Protein folding and quinary interactions: Creating cellular organization through functional disorder

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Abstract

The marginal stability of globular proteins in the cell is determined by the balance between excluded volume effect and soft interactions. Quinary interactions are a type of soft interactions involved in the intracellular organisation and that are known to have stabilising or destabilising effects in globular proteins. Recent studies suggest that globular proteins have structural flexibility, exhibiting more than one functional state. Here, we propose that the quinary-induced destabilisation can be sufficient to produce functional partial unfolded states of globular proteins. The biological relevance of this mechanism is explored, involving intracellular phase separation and regulatory stress-response mechanisms.

Keywords

Macromolecular crowding, Protein stability, Membraneless organelles, Phase separation, Intracellular organisation

Abbreviations

PTM, Post-translational modifications; IDP, Intrinsically disordered proteins; IDPR Intrinsically disordered protein regions; LCD, Low complexity domains; OA, aggregates of thermally denatured ovalbumin; Tc, tetracycline

1. Intracellular spatial organisation: the emerging role of quinary interactions

One of the greatest challenges in protein science is to understand the relationship between structure and function. Proteins are defined by specific amino acid sequences (primary structure), whose intramolecular interactions lead to the formation of α-helices, turns and β-sheets (secondary structure) that rearrange into a defined 3D architecture (tertiary structure) [1]. This folding process results in functional protein structures that act individually or collectively in multiunit complexes (quaternary structure) [2]. Proteins also interact with each other and with other biomolecules, like DNA, RNA, lipids, and carbohydrates to form macromolecular assemblies beyond quaternary structure [3–7]. These so-called quinary interactions constitute the fifth level of protein organisation inside the cell and so are also designated as quinary structure [3,8,9]. Distinct from the interactions that maintain quaternary organisation, quinary interactions are transient and weak, and are therefore undetectable when macromolecules or their assemblies are isolated with traditional cellular disruptive protocols [3,8]. For instance, the interaction free energy involved in quinary interactions is approximately 1 kcal mol^{-1} [10], which is two to seven times less than the interaction free energy found in pair residues of the well-known barnase-bastar quaternary complex [11]. Moreover, quinary interactions evolved to organize and regulate biomolecular functions in response to variable cellular conditions [12] and so are considered specific. In contrast, non-specific interactions occur because biomolecules exist in densely crowded environments and therefore intrinsically interact with other macromolecules in their vicinity (for further discussion of these terms see [13]).

The major function of quinary interactions is the spatial organisation of the intracellular environment [3,8,9]. The formation of reversible multi-enzyme complexes, such as purinosome and tricarboxylic acid (TCA) cycle metabolon is thought to be driven by this kind of interaction [9,14]. Quinary interactions were also considered to have a major role in the formation of membraneless organelles [8,12,15]. These structures correspond to cellular regions of different composition and density that create a microenvironment with a defined functionality [16,17]. Some examples include Cajal bodies, nucleolus and paraspeckles (localised in the nucleus) and the stress granules and P-bodies (localised in the cytoplasm) [18–21]. At the molecular level, the formation of these organelles involves intermolecular interactions among intrinsically disordered proteins (IDPs) and/or RNA molecules [22].

In addition to their role in the spatial arrangement of the cellular milieu, quinary interactions modulate protein folding and stability [1,10,23–30]. In-cell studies showed that quinary interactions can have both stabilising [24,29–33] and destabilising [10,24–30,33] effects on globular proteins. The role of these opposite effects is still not clear but preliminary evidences suggests a dependence on protein sequence, function and location [24,31,33]. Here, it is proposed that the destabilisation of globular proteins induced by quinary interactions can lead to the formation of functional partially unfolded states. It is a further hypothesis that as in the cases of IDPs and RNA, such states may drive the formation of membraneless organelles with a significant role in stress response.

2. Physicochemical properties of the intracellular environment and marginal stability of proteins

The interior of the cell is characterised by a high concentration of macromolecules (200 to 400 g L^{-1}) [34], which results in a crowded environment that modulates macromolecular structure, diffusion, association, and function [35,36]. Two molecules cannot occupy the same space at the same time, and so they sterically exclude volume from the neighbouring molecules. Macromolecular crowding refers to the situation where each molecule excludes volume from the surrounding molecules [35]. The available volume for each molecule is reduced, consequently leading to more compact macromolecular conformations. For proteins, assuming that the native state is more compact than the unfolded state, crowding favours folding and stability [31,37–40]. However, the presence of large amounts of macromolecules not only creates an excluded volume effect but also promotes intermolecular interactions between neighbouring molecules [35,41]. These interactions, also known as non-steric or soft interactions, are those that a protein can perform with its surroundings beyond the steric interactions that result from the excluded volume effect [12,41]. They stabilise or destabilise globular proteins through charge-charge, polar, hydrogen bond and hydrophobic contacts with both the native and unfolded states (for more details see Appendix 1) [10,24–30,33,42–47]. The magnitude of this modulation ranges on average from 0.1 to 1.1 kcal mol^{-1} [10,43–47]. Within soft interactions, quinary interactions have received considerable attention, with the majority of the studies focusing on their ability to induce protein destabilisation [10,24–30,33].

The folding landscape of proteins inside the cell does not differ significantly from that observed in dilute solutions, suggesting that the alterations induced by crowding or soft interactions are energetically small [1,12,23]. Nevertheless, the majority of globular proteins are marginally stable, presenting low energetic transitions between the folded and unfolded states (5 to 15 kcal mol^{-1}) [1,48,49]. This situation leads to protein native states only slightly more stable than the corresponding unfolded states, and so highly sensitive to alterations in their surroundings [1,12,29]. Therefore, although modest, the changes induced by the intracellular crowded milieu can impact the function of proteins [1,12,23]. For example, compaction of the phosphoglycerate kinase (PGK) native state in a crowded environment leads to a structure with higher enzymatic activity [37]. Another example is the destabilisation of cell surface antigen VlsE in the cytoplasm of mammalian cells, that may be related to the need of this protein to unfold in order to translocate to the outer membrane [24,50]. More general functions can involve the maintenance of an adequate protein turnover rate [44,50] as the cell is continuously producing and degrading proteins. Fast protein degradation requires destabilisation, because the proteolytic machinery acts on the unfolded states [49,51]. Marginally stable proteins populate unfolded conformations more often, increasing their proteolytic sensitivity and consequent degradation [49,51].

Globular proteins evolved to function in a crowded environment, which leads them to suffer constant encounters with their surroundings, that may or may not result in functional partnership [52–55]. This situation was highlighted in a recent study by Oliveberg and co-workers, where quinary interactions were able to modulate the binding affinity of proteins to form encounter complexes in different environments [55].

Using surface mutations, the authors were able to tune the ability of bacteria and human proteins to move freely or stick to the bacteria cytoplasm [55].

In conclusion, the modulation of protein folding stability or binding affinity by the intracellular milieu is not a rare event, and may result from evolutionary adaptation.

3. Quinary interactions as the new modulators of globular protein´s structural flexibility

Recent experimental evidence showed that globular proteins have a certain degree of structural flexibility [56,57]. More importantly, this increased conformational flexibility is functional and depends on specific regulatory mechanisms or simple physico-chemical alterations in the surrounding environment [57,58]. For instance, there are certain globular proteins, called metamorphic proteins, that display more than one native conformation [58–63]. Lymphotactin is a good example. This chemokine displays two distinct native conformations with different biological functions: a monomeric form, composed by a three-stranded β-sheet and a α-helix carboxyl-terminal, that binds to the XRC1 receptor and a dimeric all-β-sheet conformation that binds to heparin, an glycosaminoglycan localised in the extracellular matrix [58]. Depending on the conditions (temperature and ionic strength), lymphotactin can adopt one conformation over the other, involving a cooperative global unfolding transition [58,60]. Several other examples of these metamorphic proteins are reported, in which folding/unfolding events modulate their biological function [61,62,64,65]. In fact, Porter *et al.* predicted that up to 4% of the proteins in Protein Data Bank (PDB) present some degree of metamorphism, reinforcing the increased relevance of functional flexibility of globular proteins [63].

Another phenomenon that corroborates the increased flexibility of globular proteins is regulated unfolding [57,66]. This process involves the formation of functional partially or globally unfolded states induced by factors, including alterations in pH, temperature, post-translational modifications (PTMs) and ligand binding [57,66]. The newly formed states display more residues to perform additional interactions, providing alternative mechanisms of cellular signalling [57,66]. Following this line of reasoning, Jakob *et al.,* proposed a framework to understand these order-to-disorder transitions [57]. They proposed that globular proteins could comprise modules [57]. This suggestion was based on the experimental evidence that the folding of many globular proteins occurs through a step-wise pathway model, by the cooperative unfolding and refolding of modular units designated as foldons (~15 to 35 residues) [67,68]. In this model, unfoldons correspond to less stable regions of globular proteins that undergo transient order-to-disorder transitions to perform their biological function [57,69]. Further thermodynamic detail regarding these conformational changes can be achieved if the folded state of a globular protein is considered as an ensemble of microstates and not as an individual 3D structure [70,71]. This concept of conformational ensembles and its application to the study of protein´s conformational transitions are discussed in Appendix 2.

Crowding and soft interactions usually affect folding and stability by modulating protein populations (i.e. favouring folded or unfolded states), without causing partial or global unfolding [10,26,38,39]. However, partial unfolding in crowded environments has been reported *in silico* [72,73]. For example, Feig and co-workers observed the partial unfolding of villin, induced by intermolecular interactions between villin and neighbouring proteins [72]. As soft interactions, quinary interactions destabilise globular

proteins enthalpically, through attractive hydrophobic or electrostatic interactions with the native or the unfolded state [10,24–26,41]. In certain situations, therefore, the destabilisation induced by quinary interactions could be sufficient to partially unfold the less stable segments (unfoldons) of globular proteins, consequently leading to the formation of partially unfolded states with further biological function (Figure 1). Although the energy involved in quinary interactions is considered to be low (~1 kcal mol^{-1}), many globular proteins are marginally stable, and at the lower limit (5 kcal mol^{-1}), this energy represents one-fifth of the total protein stability [10]. Such energetic magnitude is not usually sufficient to produce significant structural changes in globular proteins [1]. However, previous studies reported that under physiological conditions, the simple occurrence of thermal fluctuations can be sufficient to induce partial unfolding in some globular proteins [74–77]. The energy of thermal fluctuations is generally of the order of k_BT (~0,6 kcal mol^{-1} at 298 K)[78] and therefore within the range of quinary interactions energy (~1 kcal mol^{-1}) [10,23]. Moreover, the partially unfolded states (N*) that result from the action of these thermal fluctuations are characterised by Gibbs free energies slightly higher than those of the native state (N) and separated from them by small kinetic barriers (Figure 2-A) [74]. This energetic profile is similar for quinary interactions that also imply low thermodynamic and kinetic variations [8,15]. Thus, the native states and the partially unfolded states resulting from quinary-induced destabilisation can present similar thermodynamic stability, separated by low kinetic barriers (Figure 2-B). Consequently, small energetic expenditures are necessary when returning to the folded state. In other words, quinary interactions could render globular proteins more flexible and act as functional conformational switches. This means that, in addition to PTMs, temperature, pH and other factors responsible from regulated

unfolding, quinary interactions could present an extra mechanism to regulate the protein folding dynamics in the cell.

4. Quinary-induced conformational switch in membraneless organelles formation

The biological relevance of the increased globular proteins structural flexibility, induced by quinary interactions may be appreciated by comparing it with IDPs and hybrid proteins with intrinsically disordered protein regions (IDPRs). These proteins are known to have specific functions due to their high structural flexibility [79]. One of these functions that has recently received great attention is the formation of membraneless organelles [80–83]. This way of arranging the intracellular environment is formed by phase separation and exhibit varied biological functions including regulation of signalling pathways [84], gene expression [18,85], RNA processing [86] and stress response [87–89]. Some of these structures also include RNA that is thought to have mainly a regulatory role [90,91]. However, in some cases, RNA by itself may be sufficient to form phase-separated regions in the cell [92].

Most of the mechanisms proposed for phase separation are based on the self-assembly of proteins creating a protein-dense phase in equilibrium with a protein-depleted phase [93]. In IDPs, this process takes place through disordered low complexity domains (LCD). The patterned self-interactions between these motifs, involving electrostatic, dipole-dipole, cation-π, π-π and hydrophobic contacts are sufficient to induce phase separation [80,81,94–97]. Additionally, hybrid proteins with both LCDs and folded domains can participate in multivalent interactions that drive phase separation,

with contributions from electrostatic and hydrophobic interactions [86,98–102]. The key feature appears to be the increased structural flexibility of these proteins that allows a greater number of intermolecular interactions to occur with surrounding molecules. These interactions result in the formation of overcrowded regions, and above a certain threshold can give rise to phase separation [103,104].

What are the physico-chemical properties of IDPs compared to globular proteins that make them crucial for phase separation? Globular proteins have long been known to phase separate *in vitro* [105–112]. Examples include lysozyme [106,109,110,112–114], γ crystalline [107,115–118], arachin [105], bovine pancreatic trypsin inhibitor [111,119], and hemoglobin [120,121]. Globular proteins are also reported to phase separate in the presence of other globular proteins or IDPs [122,123]. Nevertheless, the intracellular phase separation of globular proteins remains largely unreported. A framework to understand this different behaviour of IDPs and globular proteins in phase separation was recently proposed by Zhou *et al.* [124]. Phase separation is tuned by the balance between the steric (excluded volume) and attractive intermolecular interactions [124]. Modelling globular proteins as colloids and IDPs as polymers, the authors suggested that phase separation of IDPs occurs at much lower concentrations due to their greater ability to form attractive intermolecular interactions as well as the lower energetic cost associated with the steric repulsion [124]. This suggestion is consistent with the experimental studies reporting phase separation in aqueous solutions of two globular proteins, two IDPs or one globular protein and one IDP [122]. For example, the phase separation threshold for the mixture of two IDPs, such as casein and gliadin, is around 5.5 % (w/v) of total protein. This threshold is higher with a globular protein and an IDP, such as ovalbumin and casein (19.6 % (w/v)), whereas two globular

proteins, for example ovalbumin and bovine serum albumin (BSA), do not phase separate under similar conditions [122]. Remarkably, the phase separation between globular proteins and their denatured forms was also found to occur at relatively low concentration. For instance, mixtures of ovalbumin and aggregates of thermally denatured ovalbumin (OA) or BSA and OA phase separate at a total protein concentration threshold of 13.4% (w/v) and 16.4% (w/v), respectively [122,125]. Unfolded proteins expose a larger surface to form attractive intermolecular interactions in comparison to their native counterparts. This behavior is closer to IDPs than globular proteins, which could explain, in part, why OA phase separate from BSA but the latter does not phase separate from the ovalbumin native form. Following this line of reasoning, we propose a similar role for the partially unfolded states that result from quinary modulation. These states expose more surface to form additional intermolecular interactions (also quinary), therefore increasing their potential to multivalency. Above a critical concentration, the consequent association with surrounding molecules leads to phase separation, producing a different phase (environment) from the neighbouring crowded milieu (Figure 3).

Although cellular observation of this process is lacking, previous work suggests the involvement of unfolded states in the dynamics of protein complexes found in stress granules (eIF3, PeBoW, methionyl/glutamyl t-RNA synthetase) [126]. The authors proposed that the partial unfolding of one of the thermally sensitive components of these complexes by heat could be sufficient to provoke the co-sequestration of the associated-components to stress granules [126]. In addition, the observation of phase separation between native ovalbumin/BSA and OA provides chemical evidence that above certain concentrations, the intermolecular interactions between unfolded

proteins produce clusters constituting regions with different physicochemical properties (OA), that then phase separate from the surrounding molecules (native ovalbumin/BSA).

5. Quinary-modulation of proteins folding dynamics as an additional mechanism to stress response?

There is increased evidence that, under stress conditions, quinary interactions organise the intracellular machinery (proteins, nucleic acids) in different quinary assemblies, like reversible protein aggregates and membraneless compartments [87,126–128].The formation of these assemblies can be tightly regulated by pH, temperature or PTMs, such as, phosphorylation [127]. Similarly, we propose that quinary interactions could by themselves, be the triggers of these adaptive assemblies, with the globular partially unfolded states exposing additional hydrophobic residues. The increased surface promotes attractive interactions with neighbouring molecules that could be, for example, enzymatic partners or multiprotein complexes. These interactions could lead to the formation not only of membraneless organelles, but also reversible protein aggregates or filaments. Overall, quinary interactions could organise globular proteins into multiple transient adaptive assemblies, which in turn would present an advantageous strategy in stress response comparing to the detrimental misfolding [127]. Remarkably, a recent study highlighted the importance of surface hydrophobic patches in quinary interactions [129]. The authors considered that the low number of hydrophobic residues in the surface of globular proteins suggests a specific and non-random quinary organization of the intracellular environment [129]. Could quinary interactions have evolved to sense modifications in the intracellular milieu?

Recent findings suggest that modifications in cellular volume can tune quinary interactions between two enzymes involved in the glycolytic pathway [130]. Cellular volume changes are observed in many situations, particularly in starvation stress [131]. In conclusion, uncovering the molecular principles that govern quinary interactions between globular proteins and the cellular milieu will allow us to understand how this adaptive interplay responds to the severe modifications occurring during stress.

6. Concluding remarks and future perspectives

Quinary interactions are emerging as crucial role-players in protein dynamics and intracellular organisation [9,12,132,133]. Because of this quinary interplay, the cell is no longer “a bag full of molecules”, but rather a highly organised environment, optimized to function and survive in diverse conditions [132]. However, many of the fundamental functions are waiting to be uncovered.

Here, the role of quinary interactions in the modulation of the structural flexibility of globular proteins, to the extent that allows these proteins to perform some biological functions usually attributed to IDPs, was explored. This function does not apply to all globular proteins, but could increase the protein´s functional states under evolutionary quinary control, opening new mechanisms to regulate the enormous number of biochemical processes that enable the cell to function.

Another seminal idea relies on the competition between the functional or deleterious protein-protein interactions in the crowded intracellular environment [134]. Disruption of protein homeostasis is often associated with the formation of partially unfolded states because these states display a larger surface capable of forming

attractive interactions, and increasing the probability of detrimental aggregation [135]. The mechanism proposed here is based on functional partially unfolded states, and so its disregulation could lead to the accumulation of dysfunctional partial unfolded states and consequent protein aggregation. In addition, there is evidence that an imbalance in the proteome of membraneless organelles, for example, as a result of cellular ageing, leads to aggregation and pathological amyloid fibrils [136–138]. Thus, understanding how the quinary interplay of proteins and the cellular milieu modulate their folding dynamics and stability to prevent detrimental aggregation, will open perspectives on the molecular mechanism behind protein misfolding diseases.

Future experiments need to consider *in vivo* approaches (NMR [139], FReI [140]), where the weak associations between proteins and the differently crowded intracellular environments are preserved. These methods must be complemented with *in vitro* analysis because the *in vivo* approaches mask the individual function of crowding molecules [40,43,141,142]. In addition, sophisticated all-atom simulations of proteins in the crowded environments [73] will contribute to an understanding of the molecular detail of protein conformational dynamics, providing insight into the proposed conformational switch mechanism.

It could also be useful to consider directed evolution assays as a mean to identify the specificity associated with the functional role of quinary interactions detected by in-cell spectroscopy [12,55]. In addition, many cellular processes involve a dominant role of kinetics. The reported modulation of protein folding kinetics in crowded environments (either of the energetic barriers or viscosity) clearly corroborates the importance of combining equilibrium and kinetic measurements [31,33,142].

Although protein folding dynamics has been studied for more than 50 years [143], little is known about its modulation by the complex intracellular environment. The discovery of such hidden features will revolutionise our understanding of biochemical pathways and networks, and place us one step closer to decoding the intricate spontaneous appearance of cellular order in our disorder-dictated universe.

Appendix 1- The nature of soft interactions

Soft interactions involve preferential interactions between the native or unfolded states of proteins and their surrounding environment [12,41]. Preferential interactions of molecule(s) with native proteins can lead to stabilisation of proteins, as happens in ligand-binding events [144]. In contrast, preferential interactions with unfolded proteins generally involve direct interaction between the surrounding molecule(s) and the protein backbone, thus favouring unfolded states due to the increased exposed surface (e.g. chemical denaturants such as urea) [145]. Proteins can also be stabilised by preferential hydration of the protein surface, which results from unfavourable interactions with the nearby molecules (e.g. osmolytes) [146,147].

In the context of destabilising interactions, charge-charge and hydrophobic contacts have received increased attention. For instance, *in vitro* studies using proteins as crowding agents revealed destabilisation of proteins induced by charge-charge interactions [43–45]. This charge-dependent destabilisation was also detected *in vivo* [25,27]. In addition, these interactions can be tuned by pH as an additional regulation factor to modulate protein stability [27,43,148,149].

Hydrophobic interactions have been less explored, with fewer studies mentioning their possible involvement in the destabilisation of protein native states [24,33,43]. Recent *in silico* approaches studied the influence of crowding agent hydrophobicity on the stability of a small model protein [150]. The results showed that with the increase of hydrophobicity, attractive side chain-side chain interactions started to form between the unfolded state and the crowding agent, destabilising the protein´s native state [150].

Finally, the pattern of interactions can also be altered by the differential distribution of residues on a protein´s surface. This alteration was observed with weak, transient interactions between *Escherichia coli* cytoplasm and ubiquitin or between *E. coli* cytoplasm and the N-terminal metal-binding domain of mercuric reductase NmerA [151]. Although these proteins have almost the same number of hydrophobic surface residues, in NmerA these residues are dispersed whereas in ubiquitin they are clustered in a hydrophobic pocket at the C-terminus. This different distribution is crucial because weak interactions between ubiquitin and cytoplasmic molecules are 100-fold stronger than those in NmerA [151].

Appendix 2- The structural flexibility of globular proteins and the native ensemble model

A useful approach to understand the conformational changes associated with metamorphic proteins and partial unfolding of globular proteins relies on the concept of conformational ensembles, i.e., native proteins do not populate a single structure but exist as an ensemble of transient microstates, including partially unfolded states [70,71].

Factors such as ligand binding, mutations or modifications in the environmental conditions (pH, temperature) remodel the energy landscape of the ensemble, affecting the distribution of microstates and consequently allowing partially unfolded states to be populated more often [70,71]. An example is the homodimeric transcription factor tetracycline (Tc) receptor (TetR), a regulator of the Tc resistance mechanism in gram-negative bacteria. When Tc is present, it binds to the Tec binding domain (TBD) of the TerR protein, reducing the ability of the protein to bind to DNA and consequently allowing the expression of antiporter protein TetA that exports Tc to the exterior of the bacterial cell [152]. When Tc is absence, DNA binds to TetR through the DNA binding domain (DBD), preventing the transcription of *tetA* gene and consequent expression of TetA protein [152]. This process happens because DBD native ensemble of microstates more frequently populate locally unfolded states, which increases the DNA binding affinity [71,153]. On the other hand, the binding of Tc to TBD stabilizes DBD, reducing its flexibility, explained by a redistribution of the ensemble to conformations with lower binding affinity for DNA [71,153]. This framework has been successfully applied to other situations of allosteric regulation involving partial unfolding of globular proteins [154,155] and also to rationalise protein fold evolution [156]. Further development of this model may provide new perspectives regarding how external factors, particularly quinary interactions, can modulate the landscape of conformational ensembles of globular proteins.

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Figure 1. Quinary modulation of protein´s marginal stability in the intracellular environment. Quinary interactions (1) between a hypothetical protein and surrounding molecules (in this case represented by yeast aminoacyl-tRNA synthetase complex (PDB. 2HSM (left) and yeast Hsp90-Sba1 closed chaperone complex (PDB. 2CG9) (right)) lead to the destabilization of the hypothetical protein, producing a partially unfolded state that could have a further biological role (2). The proteins representing the surrounding molecules were chosen for visualization purposes, and may not correspond to biological relevant interactions.

Figure 2: Thermodynamics and kinetics of quinary-induced partial unfolding of globular proteins. A: At physiological temperature, thermal fluctuations can lead to the formation of partially unfolded states (N*), slightly less stable than the native ones with small kinetic barriers between them. **B:** Similarly, quinary-induced partial unfolding can promote the formation of partially unfolded states (P) with similar characteristics. These two states (N* and P) are energetically similar but result from different phenomena. The second case results in an advantage switch mechanism, where the cell may increase a globular protein´s functional state with low energetic expenditure.

Figure 3. The involvement of globular proteins in intracellular phase separation. Quinary interactions between hypothetical proteins and neighbouring molecules destabilize protein native states (A), resulting in their partial unfolding (see Figure 1). The consequent association with surrounding macromolecules (1) leads to the creation of a different overcrowded environment (B). The proteins representing the crowding molecules were chosen for visualisation purposes and may not correspond to biological relevant interactions.