulsion. This last requirement is the most difficult to meet and, in practice, it is necessary to use a variety of different crowding agents to reduce the risk that observed effects are caused by specific interactions. It is also essential to establish that any effects observed when using crowding agents are not the result of inadvertent changes in other factors, such as pH, ionic strength or redox potential. The use of highly concentrated cell extracts as crowding agents might seem appropriate, but is not recommended because of the high probability that any interpretation will be complicated by specific interactions, hydrolyase activity and the presence of denatured proteins.

Crowding effects caused by high concentrations of haemoglobin, serum albumin and dextran can be quantitatively accounted for by hard particle excluded volume models[22], but the effects of polyethylene glycol cannot, as this polymer appears to have additional interactions with proteins that are neglected in excluded volume models[15]. Haemoglobin suffers from the severe disadvantage that its colour prevents the use of optical assay methods, so colourless proteins, such as ovalbumin and bovine serum albumin, are preferred in practice. Commonly used synthetic crowding agents include Ficolls, dextrans, polyethylene glycol and polyvinyl alcohol. Ficoll 70 is the most convenient because it has a

relatively low viscosity. Recent studies show that molecules of Ficoll 70 have an average radius of 55 Å and an average molecular weight of 74 000, but behave as relatively open structures rather than as hard-packed spheres[23].

A general technique has recently become available for measuring the thermodynamic activity and state of association of a dilute macromolecular solute in the presence of high concentrations of a second macromolecular solute[24]. This technique uses sedimentation equilibrium centrifugation to study the thermodynamic activity of macromolecules in solution. The dilute macromolecular solute, suitably labelled with fluorescent or radioactive tags, is centrifuged in a solution of crowding agent such as bovine serum albumin or dextran until sedimentation equilibrium is reached. Analysis of gradient fractions allows the distribution of the tagged molecules to be determined, from which their activity coefficient can be calculated at different concentrations of crowding agent. For example, the activity coefficient of fibrinogen is tenfold higher in a solution of 80 g l^{-1} bovine serum albumin[24]; this concentration of crowding agent is close to the total protein concentration in blood plasma, so the activity of fibrinogen in its natural environment could be an order of magnitude larger than that in the uncrowded buffers in which its properties are commonly studied. The tracer sedimentation equilibrium method has also been used to show that monomers of the bacterial cell division protein FstZ associate with one another in the presence of crowding agents in a manner quantitatively described by a hard-sphere model for the effects of excluded volume[22].

Another recent development provides a method for mimicking the effects of confinement and crowding on the structure and stability of proteins by enclosing the proteins within the pores of silica glass[25]. Small molecules can diffuse through the glass, which is also optically transparent. There are two advantages of this approach over the use of soluble macromolecules as crowding agents: (1) it provides a means of perturbing the structure of the test protein, for example by heat denaturation, without also affecting the properties of the crowding agent; and (2) it prevents aggregation because the glass matrix shields the protein molecules from one another. The thermal stability of α-lactalbumin is increased by 25–30°C by encapsulation in a silica matrix[25], confirming the theoretical prediction that crowding should enhance the stability of the folded, relative to the unfolded, state[26]. Earlier studies demonstrated that the addition of high concentrations of heat stable proteins such as ribonuclease stabilizes other proteins against denaturation by heat or ethanol[27].

Crowding and molecular chaperones

Crowding could explain why molecular chaperones exist despite the ability of many proteins to assemble themselves. Since pioneering studies in the laboratories of Christian Anfinsen[28] and Aaron Klug[29], many cases have been reported in which purified denatured proteins spontaneously refold and reassociate in the test tube in the absence of either other macromolecules or exogenous energy input. These observations led to the view that primary translation products emerging from polysomes inside cells fold and associate into their functional conformations in a spontaneous manner. This view was challenged by the discovery of molecular chaperones, a class of unrelated proteins that share the property of assisting the assembly and disassembly of other protein-containing structures without being present in the final functional form of these structures[30,31].

Molecular chaperones can be divided into two subclasses, termed steric and nonsteric[32]. There are only two known classes of steric chaperone and these provide structural information essential for correct assembly of their target proteins[33,34]. Nonsteric chaperones comprise a bigger group and act differently. They do not provide structural information but prevent and, in some cases, reverse, the aggregation of a subset of partly folded polypeptide chains into nonfunctional structures[35–37]. Partly folded polypeptide chains arise either during protein synthesis or as a result of denaturation of mature proteins caused by stresses such as heat shock. Chaperones prevent aggregation by binding transiently to hydrophobic regions exposed on the polypeptide surface, thus decreasing the propensity of these surfaces to bind to one another. Aggregation is a high-order process whose rate is very sensitive to concentration. It is commonly observed that aggregation occurs during the refolding of purified denatured proteins, indicating that it is a specific process involving identical polypeptide chains. This problem can be reduced in the test tube by lowering the protein concentration, but how does the cell tackle this problem?

The application of crowding theory to protein aggregation makes two predictions:

- Crowding should favour aggregation because of its effect of increasing the thermodynamic activity of partly folded polypeptide chains. This effect will be greater for small polypeptides as large polypeptides will have their diffusion slowed and, thus, have a reduced encounter rate. In particular, crowding should enhance the aggregation of slow-folding chains, as fast-folding chains can internalize their hydrophobic surfaces before these can bind to those in other chains.
- Crowding should enhance the functional activity of chaperones by stimulating their association with partly folded chains; thus, these chains should have less time to encounter one another.

Recent evidence provides some support for both these predictions. Hen lysozyme provides a good test system because it can be produced in two states with identical amino acid sequences, but with very

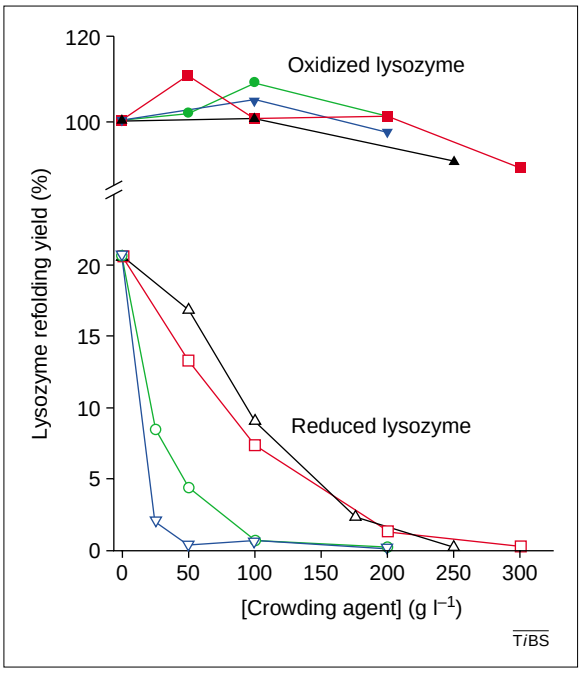

Fig. 4. Effects of four different crowding agents on the refolding of denatured hen lysozyme. Oxidized (closed symbols) or reduced (open symbols) pure denatured lysozyme was diluted in refolding buffer containing varying concentrations of a single crowding agent (Dextran 70, black; Ficoll 70, red; BSA, green; ovalbumin, blue) at 37°C, and enzymic activity measured after 4 h. The greater effect of the two protein crowding agents, compared with that of the polysaccharide crowding agents, indicates that other interactions in addition to steric exclusion might be contributing to the prevention of correct refolding by these particular agents. Abbreviation: BSA, bovine serum albumin. Reproduced, with permission, from Ref. 38.

different aggregation tendencies. The two states are termed oxidized (as two disulfide bonds are present) and reduced (where these bonds are in the thiol form). When unfolded oxidized lysozyme is diluted from denaturant it refolds into active enzyme with 100% efficiency within one second. Addition of natural or synthetic crowding agents to the refolding buffer has little effect on either the rate of folding or its yield (Fig. 4). By contrast, reduced lysozyme refolds very slowly with poor efficiency: only 20% of the chains produce active enzyme within 30 minutes; the remaining 80% aggregate into insoluble complexes. Addition of crowding agents completely abolishes the production of active enzyme from refolding reduced lysozyme, all the chains ending up instead as insoluble complexes (Fig. 4). Because hen lysozyme is synthesized naturally by cytosolic ribosomes in oviduct cells in the reduced form before transfer into the lumen of the endoplasmic reticulum, the question arises as to how the strong tendency of reduced lysozyme to aggregate under crowded conditions is combated.

A plausible answer is provided by the observation that the aggregation produced in both the presence and absence of crowding agents is decreased more than tenfold when the refolding buffer is supplemented with protein disulfide isomerase[38], the most abundant protein in the endoplasmic reticulum lumen. This protein acts both as an enzyme to speed the formation of disulfide bonds and as a molecular chaperone; both these activities reduce aggregation. Other experiments show that the chaperone activity of protein disulfide isomerase is enhanced by the presence of crowding agents[38]. An interesting observation is that at concentrations of crowding agent low enough to permit some reduced chains to escape aggregation, the rate of correct folding of these chains is increased by up to fivefold compared with the rate in uncrowded buffers. This increase in the intrinsic folding rate can be explained in terms of the stabilization of compact refolding intermediates by the effect of excluded volume[39]. Such experiments need extending to other proteins, but the implication is that some polypeptides might fold faster inside the cell than they do in the test tube because of this effect of crowding. Thus, crowding is not all bad – it has positive as well as negative effects on the assembly of functional proteins.

Other examples of the positive effects of crowding are provided by studies on the assembly and functioning of GroEL. This molecular chaperone assembles from 56-kDa ATPase subunits into two stacked rings, each containing seven subunits, enclosing a cavity large enough to contain a polypeptide chain of up to 60 kDa in size. Each cavity acts as an 'Anfinsen cage'; that is, as a molecular test tube inside which a single polypeptide chain can continue to fold as in a classical Anfinsen refolding experiment, but with no danger of aggregation[40–42]. The functional GroEL tetradecamer reassembles to ~20% recovery after complete denaturation in urea, provided adenine nucleotides are present in the refolding buffer. This recovery increases to 70% when Ficoll 70 at 300 g l^{-1} is added to the refolding buffer as a crowding agent[43]. This stimulation of correct assembly can be interpreted in terms of the excluded volume favouring the most compact state, and supports earlier studies with other proteins and nucleic acids indicating that crowding favours the association of macromolecules[13]. The observation that crowding agents do not enhance aggregation in this system, unlike the case for lysozyme, could be explained by the twofold-slower diffusion rate of the 56-kDa GroEL chains compared with that of the 14-kDa lysozyme chains. This reduction of diffusion by crowding might allow the slower-moving GroEL chains to fold correctly before they can encounter other partly folded GroEL chains; this interpretation is supported by the fact that when folding is slowed by omitting nucleotides, Ficoll 70 causes the chains to aggregate[43].

An essential partner for the correct functioning of GroEL is the heptameric oligomer GroES that acts as a cap on the Anfinsen cage to prevent the folding polypeptide from diffusing out into the fluid phase of the cytoplasm, which it would otherwise do at a rate much faster than the rate of folding. Inside the closed cage, folding proceeds for a length of time that is set by an ATPase activity that releases GroES; this time

is ~10 seconds at 37°C. But, what about polypeptides that fold slowly, which have not internalized all their hydrophobic regions within this time? Release of such partly folded polypeptides into the fluid phase would run the risk of causing aggregation. Such release has indeed been observed when the GroEL–GroES system is studied in uncrowded buffers *in vitro*, but it is prevented by the addition of crowding agents at 200 g l^{-1}; instead, the polypeptide rebinds to the same GroEL oligomer it has just left[44]. The promotion of protein association by crowding thus overcomes the problem presented by aggregation-prone polypeptides that fold on a time scale longer than one sojourn in the Anfinsen cage[45].

Conclusions

Both experiment and theory support the view that crowding exerts profound quantitative effects on macromolecular interactions in living systems. Because crowding influences macromolecular association and conformation, it will play a role in all biological processes that depend on noncovalent associations and/or conformational changes, such as protein and nucleic acid synthesis, intermediary metabolism and cell signalling[46], gene expression and the functioning of dynamic motile systems. Thus, the persistent neglect of this property by biochemists should be remedied. There is a need to change the mindset that regards crowding as an obscure phenomenon that can safely be ignored, and that asks why elegant *in vitro* experiments should be complicated by the addition of apparently inert and irrevelant macromolecules to simple dilute buffer solutions.

The answer to this question is that living systems are ubiquitously crowded at the biochemical level. This crowding does not seem to be an epiphenomenon because the high non-linearity of crowding effects generates an exquisite sensitivity of macromolecular properties to small changes in their environment that is plausibly a *sine qua non* for the living state[13]. It is hard to imagine that biochemical systems could ever operate effectively if the total concentration of macromolecules inside cells was only 1–10 g l^{-1}. The increasing number of new proteins being uncovered by genome sequence analyses makes this a particularly appropriate time for more researchers to add crowding to the menu of concepts upon which they base their studies of the macromolecular constituents of living cells.

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Structural properties of lipid-binding sites in cytoskeletal proteins

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Several cytoskeletal proteins have been shown to interact *in vitro* with, and in some cases are regulated by, specific membrane lipids. In some cases, evidence for *in situ* interactions has been provided. The molecular basis for such interactions is now being unravelled. At least five structurally distinct types of lipid-binding sites in cytoskeletal proteins have been identified. However, our understanding of the physiological role of such interactions is still limited. Precise knowledge about the binding-site structures and the actual amino acid residues involved should now enable the expression of mutant proteins that specifically lack the ability to interact with lipids. The impact of these mutations on protein location and function can then be assessed.

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The cytoskeleton, including actin filaments, intermediate filaments, microtubules and associated proteins, is an important ubiquitous cellular component that determines cell shape and is actively involved in motile events such as cell locomotion and adhesion. To influence these processes, the cytoskeleton must be anchored reversibly to the plasma membrane in a manner that is regulated by signalling events. Indeed, evidence for specific interactions with cytosolic domains of transmembrane proteins has been provided for several cytoskeletal proteins[1,2]. Evidence is also accumulating for the role of membrane lipids in directly recruiting cytoskeletal proteins to membrane domains. A variety of cytoskeletal proteins have been shown to associate *in vitro* with, and in some cases to insert into, lipid bilayers, without expressing secondary structures comparable with that of membrane-inserted domains of transmembrane proteins[3]. In a few studies, the existence of such interactions has been demonstrated in intact cells using hydrophobic photolabelling or immunoprecipitation followed by lipid extraction[4–6].

The *in vivo* relevance of such direct interactions is still far from clear. Relatively weak interactions with negatively charged phospholipids such as phosphatidylserine (PtdSer) could serve to enrich cytoskeletal proteins at the membrane, and to facilitate interactions with other proteins by converting a three-dimensional search of interaction partners into a two-dimensional process[7]. Tighter, more specific interactions could directly regulate protein function. This has been demonstrated *in vitro* for gelsolin and profilin. Gelsolin nucleates, caps and severs actin filaments, and profilin sequesters actin monomers. The activity of both proteins is regulated by phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2][8,9]. This regulation also appears to be relevant in intact cells[10]. Specific interactions with phosphoinositides (Box 1), whose level is regulated by signal transduction, could serve to recruit cytoskeletal proteins to specific membrane domains, so called 'lipid rafts', in which specific lipids and signalling enzymes are thought to accumulate[11]. This could ensure local activation of cytoskeletal proteins, for example by phosphorylation. To obtain more insights into the physiological role of interactions between cytoskeletal proteins and specific lipids, we need to obtain detailed information on the molecular structure of specific lipid-binding sites. This would enable the creation, by site-directed mutagenesis, of proteins lacking the ability to interact with lipids. Cellular expression of these mutated proteins should then provide information on the functional role of the lipid-binding sites. This article focuses on recent advances in the characterization of specific lipid-binding sites in cytoskeletal proteins at the molecular level and also on what these results reveal about the possible relevance of such interactions. At least five different types of binding sites have been identified so far. Features of these sites include clusters of basic amino acid residues without, or in conjunction with, a covalently bound fatty acid, classical

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