Toward an understanding of biochemical equilibria within living cells

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Abstract

Four types of environmental effects that can affect macromolecular reactions in a living cell are defined: nonspecific intermolecular interactions, side reactions, partitioning between microenvironments, and surface interactions. Methods for investigating these interactions and their influence on target reactions in vitro are reviewed. Methods employed to characterize conformational and association equilibria in vivo are reviewed and difficulties in their interpretation cataloged. It is concluded that, in order to be amenable to unambiguous interpretation, in vivo studies must be complemented by in vitro studies carried out in well-characterized and controllable media designed to contain key elements of selected intracellular microenvironments.

Keywords Intermolecular interactions · Excluded volume · Macromolecular crowding · Surface interactions · Partitioning · Conformational equilibria · Association equilibria

Introduction

Over 10 years ago, one of us (APM) wrote a commentary entitled “How can biochemical reactions within cells differ from those in test tubes?” (Minton 2006), in which several physical-chemical mechanisms demonstrated to affect the kinetics and equilibria of macromolecular reactions in thermodynamically nonideal and heterogeneous media were described and discussed. Since that time, a substantial number of experimental and simulation studies have been conducted with the aim of answering, at least in part, the corollary and more immediate biological questions of *how much* and *why* reactions in cells differ from those measured in test tubes.

In the first part of this review, we summarize the present knowledge about environmental factors influencing macromolecular equilibria in vitro. These factors include: (1) non-specific repulsive and attractive solute–solute interactions that do not lead to complexation, (2) stronger attractive solute–solute interactions leading to side reactions, (3) the partitioning of reactants and products between different microenvironments, and (4) interaction of soluble macromolecules with surfaces. In the second part of this review, we discuss recent experimental and simulation studies of macromolecular equilibria in vivo and examine whether and to what extent these studies provide information about the underlying basis of observed phenomena.

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Definition and classification of interactions

It is facile to declare that the intracellular environment influences a particular macromolecular reaction through various interactions between constituents of the environment and the designated reactants and products. But what do we mean by the term “interaction”? The most general definition of intermolecular interaction is the sensitivity of one molecule to the presence of a second molecule. An interaction so defined may be either repulsive or attractive, specific or nonspecific, result in complex formation or not, and derive from one or more elementary physical forces, as shown in Fig. 1.

Fig. 1 A schematic illustration of how noncovalent interactions may be classified along the interaction free energy scale

The term *nonspecific* is used here to denote those interactions that are not directly related to the biological functions of the interacting partners, but arise from general physical properties, such as molecular size and shape, the magnitude and disposition of residue charges, and the disposition of polar and nonpolar residues on the surface. Nonspecific interactions, if attractive, may lead to transient clustering of proteins but do not lead to well-formed complexes. The cumulative effect of nonspecific interactions at high macromolecular concentration is often referred to as *macromolecular crowding* (Gnutt and Ebbinghaus 2016; Kuznetsova et al. 2014; Shahid et al. 2017; Rivas and Minton 2016; Theillet et al. 2014; Zhou et al. 2008). The terms *site-specific* or *specific* denote those interactions—typically multivalent—leading to well-formed complexes, both stable and transient, essential to biological function, such as those formed between a protein and a targeted kinase or phosphatase, or two proteins acting sequentially in an electron transport chain. The term *quinary interaction* is discussed in the [Appendix](#).

As the free energy of attractive interaction between a designated reactant or product and a species of molecule in the environment becomes increasingly negative, at some point—to some degree, a matter of definition (Hill and Chen 1973)—the complex formed between the two species may be regarded as an additional reactant or product. In that event, the interaction between the environmental component and the designated reactant may be considered a *side reaction*, and the formalism by which one analyzes modulation of the designated reaction in the presence of that environmental species should take into explicit account the additional complex or complexes. We shall discuss nonspecific interactions and side reactions separately.

How can nonspecific solute–solute interactions affect a specific macromolecular reaction in living cells?

Most of the following summary is derived from general relations first presented by Minton (1981, 1983) and is included

here as a tutorial guide to understanding topics to be discussed subsequently.

Conformational equilibria

Consider the interconversion between two conformational states O (open) and C (closed). These might correspond to two conformations of a native protein, or the unfolded and native states of a small two-state folding protein or RNA.

In a dilute solution under a defined set of conditions (i.e., fixed temperature, pressure, and solvent), we define a standard state free energy change and associated equilibrium association constant:

$$\Delta G_{OC}^0 = -RT \ln K_{OC}^0 \quad (2)$$

where R denotes the molar gas constant and T the absolute temperature. Now we consider a second solution under the same conditions, containing the same dilute reactant and product, but containing, in addition, an arbitrary (but defined) amount of one or more macromolecular cosolutes, which we shall refer to as the crowded medium. In this solution, the standard state free energy change accompanying the conformational change is:

$$\Delta G_{OC} = -RT \ln K_{OC} \quad (3)$$

These two free energy changes are linked by the thermodynamic cycle shown in Fig. 2a, where $\Delta G_i^{\text{transfer}}$ denotes the free energy associated with the transfer of a molecule of species i from the dilute to the crowded medium, expressed in units of energy/mol.

According to this cycle:

$$\ln \left(\frac{K_{OC}}{K_{OC}^0} \right) = \Delta G_{OC} - \Delta G_{OC}^0 = \Delta G_O^{\text{transfer}} - \Delta G_C^{\text{transfer}} \quad (4)$$

when free energy changes are specified in units of RT .

Fig. 2 Thermodynamic cycles illustrating the effect of nonspecific interactions on a conformational equilibrium (a) and an association equilibrium (b)

Association equilibria

For pedagogic reasons, we shall present here the thermodynamic formalism appropriate to the simplest reaction involving two reactant species, namely the formation of a single binary complex. The interested reader is referred to Minton (1981, 1983) for a more general treatment.

As in the case of conformational equilibrium, we define the free energies of association in the dilute and crowded media:

$$\Delta G_{AB}^0 = -RT \ln K_{AB}^0 \quad (6)$$

and:

$$\Delta G_{AB} = -RT \ln K_{AB} \quad (7)$$

respectively. These two equilibrium constants are linked according to the thermodynamic cycle illustrated in Fig. 2b. According to this cycle:

$$\ln \left(\frac{K_{AB}}{K_{AB}^0} \right) = \Delta G_{AB} - \Delta G_{AB}^0 = \Delta G_A^{\text{transfer}} + \Delta G_B^{\text{transfer}} - \Delta G_{AB}^{\text{transfer}} \quad (8)$$

when free energies of transfer are specified in units of RT .

Thermodynamics of transfer

The free energy required to transfer a molecule of solute species i from a highly dilute solution of that species alone to the crowded medium may be decomposed to its enthalpic and entropic contributions:

$$\Delta G_i^{\text{transfer}} = \Delta H_i^{\text{transfer}} - T \Delta S_i^{\text{transfer}} \quad (9)$$

In Table 1, the contributions of the various physical mechanisms of interaction between the molecule of species i and constituents of the medium contributing to the free energy of transfer of species i are enumerated and their respective

influences tabulated. We note that *any kind of interaction*, whether it be attractive or repulsive, will result in a reduction of entropy relative to the reference state of no interaction, since the interaction provides an increase in the order of the system.

The total free energy of transfer may be accordingly partitioned into contributions from various types of interactions:

$$\Delta G_i^{\text{transfer}} = \Delta G_i^{\text{excluded volume}} + \Delta G_i^{\text{electrostatic}} + \Delta G_i^{\text{H-bonding}} + \Delta G_i^{\text{solvent mediated}} \quad (10)$$

As may be seen from Table 1, $\Delta G_i^{\text{excluded volume}}$ will always be positive, $\Delta G_i^{\text{H-bonded}}$ and $\Delta G_i^{\text{solvent mediated}}$ will generally be negative, and $\Delta G_i^{\text{electrostatic}}$ may be positive or negative. Thus, the transfer free energies appearing in Eqs. (4) and (8) may, in principle, be greater than or less than zero, and interactions between the reactant and product species in reaction (1) may shift the equilibrium toward reactant(s) or product(s), depending upon the sum of free energies of the interaction indicated in Eq. (10).

In summary, if we want to know *how much* nonspecific interactions between nonreacting background molecules and the reactants and products of a designated target reaction affect the equilibrium of that reaction, Eqs. (4) and (8) tell us that we have to be able to measure both K_{OC} and K_{OC}^0 in the case of conformational equilibria, and both K_{AB} and K_{AB}^0 and in the case of association equilibria. But if we want to know *why* the reactions differ, Eq. (10) tells us that we must identify and quantify the most significant interactions between constituents of the background and each reactant and product of the target reaction.

How can side reactions in solution affect a specific macromolecular reaction in living cells?

For simplicity, we treat the example reactions (1) and (5) in the ideal limit, i.e., in the absence of nonspecific interactions.

Table 1 Contributions of various types of interactions to the free energy of transfer (modified from Minton 1983)

Type of intermolecular interaction	$\Delta H_i^{\text{transfer}}$	$\Delta S_i^{\text{transfer}}$	$\Delta G_i^{\text{transfer}}$
No interaction (ideal)	0	0	0
Attractive (electrostatic, H-bonding, hydrophobic/hydrophilic)	< 0	< 0	< 0, ~ 0, > 0
Repulsive (electrostatic)	> 0	< 0	> 0
Excluded volume	0	< 0	> 0

Conformational equilibrium

It is conceivable that both O and C could form multiple complexes with additional components of the immediate environment. In a simple example, let us postulate that each of these species can associate with a single molecule of a third species present in the microenvironment, denoted X, as in Fig. 3a. In the absence of X, the equilibrium between O and C is specified by:

In the presence of X, the following additional side reactions must be specified:

Combination of the three equilibrium relations leads to an expression for the experimentally measured ratio of product and reactant concentrations:

$$K_{OC} \equiv \frac{[C]_{\text{total}}}{[O]_{\text{total}}} = K_{OC}^0 \frac{1 + K_{CX}[X]}{1 + K_{OX}[X]} \quad (13)$$

It is apparent that the ratio K_{OC} is not a true equilibrium constant, as it depends upon the concentration of the side reactant X (which may depend upon the microenvironment), if K_{CX} differs significantly from K_{OX} .

Association equilibrium

In the absence of side reactants, the association equilibrium is specified by the relation:

In a simple example of side reactions, let us postulate that each of the reactant and product species may associate reversibly with a single molecule of the background species X, as illustrated in Fig. 3b. In the presence of X, the following additional side reactions must be specified:

Combination of relations (14) and (15) leads to the following expression for the experimentally determined ratio of product to reactant concentrations:

$$K_{AB} \equiv \frac{[AB]_{\text{total}}}{[A]_{\text{total}}[B]_{\text{total}}} = K_{AB}^0 \frac{1 + K_{ABX}[X]}{(1 + K_{AX}[X])(1 + K_{BX}[X])} \quad (16)$$

As in the case of the conformational equilibrium, the ratio K_{AB} is not a true equilibrium constant, since it will vary with [X] (which may depend upon the microenvironment) unless all three of the quantities $K_{AX}[X]$, $K_{BX}[X]$, and $K_{ABX}[X]$ are $\ll 1$, i.e., binding of any reactant or product to X is negligible.

How can the distribution of reactants and products in an inhomogeneous medium affect a specific macromolecular reaction in living cells?

When one looks closely at subcellular regions of the cellular interior, it is evident that these regions may vary

Fig. 3 Reaction schemes illustrating the effect of side reactions on a conformational equilibrium (a) and an association equilibrium (b)

significantly in their local composition (Luby-Phelps 2013; Rivas and Minton 2016). We refer to subcellular regions that are small enough to be relatively homogeneous in content, yet large relative to molecular dimensions as *microenvironments*. The following discussion focuses on those microenvironments that are open to the exchange of macromolecular reactants and products.

Conformational equilibrium

Let each of the reactant species distribute between two microenvironments, denoted 1 and 2. For example, microenvironment 1 might be an aqueous phase and microenvironment 2 might be a lipid phase. In the ideal limit, we define the partition coefficients of each species:

$$K_Z^{(1 \rightarrow 2)} \equiv [Z]_{(2)} / [Z]_{(1)} \quad (17)$$

where $[Z]_{(i)}$ denotes the equilibrium concentration of species Z in microenvironment i . The partition coefficient of species Z is a function of the free energy change associated with transfer of a molecule of Z from microenvironment i to microenvironment j :

$$\Delta G_Z^{(i \rightarrow j)} = -RT \ln K_Z^{(i \rightarrow j)} \quad (18)$$

The thermodynamic cycle relating concentrations of each species in each of the two microenvironments is illustrated in Fig. 4a. The experimentally measured ratio of product to reactant concentrations is then given by:

$$K_{OC} \equiv \frac{[C]_{total}}{[O]_{total}} = K_{OC}^{(1)} \frac{1 + f_2 (K_C^{(1 \rightarrow 2)} - 1)}{1 + f_2 (K_O^{(1 \rightarrow 2)} - 1)} \quad (19)$$

where $K_{OC}^{(i)}$ denotes the value of K_{OC} measured in microenvironment i and f_2 denotes the fraction of the total system

volume occupied by microenvironment 2. Clearly, the measured ratio K_{OC} is not a true equilibrium constant, as it will depend upon f_2 if $K_O^{(1 \rightarrow 2)}$ differs significantly from $K_C^{(1 \rightarrow 2)}$. It follows from Eq. (17) that:

$$\frac{K_{OC}^{(2)}}{K_{OC}^{(1)}} = \frac{K_C^{(1 \rightarrow 2)}}{K_O^{(1 \rightarrow 2)}} \quad (20)$$

where $K_{OC}^{(2)}$ denotes the conformational equilibrium constant measured in microenvironment 2.

Association equilibrium

Let each of the reactant and product species distribute into microcompartments 1 and 2, as illustrated in Fig. 4b. In the ideal limit, the partition coefficients of each species are specified by Eq. (17). Combination of Eqs. (17) and (18) leads to the experimentally measured ratio of the concentrations of product and reactants:

$$K_{AB} \equiv \frac{[C]_{total}}{[A]_{total}[B]_{total}} = K_{AB}^{(1)} \frac{1 + f_2 (K_{12,AB} - 1)}{[1 + f_2 (K_{12,A} - 1)][1 + f_2 (K_{12,B} - 1)]} \quad (21)$$

As is the case of conformational equilibria, the measured ratio of product and reactant concentrations, K_{AB} , is not a true equilibrium constant, as it may vary significantly with f_2 if one or more of the partition coefficients differs significantly from 1. It follows from Eq. (21) that:

$$\frac{K_{AB}^{(2)}}{K_{AB}^{(1)}} = \frac{K_{AB}^{(1 \rightarrow 2)}}{K_A^{(1 \rightarrow 2)} K_B^{(1 \rightarrow 2)}} \quad (22)$$

Both Eqs. (20) and (22) indicate that the equilibrium constant for a designated reaction may, in principle, vary widely between different microcompartments, depending upon the distribution of reactants and products between the compartments.

Fig. 4 Reaction schemes illustrating the effect of partitioning between microenvironments on a conformational equilibrium (a) and an association equilibrium (b)

How can surface interactions affect a specific macromolecular reaction in living cells?

Surface interactions represent a special case of partitioning, as illustrated in Fig. 5. In the ideal limit, we may define the adsorption coefficient, relating the concentrations of a given species in the bulk and on the surface:

$$K_X^{adsorb} = [X]_{surface} / [X]_{bulk} \quad (23)$$

The adsorption coefficient so defined is equivalent to the partition coefficients defined in Eqs. (15) and (19), and the reaction schemes for conformational and association equilibria, illustrated in Fig. 5, are thermodynamically equivalent to the analogous schemes illustrated in Fig. 4.

A macromolecule in the immediate vicinity of a membrane surface is in a “surface microenvironment” significantly different from that remote (relative to molecular dimensions) from the surface. For example, the surface microenvironment adjacent to a biological membrane is highly enriched in phospholipid headgroups and the cytoplasmic domains of intrinsic membrane proteins. The membrane itself may consist of various domains of distinct composition (Carquin et al. 2016) and present multiple surface microenvironments. The same general concept applies to the surface of a large quasi-static structure, such as a microtubule or an actin fiber. It follows that, in a medium containing significant surface area, the experimentally measured ratio of product to reactant concentrations is not a true equilibrium constant, as it may vary substantially with the amount(s) and type(s) of accessible surface.

The volume of a surface microenvironment may be estimated as the product of the area of the surface and the range of interaction between the membrane and the soluble macromolecule of interest (Hoppe and Minton 2015; Minton 1995a). The fraction of soluble cytoplasm that might be considered to lie within a surface microenvironment has been crudely estimated. It is dependent upon the cell type and the size of the macromolecule, and in the case of large macromolecules or macromolecular complexes, may approach

unity (Minton 1990). Depending upon whether the interaction between the surface and the soluble macromolecule of interest is net attractive or net repulsive, the partition coefficient relating bulk to surface concentration may be greater than or less than unity.

The nonnegligible contribution of surface interactions to the distribution of protein within an intact cell is illustrated by several observations. The association of several glycolytic proteins with cytoskeletal filaments in vitro and in intact cells is well documented (Clegg 1984; Knull and Minton 1996). Green et al. (1965) reported that most or all of the glycolytic enzymes in erythrocytes and in yeast were membrane-associated. Kempner and Miller (1968) reported the remarkable observation that, following gentle centrifugation of the intact one-celled organism *Euglena gracilis*, all of the enzymes assayed were associated with sedimented particulates—no measurable enzyme activity remained in supernatant aqueous cytosol.

The phenomenon of protein denaturation at surfaces (Gray 2004) is just one example of how surface interactions can affect conformational equilibria. Theoretical models predict enhancement of protein association and oligomerization at surfaces (Hoppe and Minton 2015; Minton 1995a) and has been observed experimentally in a variety of protein–surface model systems (Burke et al. 2013; Herrig et al. 2006; Knight and Miranker 2004; Langdon et al. 2013; Melo et al. 2014; Risør et al. 2017). These examples of macromolecular associations that proceed more rapidly or to a greater extent on surfaces than in solution suggest that the consequences of localization via adsorption may be a general phenomenon with important implications in heterogeneous physiological environments.

Combination of environmental effects

In the preceding sections, each category of environmental effect on archetypical reactions was considered separately. In a real cellular interior, one would expect a combination of these effects to influence a studied biomolecular reaction. Consideration of multiple effects poses a theoretical challenge,

Fig. 5 Reaction schemes illustrating the effect of surface interactions on a conformational equilibrium (a) and an association equilibrium (b)

and attempts to treat combinations of effects have, so far, assumed that the free energy change brought about by a combination of effects (for example, excluded volume and longer ranged attractive or repulsive interactions) is the sum of the free energy changes brought about by each effect individually (Jiao et al. 2010; Minton 2013; Shkel et al. 2015). Minton and co-workers have introduced approximate analytical models according to which repulsive and weak attractive nonspecific interactions between molecules are treated as the interaction between equivalent hard convex particles, the dimensions of which are a parameterization of the weak nonspecific interactions (Minton 1995b, 2007; Minton and Edelhoch 1982), and stronger attractive interactions are treated as the formation of complexes of the equivalent particles (Jiménez et al. 2007; Rivas et al. 1999). More recently, repulsive and weakly attractive interactions have been treated in the square well approximation (Hoppe and Minton 2016; Minton 2017). Utilizing these approximate models, the combined effects of excluded volume and binding upon self-association and hetero-association have been explored (Fodeke and Minton 2011; Jiao et al. 2010; Minton 2013). Calculation of the combined effects of excluded volume and surface interaction upon protein fibrillation have been recently reported (Hoppe and Minton 2015). It was predicted that excluded volume effects in solution will strongly enhance both protein adsorption and fibrillation.

How are environmental effects on macromolecular interactions and reactions studied in vitro?

Effects of environment on the free energy of solute transfer

The total free energy of transfer of a solute from a dilute solution to a complex medium was partitioned into contributions from different physical forces in Eq. (10). From another point of view, we may partition that free energy into contributions from solution interactions and surface interactions:

$$\Delta G_i^{\text{transfer}} = \Delta G_i^{\text{solution}} + \Delta G_i^{\text{surface}} \quad (24)$$

If the free energy of interaction between two macromolecular species is attractive and sufficiently strong under a particular set of experimental conditions, it may be evaluated through analysis of the composition dependence of reversible association. Methods for the characterization of associations in dilute or near-ideal solutions are numerous and have been widely reviewed (Monterroso et al. 2013; Podobnik et al. 2016; Zhou et al. 2016). Similarly, if the free energy of interaction between a soluble macromolecule and a surface is attractive and sufficiently strong under a particular set of experimental conditions, it may be evaluated through analysis of the

composition dependence of adsorption (Chatelier and Minton 1996). Methods for characterizing adsorption equilibria and kinetics in dilute or near-ideal solutions are reviewed elsewhere (Gray 2004; Rabe et al. 2011; Ramsden 1994).

Obtaining information about weak interactions in solution is more challenging to the experimenter, as such interactions may not lead to complexes and, in general, are only manifest in solutions that are highly concentrated. Under these conditions, large deviations from thermodynamic ideality must be taken into account. The composition-dependent free energy of transfer of proteins from dilute to concentrated or crowded solutions may be obtained by analyzing the concentration dependence of thermodynamically based colligative properties, e.g., sedimentation equilibrium (SE), static light scattering, and osmotic pressure (Fodeke and Minton 2010; Jiménez et al. 2007; Minton and Edelhoch 1982; Rivas et al. 1999; Ross and Minton 1977; Wu and Minton 2015; Zorrilla et al. 2004a).

Obtaining information about weak and possibly repulsive interactions between macromolecules and surfaces is even more challenging, as the high bulk solution concentrations that may be required to achieve measurable occupancy of the surface layer can interfere with conventional methods of measuring adsorption cited above. One promising method is total internal reflection fluorescence microscopy, which permits the amount of a fluorescently labeled trace species within a defined distance of a surface to be measured, independent of the amount of that species far from the surface (Boehm et al. 2016; Jung et al. 2009). In order to interpret results obtained from measurements of macromolecular adsorption, it is also necessary to obtain independent information about self-interaction of the macromolecule in bulk as well as with the surface (Chatelier and Minton 1996; Monterroso et al. 2013).

Effects of environment on conformational and association equilibria of tracer reactions

The characterization of dilute macromolecules and their reactions in a solution containing high concentrations of other macromolecules presents an even greater experimental challenge, as the behavior of the dilute species must be monitored in the presence of far higher concentrations of crowding species. Changes in UV-visible absorbance, circular dichroism, and native fluorescence intensity have been used to characterize macromolecular conformational stability (see, for example, Christiansen et al. 2010; Sasahara et al. 2003; Tokuriki et al. 2004) and protein self-association (Aguilar et al. 2011) in the presence of optically transparent crowding agents.

When the crowding agent or agents absorb UV light, tracer methods must be employed to study environmental effects on macromolecular reactions. A particular component can qualify as a tracer if it has a uniquely detectable signal (i.e., enzymatic activity) or if it can be provided with a unique signal by means of labeling (fluorescent, isotopic). It is incumbent upon

those employing tracer methods to investigate whether the labels used to identify the dilute species in the concentrated medium affect the interaction between the labeled species and the unlabeled components of the medium and the chemical reactions in which the labeled species participate. This may be done by varying the ratio of labeled to the unlabeled parent species and investigating the possibility of different behavior, or by varying the label. If (*and only if*) the labeled tracer has been found to be nonperturbing, then several techniques may be used to characterize the behavior of labeled species in concentrated solutions.

Changes in the specific activity of an enzyme have been used to characterize crowding effects on conformational equilibria (Dhar et al. 2010; Paudel and Rueda 2014) and self-association (Minton and Wilf 1981). Changes in fluorescence resonance energy transfer (FRET) have been used to characterize crowding effects on conformational equilibria (Dhar et al. 2010; Nagarajan et al. 2011; Paudel and Rueda 2014; Tai et al. 2016). Changes in fluorescence intensity (Kozer et al. 2007), fluorescence depolarization (Reija et al. 2011; Wilf and Minton 1981; Zorrilla et al. 2004b), and fluorescence correlation spectroscopy (Reija et al. 2011) have been employed to detect and measure protein association equilibria in crowded solutions. Neutron diffraction was utilized to characterize changes in the conformation of deuterium-labeled polyethylene glycol as a function of the concentration of unlabeled solutions of the synthetic polymer Ficoll 70 (Le Coeur et al. 2009, 2010). Changes in the weight-average molar mass, as monitored by nonideal tracer sedimentation equilibrium (Rivas and Minton 2004), were used to characterize the extent of self-association of isotopically labeled tracer proteins in crowded solutions as a function of crowder concentration (Rivas et al. 1999, 2001; Rivas and Minton 2004). Changes in NMR-detected amide deuterium–proton exchange rates have been used to monitor the stability of deuterium-labeled proteins in crowded solutions (Miklos et al. 2009, 2010; Wang et al. 2012) and the self-association of labeled ribonuclease in crowded solutions (Ercole et al. 2011).

How are macromolecular reactions and interactions detected and characterized within a living cell?

Almost all of the studies of “in-cell crowding” reported to date follow the overall strategy summarized below:

1. *A labeled macromolecule, or a pair of fluorescently or isotopically labeled macromolecules, are introduced into the cell via expression of recombinant genes, transfection, or microinjection.* Fluorescent labels are either dyes that are covalently attached to the macromolecule (Gao et al. 2016; Gnutt et al. 2015; Ignatova and Gierasch 2004) or fluorescent proteins fused to the N- and/or C-terminal of the target macromolecule (Boersma et al. 2015; Ebbinghaus et al. 2010; Ebbinghaus and Gruebele 2011; Phillip et al. 2012; Shi et al. 2009). NMR studies are carried out using N^{15} - and F^{19} -labeled proteins (Miklos et al. 2011; Smith et al. 2016).
2. *A signal is monitored that changes with the conformation or association state of the target protein(s).* Signals monitored in vivo have been changes in fluorescence intensity and emission wavelength (Ignatova and Gierasch 2004; Phillip et al. 2012), FRET (Ebbinghaus et al. 2010; Ebbinghaus and Gruebele 2011; Gao et al. 2016; Gnutt et al. 2015; Guzman and Gruebele 2014), fluorescence cross-correlation (Shi et al. 2009; Sudhakaran et al. 2009), chemical shift (Smith et al. 2016) and hydrogen–deuterium exchange kinetics (Miklos et al. 2011; Monteith et al. 2015).
3. *A global perturbation of the cell is applied.* These perturbations have included changes in the intracellular concentration of chaotropes (Ignatova and Gierasch 2004), changes in temperature (Ebbinghaus et al. 2010; Guzman et al. 2014; Smith et al. 2016), and changes in cell volume (Boersma et al. 2015; Gnutt et al. 2015; Konopka et al. 2009; Sukenik et al. 2017).
4. *The change in monitored signal in response to the applied perturbation is interpreted within the context of a model relating signal change to the underlying change in the state of the observed reaction.* Examples are models for two-state folding (Gao et al. 2016; Monteith et al. 2015; Smith et al. 2016) and models for binary heteroassociation (Phillip et al. 2012; Shi et al. 2009).

How “dark matter” complicates analysis of weak interactions in intact cells

Ross (2016) defined the dark matter of biology as components of the intracellular milieu that “we cannot or do not detect”. If a particular experiment is monitoring only the behavior of one or two tracer species, and those species are interacting with a variety of undetectable and, hence, uncharacterized background species (i.e., the “dark matter”), it is fundamentally impossible to understand the interactions between them, since the interaction depends upon the properties of both the labeled species and the unlabeled background species with which they interact. Experiments conducted by monitoring tracer signal changes in the intracellular milieu brought about by global changes in chaotrope concentration, temperature, or cellular volume provide no information about how those perturbations alter the composition and properties of background species, and how these alterations affect interactions between the background species and the labeled species. If a change in temperature or

denaturant concentration alters the stability of a tracer species, it is likely to alter the stability of a variety of other macromolecular species in the cell and, hence, the interaction between those species and the tracer species. If a change in the global concentration affects the concentration of labeled reactants of a studied reaction, it will also affect the concentrations of the unlabeled species and, hence, the interactions between these species and the tracer species. Global dilution and/or concentration is particularly problematic, since a relatively small fractional change in the volume of a crowded medium has a much larger effect on excluded volume interactions and consequent changes in macromolecular reactivity than the same fractional change of volume in a dilute medium (Minton 1981, 1994). Change in macromolecular crowding has been invoked as an essential factor in the activation of volume regulatory channels (Parker and Colclasure 1992; Rowe et al. 2014). Global perturbations achieved by variation of total cellular volume, temperature, or concentration of intracellular chaotrope confound the various physical factors influencing the studied reaction, thus preventing an unambiguous interpretation of the results.

Advances in fluorescence imaging have permitted studies of protein stability as a function of position in the cell (Ebbinghaus et al. 2010; Ebbinghaus and Gruebele 2011). The results obtained show clearly that stability of the studied protein, as measured by melting temperature (T_m), varies nonrandomly with position within the cell. Moreover, protein molecules with similar T_m appear to cluster in domains that are much larger than molecular dimensions (Ebbinghaus and Gruebele 2011), indicating that stability of the labeled protein may depend upon association of the tracer protein with large structural elements. These results indicate that treatment of stability data as a whole cell average (see Smith et al. 2016 for an example) is an oversimplification that may provide little or no information about the effect of any particular microenvironment upon stability.

Shedding light on dark matter

Several attempts have been made to characterize the behavior of macromolecules in cytoplasm via Monte Carlo, Brownian dynamics, and molecular dynamics simulations of fluid media containing coarse-grained and atomistically detailed models of macromolecules (Feig and Sugita 2013; McGuffee and Elcock 2010), and, in one ambitious attempt (Yu et al. 2016), small molecules and water as well. It is argued that the more structurally detailed the model becomes, the more “realistic” and more reliable the results of the simulation become. However, structural detail by itself does not ensure realism. Such models cannot be considered realistic until the model potentials or potentials of mean force of intermolecular interaction employed in the simulation have been validated. Validation requires a demonstration that the combination of structural and energetic models can reproduce experimentally

measured composition-dependent solute activities and/or colligative properties in simple solutions of one and two proteins at high concentration (Fernández and Minton 2009; Fodeke and Minton 2010; Minton 2008; Wu and Minton 2015). Preliminary steps toward this goal have been taken by Elcock, Smith and coworkers (Miller et al. 2016; Ploetz et al. 2010).

It is our considered opinion that experiments aimed at elucidating the effect of the cellular interior upon selected macromolecular reactions will be amenable to unambiguous interpretation only when in vivo experiments are supplemented by carefully designed in vitro experiments. The reaction of interest should be studied within homogeneous media containing known compositions of known constituents of defined intracellular microenvironments. It is important to determine the association state(s) of the constituents of each microenvironment and their sensitivity to changes in the composition of the medium, as the interaction between background species may affect the interaction between background and tracer species (Hall 2002, 2006; Hall and Dobson 2006; Hall and Minton 2003; Minton 2017).

The use of cell lysate as a mimic of cytoplasm does not seem, to us, to be especially informative for three reasons: (1) the lysate does not contain membranes contributing to potentially important surface–tracer interactions, (2) cellular lysis mixes macromolecules that may not occur together within the intact cell, and (3) intermolecular interactions within and between the various lysate constituents, which are known to influence tracer–crowder interactions (Hall 2002, 2006; Hall and Dobson 2006; Hall and Minton 2003; Minton 2017), have not been characterized.

We conclude with a list of questions that, in our opinion, must be answered before one can unambiguously interpret the results of tracer experiments carried out in the intracellular milieu:

1. Where are the labeled proteins in the cell?
2. What is the composition of the microenvironment(s) in which the labeled proteins are located?
3. How do the labeled proteins interact with their unlabeled nearest neighbors in each microenvironment, and how do these interactions affect the monitored properties?
4. In the case of fluorescent labels, to what extent do these labels and their interactions with unlabeled neighbors influence the studied reaction?
5. How does global perturbation affect the distribution of the labeled proteins and their interactions with nearest neighbors?
6. How does global perturbation affect the composition and distribution of “dark matter” in the cell and consequent interactions between components of the dark matter and the labeled proteins?

The key to understanding how a complex microenvironment influences a particular macromolecular reaction is to study how components of the environment individually and collectively

Fig. 6 Most current *in vitro* studies of crowding effects on conformational equilibria and associations of macromolecules are carried out in model systems containing dilute tracer species in a solution of a single concentrated crowding species (*lower left*). Current *in vivo* studies of crowding effects on tracer reactions are carried out in intact cells presenting highly complex and poorly characterized microenvironments (*upper right*). Progress in understanding the origin of crowding effects *in vivo* requires the construction of model microenvironments of known and characterized composition, in which selected reactions can be studied by selective variation of composition

interact with reactant and product species. Eight years ago, Gierasch and Gershenson (2009) published a commentary entitled “Post-reductionist protein science, or putting Humpty Dumpty back together again”. We feel strongly that, if we want to understand how Humpty Dumpty—the living cell—works when put back together, we have to put it together in stages. This would seem to require the design and construction of model systems incorporating some or all of the components of an identified cellular microenvironment, in which composition may be varied in a controlled and systematic fashion (Fig. 6). We have previously advocated the use of such model systems (Rivas and Minton 2016) and are pleased to learn that others have recently recognized this imperative (Gao et al. 2017).

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Compliance with ethical standards

Conflict of interest Germán Rivas declares that he has no conflict of interest. Allen P. Minton declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix: What’s in a name?

Vainšteĭn (1973) originally defined *quinary structure* as “... [the] combination of molecules of proteins, nucleic acids and nucleoproteins into aggregates: native ones, such as the viruses, ribosomes, chromosomes, or membranes, or synthetic ones, such as planar monomolecular films, tubes, or crystals.” In our opinion, such a definition follows inductively from the canonical definitions of tertiary structure as the three-dimensional structure of an individual polypeptide and quaternary structure as the relationship between individual polypeptide subunit chains within an oligomeric protein. It follows that the term *quinary interactions* should refer to those interactions between subunits that lead to the formation of the quinary complex. Nonetheless, a number of subsequent publications have utilized the terms “quinary structure” and “quinary interactions” to mean entirely different things that are inconsistent with the original meaning of these terms.

McConkey (1982) found that the net charge of proteins was conserved during evolution more than predicted by standard evolutionary theory. He argued that this was the result of the need to minimally affect protein–protein interactions, leading to functionally related complexes. However, McConkey’s data support only the hypothesis that changing the net charge of a protein may adversely effect interactions of any type, nonspecific as well as specific, repulsive as well as attractive, with all constituents of the local environment of that protein, that have been optimized through the course of evolution for the performance of the biological function of that protein. McConkey defined the “quinary structure” of a protein as the entirety of functionally related

transient complexes formed between that protein and all other macromolecules within the cell. This nomenclature not only conflicts with the Vainshtein definition, but is misleading as well. What McConkey is referring to is not a physical structure, but, rather, the set of connections to a single node within the macromolecular interactome.

The terms quinary interactions or structure have recently been utilized to denote all weak and transient intermolecular interactions (Chien and Gierasch 2014; Wirth and Gruebele 2013). The use of the term “quinary” in this context is both redundant and misleading, as these interactions do not necessarily lead to the formation of well-formed quinary complexes, or, in the case of net repulsive interactions, any complexes at all. Wirth and Gruebele (2013) also include the metabolon (Srere 1985) and the intracellular formation of membrane-free macromolecular complexes driven by phase separation (Banani et al. 2017) as examples of quinary structures. In our opinion, while the hypothetical metabolon may correspond to the Vainshtein definition of a quinary structure resulting from quinary interactions, phase separation does not, as it represents a higher level of structural organization resulting from a variety of interactions. Finally, Cohen and Pielak (2017) have, in some instances, utilized the term “quinary structure” to describe (in our view correctly) the structure of well-formed macromolecular complexes, in accord with the original Vainshtein definition. But in other instances, they use the term to connote the influence of all noncovalent macromolecular interactions in solution and have even suggested that it applies to all intermolecular interactions contributing to the organization of the cellular interior (Cohen and Pielak 2017).

We feel that the use of a single term to denote widely disparate phenomena renders the term meaningless. Accordingly, we recommend that specific terminology, such as that employed in Fig. 1, be utilized to describe the various types of interactions. If, on the other hand, one is referring to the totality of weak intermolecular interactions, it seems to us that the adjective “weak” is sufficient.

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