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CORRECTION

How can biochemical reactions within cells differ from those in test tubes?

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There was an error published in *J. Cell Sci.* **119**, 2863-2869.

There is a typographical error in equation (2). The right-hand side of this equation should read '$\Delta F_{I,AB} - (\Delta F_{I,A} + \Delta F_{I,B})$'.

JCS and the author apologise for any confusion that this error might have caused.

How can biochemical reactions within cells differ from those in test tubes?

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Summary

Nonspecific interactions between individual macromolecules and their immediate surroundings ('background interactions') within a medium as heterogeneous and highly volume occupied as the interior of a living cell can greatly influence the equilibria and rates of reactions in which they participate. Background interactions may be either repulsive, leading to preferential size-and-shape-dependent exclusion from highly volume-occupied elements of volume, or attractive, leading to nonspecific associations or adsorption. Nonspecific interactions with different constituents of the cellular interior lead to three classes of phenomena: macromolecular crowding, confinement and adsorption. Theory and experiment have established that predominantly repulsive background interactions tend to enhance the rate and extent of macromolecular associations in solution, whereas predominately attractive background interactions tend to enhance the tendency of macromolecules to associate on adsorbing surfaces. Greater than order-of-magnitude increases in association rate and equilibrium constants attributable to background interactions have been observed in simulated and actual intracellular environments.

Key words: Protein associations, Protein stability, Protein folding, Macromolecular crowding, Macromolecular confinement, Macromolecular adsorption

Introduction

Although the time when a living cell was regarded naively as a 'bag of enzymes' is long past, the extent to which the high internal concentration of macromolecules and the constraints of cellular architecture (see Fig. 1) can influence intracellular biochemical reactions is still not generally appreciated. In this Commentary, I briefly describe three mechanisms by which features of the cellular interior can significantly alter, in a largely nonspecific manner, the equilibria and rates of biochemical reactions relative to the equilibria and rates of the same reactions in dilute solution. The three mechanisms correspond to three different types of interactions between a particular macromolecular reactant (X) and constituents of the local environment (the background).

Types of background and background interactions

Macromolecular crowding

In some intracellular compartments, X may be surrounded by a variety of other soluble macromolecules that cumulatively occupy a significant fraction of the total volume (Fulton, 1982). Since no single macromolecular species need be present at high concentration, such media are referred to as crowded or volume occupied rather than concentrated (Minton and Wilf, 1981). Since macromolecules cannot interpenetrate, the fraction of volume into which a macromolecule can be placed at any instant is much less than the fraction of volume into which a small molecule can be placed (Fig. 2A,B). The total free energy of interaction between X and all of the other molecules in the crowded medium is inversely related to the probability of successful placement of X at a random location within the crowded medium (Lebowitz et al., 1965). Hence the extra work required to transfer X from a dilute to a crowded solution resulting from steric repulsion between X and background molecules depends upon the relative sizes and shapes of X and background species, and increases in a highly nonlinear fashion with increasing size of X and with fractional volume occupancy (Minton, 1998).

Macromolecular confinement

In eukaryotic cells, much of the fluid phase of cytoplasm exists within interstices between a variety of large fibrous and membranous structures (Knull and Minton, 1996). It has been estimated that between 10% and 100% of the fluid phase of the cytoplasm lies within one macromolecular diameter of the surface of one of these structures (Minton, 1989). By analogy with size exclusion columns, we may generically treat these interstitial elements of fluid volume as 'pores'. When the size of a pore is not much larger than that of an enclosed macromolecule reactant X, steric-repulsive interactions between X and the pore boundaries result in a reduction of the volume available to X (Fig. 2C,D). Hence work (free energy) is required to transfer a molecule of X from an element of unbounded solution into a pore of equal volume (Giddings et al., 1968). The magnitude of excess work depends strongly upon the relative sizes and shapes of both the confined macromolecule and the pore (Giddings et al., 1968; Minton, 1992).

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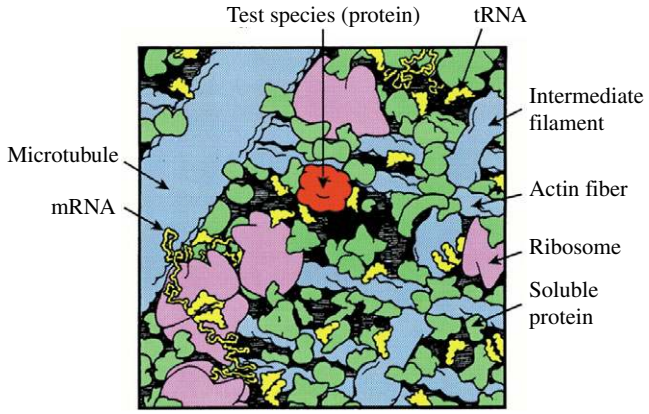

Fig. 1. Cartoon of eukaryotic cytoplasm magnified $10^6\times$. Reproduced from Minton (Minton, 2001a) and modified from Goodsell (Goodsell, 1993).

Macromolecular adsorption

When X bears a net charge opposite to that of the surface of a nearby fiber or membrane, X may be reversibly nonspecifically adsorbed onto the surface (Cutsforth et al., 1989; Knull and Walsh, 1992; Lakatos and Minton, 1991). Similarly, if X is a post-translationally modified protein bearing a lipid side chain, it may also reversibly and nonspecifically adsorb onto lipid bilayer membranes (Arbuzova et al., 1998). When surface adsorption is spontaneous, the associated free energy change is negative, but its magnitude depends on entropic factors that vary nonspecifically with the size and shape of X (Minton, 1995).

Influence of background interactions upon reaction equilibria and rates

Fig. 3 illustrates how nonspecific interactions between reactants and the background can influence the rate and/or equilibrium of a particular reaction – for example the association of two globular proteins, A and B, to form a heterodimer. In this reaction, the 'reactants' are A and B separated by a distance sufficiently large that they do not interact, and the 'product' is the heterodimer. In the 'transition state', A and B are close to each other and orientated such that they are poised to form the heterodimer, but they are still not bound to each other by short-range attractive interactions.

The free energy profile of a reaction taking place in bulk solvent may be altered when the same reaction takes place within a medium such as a cellular interior that is filled with other macromolecules, even if these macromolecules are nominally inert (i.e. they do not participate directly in the reaction. Individual pairwise interactions between a single molecule within the reaction and a molecule from the background need be neither strong nor specific. The density of macromolecules within cells is sufficiently high (~500 g/l in some compartments) that the sum of a large number of weak, nonspecific interactions can contribute substantially to the standard free energy of each state of the reaction system.

The free energy profile in green in Fig. 3 has been shifted downwards because all three states of the system interact with the background in an attractive (free energy lowering) fashion. Such nonspecific intermolecular attraction can be due to weak

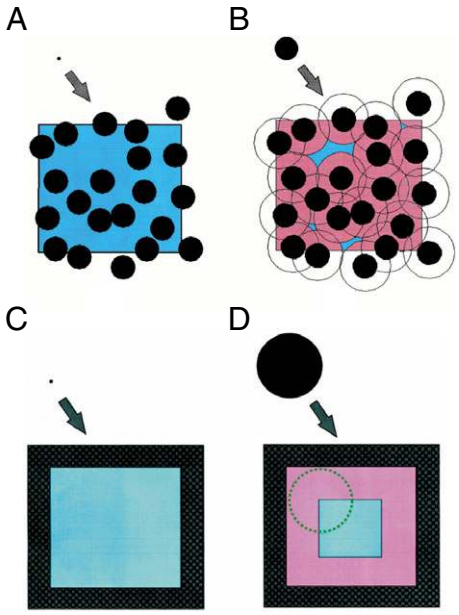

Fig. 2. Schematic illustration of volume available (blue) and volume excluded (pink and black) to the center of a (A,C) small and (B,D) large spherical molecule added to a solution containing a approximately 30% volume fraction of large spherical molecules (A,B) and the interior of a pore shown in (square) cross-section (C,D). Reproduced from Minton (Minton, 2001a).

electrostatic or hydrophobic effects and often results in the formation of weak, nonspecific complexes (e.g. the interaction of proteins with urea). The important feature of this profile is that the relative free energies of the three states of the system have also been altered: the background interactions stabilize the transition state and products more than they do the reactants. These background interactions should therefore push the equilibrium state towards product formation, primarily by enhancing the forward reaction rate.

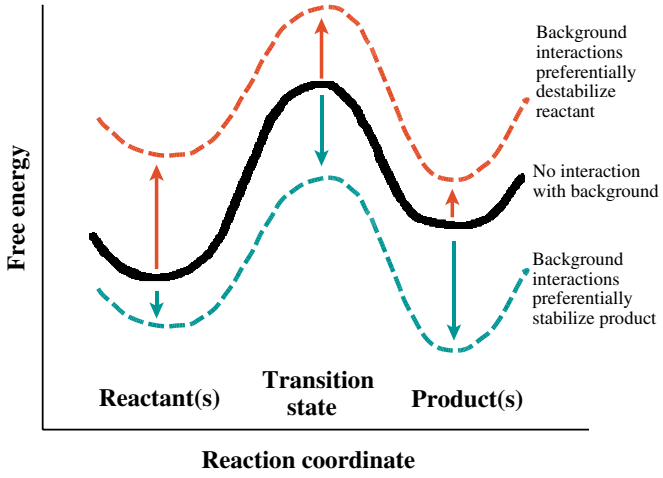

Fig. 3. Free energy profiles of a chemical reaction in bulk solution (black curve), in the presence of stabilizing background interactions (green), and in the presence of destabilizing background interactions (red).

By contrast, the free energy profile in red in Fig. 3 has been shifted upward because all three states of the system interact with the background in a repulsive (free energy raising) fashion. Nonspecific intermolecular repulsion can be due to volume exclusion (steric repulsion) or electrostatic effects and does not lead to formation of complexes between the mutually repelling species. Nevertheless, because the repulsive interactions in this example destabilize the reactant state more than they do the transition and product states, the overall effect upon the relative free energies of the three states is identical to that above. The equilibrium is shifted towards product formation, because of a preferential increase in the forward reaction rate. Note that even though the mechanisms underlying these two perturbations of the free energy profile are different, one cannot distinguish between them solely by measuring changes in reaction equilibria or kinetics.

There are many other combinations of repulsive and attractive background interactions that can lead to the same relative shifts in the free energies of the three reaction states and hence the same changes in reaction rates and equilibria. By considering the molecular composition and environmental variables characterizing a particular reaction system, one may discern whether dominant background interactions are likely to be attractive or repulsive. But in a medium as complex and heterogeneous as the cytoplasm for example, the task of identifying these and their influence on any specific reaction is extremely challenging.

A common energetic formalism

We may analyze the effects of all background interactions on a particular reaction in the context of a uniform thermodynamic picture. Consider, for example, the dimerization reaction introduced above. The apparent equilibrium constant* for association, $K_{AB} \equiv c_{AB}/c_A c_B$, is related to the standard free energy of association, ΔF_{AB}, by the thermodynamic relationship:

$$\Delta F_{AB} = -RT \ln K_{AB} , \quad (1)$$

where R denotes the molar gas constant and T the absolute temperature†. We may construct thermodynamic cycles that relate the free energies of association in the absence and presence of background interactions (Fig. 5), because free energy is conserved around the cycle:

$$\Delta F_{AB} - \Delta F^{\circ}_{AB} = \Delta F_{AB} - (\Delta F_{I,A} + \Delta F_{I,B}) , \quad (2)$$

where ΔF°_{AB} denotes the standard free energy of association of A and B in the absence of background interaction and $\Delta F_{I,X}$ denotes the standard free energy of interaction between X and the background. It follows from equations 1 and 2 that:

$$\frac{K_{AB}}{K^{\circ}_{AB}} = \exp\left(\frac{\Delta F_{I,A} + \Delta F_{I,B} - \Delta F_{I,AB}}{RT}\right) . \quad (3)$$

*The apparent equilibrium constant, defined as a function of the concentrations of solute species, is distinguished from the thermodynamic equilibrium constant, which is defined as a function of the thermodynamic activities of solute species. The relations presented in this section describe the effect of background interactions upon the apparent equilibrium constant for the selected reaction.

†Free energy changes denoted by ΔF may refer to either Gibbs or Helmholtz free energies since the relationships described here hold equally in constant pressure and constant volume systems.

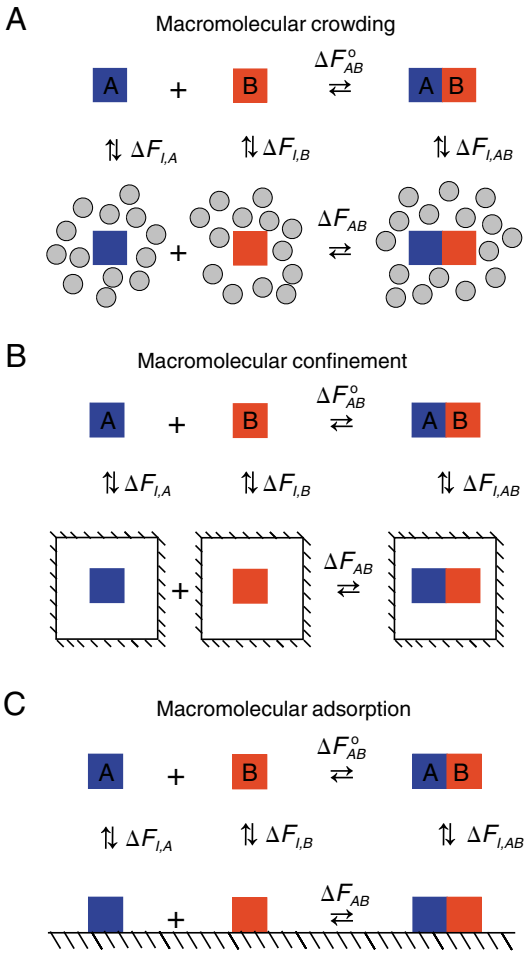

Fig. 4. Schematic illustration of how a single set of free energy relationships comprising a thermodynamic cycle may be used to analyze the influence of (A) macromolecular crowding, (B) macromolecular confinement, and (C) macromolecular adsorption upon macromolecular association equilibria.

It is evident that background interactions will increase the apparent equilibrium constant for association when the sum of free energies of the individual background interactions of A and B is more positive than the free energy of background interactions of the heterodimer AB. This result is independent of whether the absolute value of any individual $\Delta F_{I,X}$ is positive or negative (see Fig. 3).

Note that, although equations 1-3 are entirely general, each type of background interaction results in a distinctly different dependence of $\Delta F_{I,X}$ upon the size and shape of X and the sizes, shapes and fractional volume occupancies of those background constituents with which X interacts.

Predictions and observations

Approximate statistical-thermodynamic models of the effects of the background interactions upon macromolecular reactions have been developed (e.g. Minton, 1981; Minton, 1992; Minton, 1995; Zhou and Dill, 2001; Zhou, 2004). These are based upon mesoscopic or coarse-grained models that take into account the molecular nature of the individual reactant species, but adopt simplified representations of the molecules and the

Table 1. Predicted and observed effects of macromolecular crowding by high concentrations of inert macromolecules

Predicted effect	Relevant observations[§]
Enhancement of equilibrium association of dilute macromolecules (Minton, 1981; Nichol et al., 1981).	Large enhancement in the extent of sickle hemoglobin polymerization (Bookchin et al., 1999); large increase in affinity of DNA-binding proteins for DNA (Zimmerman and Harrison, 1987; Jarvis et al., 1990); enhanced self-association of spectrin (Lindner and Ralston, 1995), actin (Lindner and Ralston, 1997), fibrinogen (Rivas et al., 1999), tubulin (Rivas et al., 1999) and FtsZ (Rivas et al., 2001).
Acceleration of slow (transition state limited) protein associations (Minton, 1983; Minton, 2001a).	Large increases in the rate of fiber formation by sickle cell hemoglobin (Rotter et al., 2005), actin (Drenckhahn and Pollard, 1986), tubulin (Herzog and Weber, 1978) and fibrin (Wilf et al., 1985). Large increases in the rate of amyloid formation by apoCII (Hatters et al., 2002) and α-synuclein (Shtilerman et al., 2002; Uversky et al., 2002). Large increases in the rate of self-assembly of HIV capsid protein (del Alamo et al., 2005).
Deceleration of rapid (diffusion limited) protein associations (Zimmerman and Minton, 1993).	Reduction in rate of diffusion-limited association of TEM and BLIP (Kozer and Schreiber, 2004).
Stabilization of proteins against denaturation by heat or chaotropes (Cheung et al., 2005; Minton, 2000a).	Elevated temperatures for half-denaturation of actin (Tellam et al., 1983) and lysozyme (Sasahara et al., 2003). Elevated urea concentration for half-denaturation of FK-506-binding protein (Spencer et al., 2005). Partial restoration of catalytic activity of ribonuclease in the presence of high urea concentration (Tokuriki et al., 2004).
Acceleration of protein refolding to the native state (Cheung et al., 2005).	
Enhancement of aggregation of proteins that are partially or fully denatured (Hall and Minton, 2002; Hall and Minton, 2004).	Proteins that refold spontaneously in dilute solution aggregate in crowded solution and require chaperones to refold (Martin, 2002; van den Berg et al., 1999).

[§]Additional relevant observations reported prior to 2003 have been compiled in Minton, 1983; Zimmerman and Minton, 1993; Minton, 2001a; Hall & Minton, 2003.

Table 2. Predicted and observed effects of macromolecular confinement

Predicted effect	Relevant observations
Enhancement or inhibition of the association of confined macromolecules, depending upon complementarity of the shapes of oligomers and the confining cavity[¶] (Minton, 1992).	
Stabilization of native proteins against unfolding by heat or chaotropes. Magnitude of effect will increase as the size of the protein approaches the smallest dimension of the cavity (Zhou and Dill, 2001; Klimov et al., 2002).	Encapsulation of a variety of proteins in hydrated silica-sol-gel glasses (Eggers and Valentine, 2001) or in polyacrylamide gels (Bolis et al., 2004) increases the temperature of thermal denaturation. Encapsulation of ribonuclease T1 in reverse micelles increases temperature of thermal denaturation, and the denaturation temperature increases as the water content (volume?) of the micelle decreases (Shastry and Eftink, 1996).
Acceleration of refolding to the native state (Klimov et al., 2002).	

[¶]For example, a rod-like oligomer will fit more easily into a tubular cavity than a large spherical oligomer of the same stoichiometry (total volume).

interactions between them. Such models cannot account quantitatively for the behavior of macromolecules in environments as complex and intrinsically variable as the cytoplasm or nucleoplasm. However, they successfully predict large effects of 'inert' background substances upon a variety of reaction rates and equilibria in vitro (see Tables 1-3). In favorable cases, the magnitude of the observed effects agrees quantitatively with theoretical prediction. Data demonstrating the effects of crowding are shown in Fig. 5. Each of the effects was predicted qualitatively by one of the theories cited above.

Relevance to cell biology

Most predicted effects of macromolecular crowding, confinement, and adsorption on macromolecular isomerization equilibria and association equilibria and rates have been confirmed qualitatively (and in favorable cases quantitatively) in vitro[‡], but are such effects similarly important in a living cell? On the basis of results obtained from partitioning and size-exclusion experiments conducted with concentrated cell lysates, Zimmerman and Trach estimated that excluded volume effects in the cytoplasm of *E. coli* are comparable to those obtained in a 35% solution of a ~70 kDa globular protein, such as bovine serum albumin or hemoglobin (Zimmerman and Trach, 1991). Although this estimate might provide a useful starting point, it is clear that the answer is far more complex and elusive. Any cell is extremely large relative to any particular macromolecule of interest and is likely to contain several micro-environments, within each of which a particular macromolecule X will be subject to a different set of background interactions. For example, the cytoplasm of even an organism as simple as *E. coli* contains at least three such micro-environments: the immediate vicinity of the inner plasma membrane, within which X will encounter a high local concentration of membrane phospholipids and proteins; the interior and immediate vicinity of the nucleoid, within which X will encounter an extremely high local concentration of DNA; and the remaining cytoplasm, within which X will be

[‡]Predicted effects of macromolecular confinement on association equilibria have not yet been tested.

Table 3. Predicted and observed effects of macromolecular adsorption

Predicted effect	Relevant observations
Enhancement of equilibrium self- and/or hetero-association of adsorbed macromolecules (Minton, 1995).	Proteins that do not self-associate in solution form 2D crystals when nonspecifically adsorbed to surfaces (Darst et al., 1988).
Association of adsorbed macromolecules enhances adsorption capacity and may result in cooperative equilibrium adsorption isotherms (Chatelier and Minton, 1996; Minton, 2000b).	Equilibrium adsorption isotherms of proteins onto various types of surfaces exhibit positive cooperativity (Blanco et al., 1989; Cutsforth et al., 1989).
Association of adsorbed macromolecules can accelerate the maximum rate of adsorption and may result in cooperative adsorption progress profiles (Minton, 2001b).	Time-dependent adsorption of several proteins onto supported lipid bilayers and other surfaces exhibits a rate that increases with increasing surface occupancy (Fernandez and Berry, 2003; Nygren and Stenberg, 1990; Ramsden et al., 1994).
Adsorption can destabilize the solution conformation of a protein relative to alternate conformations that interact more strongly with the adsorbing surface (Minton, 1995; Cheung and Thirumalai, 2006).	Surface denaturation is a widely recognized obstacle to the purification and preparation of proteins at very low concentration (e.g. Edwards and Huber, 1992).

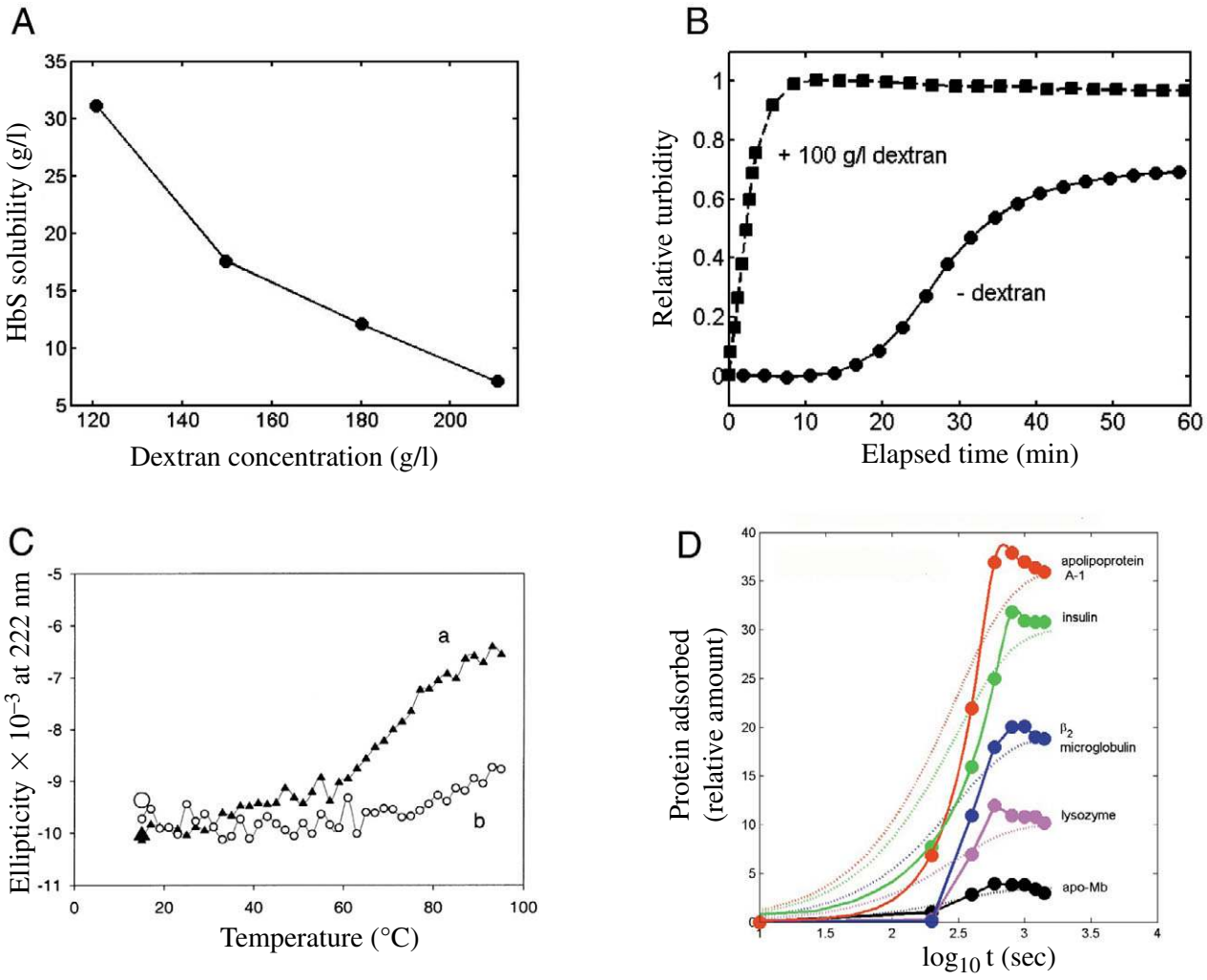

Fig. 5. (A) Dependence of the solubility of deoxy HbS upon dextran concentration (Bookchin et al., 1999). A 1.75-fold increase in dextran concentration results in a greater than four-fold increase in the equilibrium tendency of deoxy HbS to polymerize. (B) Time course of assembly of HIV capsid protein in the absence (●) and presence (■) of 100 g/l Ficoll 70 (del Alamo, 2005). The half-time for assembly of the protein is decreased ten-fold in the presence of Ficoll. (C) Temperature dependence of the ellipticity of α-lactalbumin in bulk solution (▲) and encapsulated in hydrated silica sol-gel glass (○). The confined protein behaves normally at low temperature, but exhibits only partial unfolding even at temperatures approaching 100°C. Figure reproduced with permission from Eggers and Valentine (Eggers and Valentine, 2001). (D) Time dependence of adsorption of several unrelated proteins to a supported phospholipid bilayer (symbols and solid curves) (Fernandez and Berry, 2003). The dotted curves indicate the time dependence that would be expected in the absence of interaction between adsorbed proteins. The enhanced steepness of the experimentally observed curves (solid) relative to the reference curves (dotted) indicates self-association resulting in clustering of the adsorbed proteins (Minton, 2001b).

subject primarily to the influence of other soluble proteins. One would expect the relative contributions of macromolecular crowding, confinement and adsorption to macromolecular reactivity to be rather different within each of these three micro-compartments. That complexity acknowledged, it is nevertheless true that large and unambiguous background effects have indeed been observed within certain specialized, simplified cells.

The interior of an erythrocyte is essentially a highly concentrated solution of hemoglobin. The affinity of erythrocytes containing sickle cell hemoglobin (HbS) for oxygen depends significantly upon the intracellular hemoglobin concentration, because oxygen binding is linked to the polymerization of HbS (May and Huehns, 1975). The concentration dependence of oxygen affinity may be quantitatively accounted for if, and only if, steric repulsion between hemoglobin molecules is taken into account (Minton, 1976). Ferrone has recently reviewed a large body of work demonstrating that the extensively characterized kinetics of cell sickling subsequent to deoxygenation may be well accounted for by models that take into account the substantial effect of macromolecular crowding within erythrocytes upon the thermodynamic activity (effective concentration) of monomers and each oligomer in the hemolyzate (Ferrone, 2004). By means of cleverly designed experiments, volume regulatory mechanisms in dog erythrocytes were shown to respond nonspecifically to changes in the intracellular concentration of macromolecules rather than changes in volume per se (Colclasure and Parker, 1992).

The interior of an eye lens cell consists essentially of a solution of a small number of crystallins at very high concentration (total protein concentration >500 g/l). These proteins are not translated postnatally. Thus, the crystallin molecules that an animal is born with must remain structurally intact over much of its lifetime to preserve lens transparency. The thermal stability of these proteins increases with concentration (Steadman et al., 1989), and the extraordinary thermal stability of an intact lens has been attributed in part to the stabilizing effects of macromolecular crowding inside the lens cell (Bloemendal et al., 2004).

Excluded volume theory predicts that at the high levels of fractional occupancy characterizing almost all biological fluid media, even small changes in cellular hydration will result in disproportionately large changes in the reactivity of a broad spectrum of macromolecular reactants. This prediction is consistent with and may account for the following general observations: (1) Relatively modest changes of cellular volume in animal cells are associated with changes in a wide variety of diverse intracellular processes, such as the polymerization/depolymerization of cytoskeletal filaments or the activation/deactivation of membrane ion channels, that are much too large to be accounted for on the basis of simple mass action (reviewed by Lang et al., 1998). (2) Complex and very diverse systems for maintaining the concentrations of cellular contents within narrow limits have evolved within all life forms, be they bacterial, plant or animal (Somero et al., 1992). (3) The age-related onset of a variety of protein-aggregation-linked diseases (Koo et al., 1999) correlates with significant loss of cellular and tissue hydration in the elderly and concomitant increases in the volume excluded to protein (Parameswaran et al., 1995; Hatters et al., 2002).

During the last few years several experimental techniques have been developed that enable certain aspects of the behavior of selected macromolecules – typically labeled and/or overexpressed – to be monitored within living cells (e.g. Ghaemmaghami and Oas, 2001; Ignatova and Gierasch, 2004; McNulty et al., 2006). It is hoped that techniques like these, or others yet to be devised, can be used to investigate the influence of background interactions upon the behavior of the selected species within their native environments. However, one must be extremely cautious about interpreting the results of such experiments. In particular, it is necessary to design and carry out control experiments that clearly indicate whether or not the processes of labeling and/or overexpression result in abnormal distribution of the selected protein within the cell, induction of artifactual associations, or disruption of the normal background interactions that one is attempting to investigate.

This Commentary bears as its title the provocative question "How can biochemical reactions within cells differ from those in test tubes?" When one can specify the composition of the local environment of a particular reaction at a specific intracellular location and at a particular time point in the cell cycle, studies such as those cited here will help to provide answers to the equally interesting and perhaps more biological question "By how much do biochemical reactions within cells differ from those in test tubes?"

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