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Prevalence of the Diffusion Collision Model of Protein Folding *In Vivo*: A Mechanistic Analysis of the Acceleration of Protein Folding by Peptidyl-Prolyl Isomerase and the GroEL/ES Chaperonin System

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Review Article

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ABSTRACT

This review assesses the differences between the diffusion-collision model and the extended nucleation condensation model of protein folding and attempts to determine; by analyzing the mechanisms through which peptidyl-prolyl isomerase and the GroEL/ES chaperonin system accelerate the rate of folding of their respective substrate proteins, which model of protein folding prevails *in vivo*. The difference in the kinetics between the two protein folding models was assessed in the introduction using free energy profiles which led to the identification of conditions that would favour one model over the other.

Following the justification of the choice of chaperones used for analysis, the mechanism through which both chaperones accelerated the rate of folding of their respective proteins was investigated to determine whether the conditions developed by the chaperones were consistent with one model of protein folding over another. The review concludes with a summary of the key findings gleaned from mechanistic analysis of chaperone function and highlights its relevance to the biochemical and medical fields.

Keywords: Protein; Diffusion; Nucleation; Unfolded state; Micro-domains; Peptides

INTRODUCTION

The kinetic and thermodynamic features of the protein folding process have been a matter of intense study amongst biochemists and biophysicists for the better part of a century. Yet much remains to be known about the process through which proteins fold (renature) from an unfolded (denatured) polypeptide chain into their native (functionally active) state.

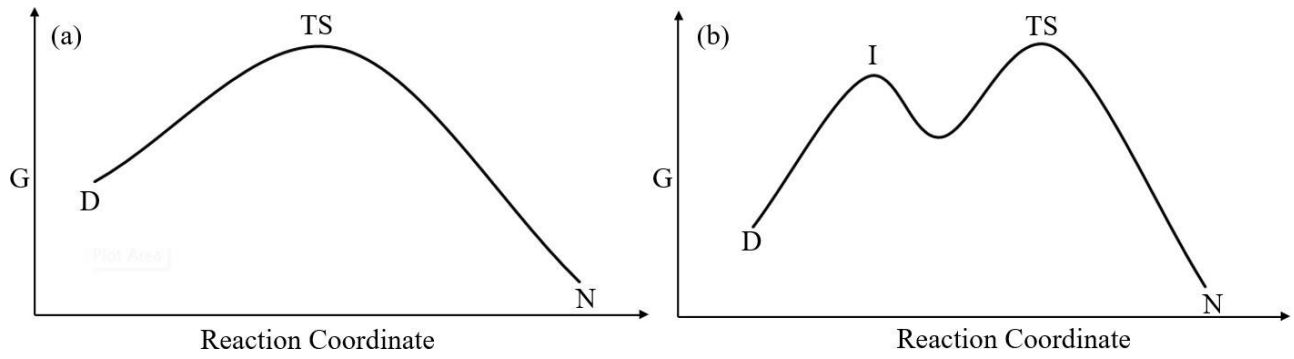
Proteins have been theorized to fold *via* one of two mechanisms that, in essence, differ in their transition states and free energy profiles: The Diffusion Collision Model (DCM) and the Extended Nucleation Condensation Model (ENCM) [1]. This review suggests that the DCM is the prevalent protein folding process *in vivo*.

The DCM postulates that segments of the unfolded polypeptide, *via* local interactions, fold rapidly to form secondary structure elements, such as α-helices and β-sheets, in a non-rate- determining step.

These marginally stable secondary structure elements, referred to as micro-domains, then coalesce in a diffusion-controlled manner to form the native state, stabilized by tertiary interactions that form when the micro-domains interact. The topology of this transition state does not necessarily resemble that of the native state [2].

The ENCM, on the other hand, posits that both local and long-range interactions concomitantly contribute to the formation of a weak, extended nucleus, to form a transition state with a topology resembling the native state [3]. The difference in free energy profiles between both models is shown in Figure 1.

Figure 1. Free energy profiles for the ENCM and the DCM of the protein folding pathway from the unfolded, Denatured state (D) to the Native state (N). **Note:** (a) Displays the free energy profile for the ENCM wherein secondary and tertiary interactions form concomitantly to form the Transition State (TS); (b) Displays the free energy profile for the DCM wherein secondary interactions form before tertiary interactions to produce Intermediates (I) in a non-rate-limiting step preceding the formation of the TS.



Molecular chaperones (referred to as only 'chaperones' in the rest of the text) are a family of proteins that assist in the folding of polypeptide chains into their native state, without being a part of that final native state [4]. Studies of the interactions between a chaperone and its substrate protein, as the protein folds, have provided insights into the mechanisms employed by the chaperone to accelerate the folding process. Examining these mechanisms could provide an insight into whether the DCM's or the ENCM's transition state and intermediates are preferentially stabilized by chaperones.

The large energetic expenditure made by cells in maintaining an intricate network of chaperones indicates that they are vital to maintaining the cellular proteome, *via* control of protein folding [4].

Studying the mechanisms through which some of these chaperones accelerate protein folding in cellular environments may provide insights as to whether the DCM or the ENCM prevails *in vivo*. Exploring the conditions that would stabilize such species for both models would provide a starting point for such an analysis.

LITERATURE REVIEW

As stated before, in the DCM the protein folds into its native state through the collision of individual micro-domains (secondary structure elements that form rapidly due to local interactions) in the correct (native) orientation, which contributes to the formation of long-range tertiary interactions that stabilize the micro-domains in their native

orientation. The rate-limiting step is not the formation of the micro-domains but rather their diffusion into the correct orientation, making it a diffusion-controlled process.

Hence, the rate of this process is inversely proportional to the viscosity (η) of the solution [5]. Furthermore, the folding pathway of the DCM is populated with multiple intermediates that may differ in topology (due to the absence of tertiary interactions in the first step of the process) but are relatively similar in their free energy contents (G).

Since micro-domains form rapidly, they would have similar levels of secondary structure which implies similar free energy content between intermediates [6]. In the ENCM, however, the formation of an extended nucleus, that precedes the transition state, requires highly specific secondary and tertiary interactions.

As a result, the number of initial conformations that can fold into an extended nucleus is minimized, resulting in minimal intermediates present in the ENCM folding pathway [3]. A decrease in viscosity and/or an increase in the rate of diffusion of micro-domains would not increase the rate of formation of the transition state in the ENCM.

Choice of chaperones for analysis

The interactions between Peptidyl-Prolyl Isomerases (PPIases), the GroEL/ES chaperonin system and their respective substrates were chosen for analysis based on their employed mechanism of accelerating the folding process: PPIases accelerate protein folding *via* chemical means (by catalyzing peptidyl-prolyl isomerization whereas the GroEL/ES chaperonin system relies primarily on mechanical means to achieve the same effect [7,8].

This ensures that both the biophysical and chemical factors of the protein folding process are considered while determining whether the DCM or the ENCM is the prevalent model for protein folding *in vivo.*

PPIase

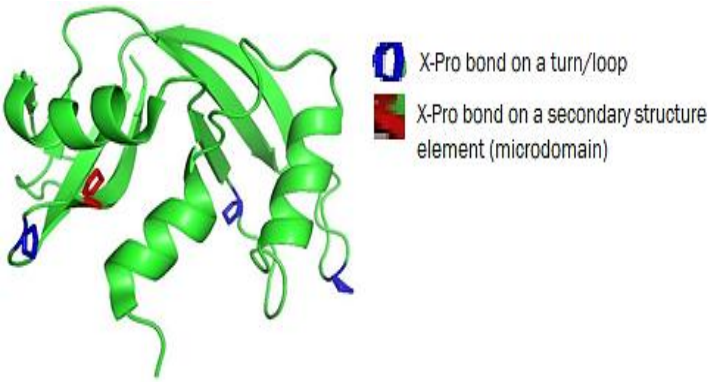
PPIase serve an essential role in the folding pathway of proline-containing polypeptides [9]. Amino acids in polypeptide chains are linked by peptide bonds, which have a partial double bond character. This introduces rigidity into the protein backbone as rotation about the carbon-nitrogen peptide bond is highly restricted, allowing it to exist only in either the cis or the *trans* conformation.

The height of the energetic barrier to rotation about the double bond to allow for *cis/trans* isomerization is influenced by the properties of the side chains of the amino acids in the peptide bond [10]. The rotational barrier about X-Pro bonds (where X is any amino acid) serves as a kinetic hurdle in the protein folding process [11].

Interactions between PPIases and their respective substrates could provide greater insight into the mechanism of protein folding. Interactions between denatured bovine pancreatic Ribonuclease A (RNase A) and PPIase isolated from pig kidney were analyzed to determine the mechanism by which RNase A-folding was accelerated.

RNase A is a 14 kDa protein that contains four X-Pro bonds within its primary structure [12]. PPIase mediated acceleration of RNase A-folding is thought to proceed *via* catalysis of *cis/trans* isomerization about the X-Pro bonds. [13]. The location of X-Pro sites on RNase A reveals the potential sites of interaction between RNase A and the PPIase isolated from pig kidney, providing insight into the mechanism through which protein folding occurs (Figure 2).

Figure 2. Cartoon representation of the bovine pancreatic ribonuclease A crystal structure (Protein Data Bank (PDB) code 1KF5) with only proline side chains visible and highlighted; blue indicates the presence of the X-Pro bond on a turn/loop whereas red indicates the presence of the X-Pro bond on a secondary structure element (microdomain). PyMol was used to make this figure.



DISCUSSION

From Figure 2, it is evident that of the four X-Pro sites on RNase A, three are present in turns and loops that connect the more regular and ordered secondary structure elements (micro-domains). Only one X-Pro site exists within a micro-domain (the X-Pro bond containing Pro117). Thus, 75% of the X-Pro sites are present on loops and turns that serve to connect micro-domains, rather than stabilize their structure. Though the loops and turns do not possess a regular, repeating structure they are not the same as a random coil and have a defined structure in the native state [14]. Catalysis of *cis/trans* isomerization about the X-Pro bond can be shown to increase the proportion of collisions between micro-domains in the correct orientation to form the long-range interactions needed to form and stabilize the native tertiary structure. The first equation that needs to be consulted is the Stokes-Einstein equation (SE equation) which allows for the calculation of the diffusion coefficient (D) as shown below.

$$D = \frac{K_B T}{3\pi\eta d} \text{.....(1)}$$

In equation (1), k_B is the Boltzmann constant, T is the temperature in kelvin, η is the viscosity and d is the diameter of the considered particle (its shape is approximated to a sphere) [15]. Given that these parameters are unaffected when RNase A-folding is accelerated by PPIase the value of D remains the same [10]. To understand the influence of the PPIase on accelerating the folding of RNase A, Fick's first law of diffusion needs to be considered.

$$J = -D\frac{ac}{ax} \text{.....(2)}$$

In equation (2), which is Fick's first law, J is the flux (amount of matter passing through the point x), C is the concentration at x and x is the given position [16]. The minus sign indicates that the flow of matter occurs from a higher concentration to a lower concentration. This can be equated to protein folding by considering concentration in terms of free energy, wherein movement across a steeper concentration gradient equates to a larger change in free energy [17]. Moving from a region with a higher concentration to a region with a lower concentration is correlated to a decrease in free energy. According to the energy landscape theory, the native state of the protein represents the global free energy minimum of the folding funnel [18]. Conformational and configurational changes to RNase A structure, such as the *cis/trans* isomerization of X-Pro bonds catalysed by the PPIase, move it further along the folding funnel, away from

its unfolded random coil state and closer towards its native state, which correlates to a decrease in free energy. This equates to movement down the concentration gradient, towards the lower concentration, which increases the value of J. This indicates that accelerating the folding of RNase A from its unfolded to its native state requires an increase in the amount of flux, which resonates with a diffusion-controlled process such as the DCM over the ENCM. Kramer's theory of diffusion over a potential energy barrier, represented by equation (3), provides further support for the DCM over the ENCM [16].

$$J = D\left(-\frac{P}{K_B T} \times \frac{du}{d\varepsilon} - \frac{dp}{d\varepsilon}\right) \text{.....(3)}$$

In equation (3), the variables J, D, k_B and T represent the same parameters as in equations (1) and (2) while U represents potential energy, p represents the probability of a molecule occupying a specific position along a reaction coordinate and ξ represents a length element (extrapolated in the equation below, where t represents the time interval of a particle with mass m to move across a given position).

$$\varepsilon = t\sqrt{\frac{K_B T}{M}} \text{.....(4)}$$

Equation (3) is of interest, however, as it replaces the concentration gradient, from equation (2), with a probability gradient while introducing a value 'U' to represent the potential energy barrier imposed onto the RNase A-folding process by the need for *cis/trans* isomerization. The PPIase would serve to increase the flux of micro-domains (J) over potential energy barriers (towards the transition state) by minimizing the height of the barriers through catalysis of *cis/trans* isomerization about the X-Pro bonds. This aligns more with the DCM than with the ENCM because (as highlighted earlier) an increase in the rate of collisions between micro-domains favours a diffusion-controlled process over a process that requires the simultaneous formation of specific local and long-range interactions to form an extended nucleus. Furthermore, the nature of the DCM implies the presence of multiple intermediates preceding the transition state that possess a similar free energy content despite differing in their overall topology (as described earlier). This would imply that the probability of a given RNase A molecule occupying a specific position immediately preceding the transition state would be greater in the DCM than in the ENCM since the formation of an extended nucleus minimizes the number of intermediates formed (as described earlier) [3]. Thus, the value of the probability gradient in equation (3) would be greater in the DCM than in the ENCM. As a result, even if an increase in the flux of micro-domains could accelerate RNase A-folding through the ENCM, the larger value of the probability gradient in the DCM (due to the presence of intermediates) would render it a more feasible pathway for accelerated RNase A-folding (following interaction with the PPIase).

Thus, interactions between the PPIase and denatured RNase A that serve to accelerate its folding into the native state provide greater support for the DCM than the ENCM as the protein folding pathway is accelerated by PPIase.

GroEL/ES chaperonin system

The GroEL/ES chaperonin system (referred to as 'GroEL/ES' in the rest of the text) is comprised of the GroEL chaperone-which consists of two 57 kDa heptameric rings that form a cylindrical structure with two cavities and an ATPase domain-and the GroES co-chaperone-which consists of a 10 kDa heptameric ring that binds to the end of the GroEL cylinder *via* an ATPase cycle that triggers structural changes in the GroEL chaperone [19]. Since GroEL/ES forms nano-cages that assist in and accelerate the folding of various polypeptides of differing sizes that do not possess an obvious common property (as opposed to the substrates of PPIases wherein they all possess X-Pro bonds), studies

detailing the kinetic data of the accelerated folding of various proteins associated with GroEL/ES were consulted for analysis [8]. These proteins were Ribulose-1,5-Bisphosphate-Carboxylase-Oxygenase (RuBisCo), Dihydrodipicolinate Synthase (DapA), Malate Synthase G (MSG), Citrate Synthase (CS) and rhodanese. Under permissive folding conditions (wherein spontaneous protein folding can proceed unhindered and off-pathway aggregation of proteins is avoided) the presence of GroEL/ES accelerated the rate of protein folding for every protein listed above, except for rhodanese [8,19-22]. A review of the different models proposed for the mechanism of function of GroEL/ES could provide insights into why that may be and could indicate which model of protein folding is best suited to the chaperone-mediated acceleration of the folding process.

It has been posited that the enclosed cavities within GroEL/ES (that arise following ATP-dependent binding) form nano-cages that can assist in protein folding through either the passive cage model (sometimes referred to as the 'Anfinsen cage' model), the active-cage model, or the iterative annealing model [8]. The passive cage model suggests that the nano-cage serves to infinitely dilute the substrate protein from other macromolecules to prevent aggregation [23]. In this model, there is no specific mechanism employed to accelerate the protein folding process; it only functions to provide permissive conditions for protein folding and, therefore, does not provide any insight into the kinetics of the protein folding process. The other two proposed models, however, are of interest as they posit that the changes that occur to the structure of the GroEL chaperone following ATP-dependent GroES binding accelerate the rate of protein folding and, thus, may provide insight into the kinetics of the protein folding process.

The active-cage model suggests that confinement within GroEL/ES serves to accelerate protein folding (beyond prevention of off-pathway aggregation) on the basis that ATP-dependent GroES binding to GroEL (following substrate protein binding) triggers several changes: A small increase in the size of the cavity and an increase in the polarity of the cavity lining, as hydrophobic residues are buried to leave a hydrophilic cavity lining [24]. Both changes serve to increase the flux of micro-domains within the solution present in the GroEL/ES cavity (referred to in the rest of the text as 'cage solution'), which would be expected to accelerate the rate of protein folding *via* the DCM, as explained previously. The small increase in the volume of the cavity (while the number of water and protein molecules present in the cavity remains constant) results in a decrease in pressure, according to equation (5).

$$p = \frac{nRT}{V} \text{.....(5)}$$

In equation (5), P is the pressure, n is the number of moles of solution, R is the gas constant, T is the temperature (in Kelvin) and V is the volume of the container. Since n, R and T remain constant in the GroEL/ES cavity, the increase in volume produces a decrease in pressure. At lower pressures, liquids display a lower viscosity which, as established earlier in relation to equation 1, corresponds to an increase in the diffusion coefficient [25]. An increase in the rate of diffusion would accelerate a diffusion-controlled reaction such as the DCM, providing further support for its prevalence *in vivo*. The change in polar character of the GroEL/ES cavity lining from hydrophobic to hydrophilic also contributes to a decrease in the viscosity of the cage solution within the GroEL/ES cavity. Transient polar interactions between the micro-domains of partially folded intermediates of the substrate protein in the GroEL/ES cavity and the hydrophilic cavity lining serve to (temporarily) decrease the effective concentration of micro-domains in the cage solution available for collision. According to the DCM, micro-domains can be considered independent units whose interactions with each other to form the native protein rely on random diffusion. Thus, these micro-domains can be viewed as independent solute molecules in the cage solution and (temporarily) decreasing their concentration (*via* transient polar interactions with the hydrophilic cavity lining) would lead to a drop in the viscosity of the cage solution [26]. The resulting increase

in the rate of diffusion in the cage solution would lead to an accelerated rate of protein folding only through the DCM and not through the ENCM, as explained earlier. The use of the DCM as the prevalent model of protein folding *in vivo* may also explain why only the rate of folding of rhodanese is not accelerated by the active-cage model (beyond prevention of off-pathway aggregation).

The hydrophilic lining of the GroEL/ES cavity (following substrate protein binding and an ATPase cycle) can interact with polar water molecules and sequester them from the cage solution. This would decrease the number of water molecules available in the cage solution to interact with the micro-domains of the substrate protein to form nonnative protein-water interactions. This drives a decrease in the number of intermediates of the substrate protein that form nonnative protein-water interactions (instead of native protein-protein interactions for example). Partition functions (Q) can be used to group and differentiate between sets of microstates (intermediates of the substrate protein) that correspond to a specific internal energy and conformational entropy. The potential energy and entropy terms can be summed up and shown using free energy and so a partition function can be defined as a sum over a set of microstates that possess the same free energy, as shown in equation (6).

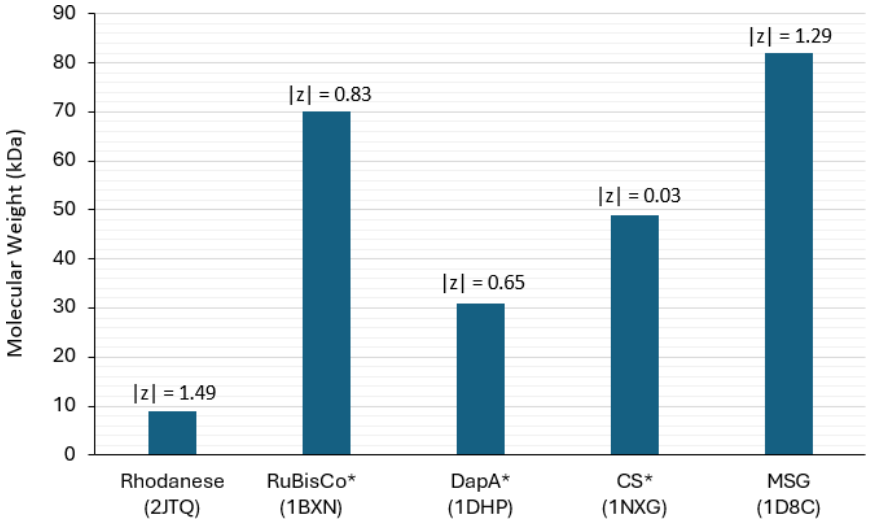
$$Q = \sum_{i}^{n_{ts}} e^{-E_i / K_B T} \text{.....(6)}$$

In equation (6), n_{ts} is the total number of microstates with a specific free energy, E_i is the free energy of a microstate with index i and k_B and T are the same values as shown in equation (1). Sequestration of water molecules from the cage solution by the hydrophilic lining of the GroEL/ES cavity serves to decrease the number of microstates wherein nonnative protein-water interactions form. This decreases the value of the partition function that represents the sum of microstates in which these nonnative protein-water interactions are formed with the same free energy (represented by Q_{pw}). A similar partition function can be used to represent the sum of microstates in which native protein-protein interactions are formed with the same free energy (represented by Q_{pp}). Q_{pp} comprises a set of intermediates that are not kinetically trapped and have the potential to form the transition state (as their free energy corresponds to that specific position along a reaction coordinate) and a simplified equation can be used to model the probability of such an intermediate molecule occupying a specific position along a reaction coordinate (P_{pp}), as shown in equation (7).

$$P_{pp} = \frac{Q_{pp}}{Q_{pp} + Q_{rs}} \text{.....(7)}$$

In equation (7), Q_{rs} represents the sum of the remaining microstates not encompassed by Q_{pp}. Q_{pw} is a subset of Q_{rs}, and the hydrophilic lining of the GroEL/ES cavity would serve to decrease the value of Q_{pw} and thus, Q_{rs}. This results in a decrease in the value of the denominator in equation (7) and an increase in the value of p_{pp}. As seen in equation (3) increasing the probability of finding a molecule at a specific position along a reaction coordinate would drive an increase in the flux of micro-domains (J) that (as explained previously in the text) is consistent with an acceleration in the rate of protein folding through the DCM rather than the ENCM. However, such an increase in the flux of domains is reliant on the value of Q_{pw} decreasing significantly following the sequestration of water molecules from the cage solution by the hydrophilic cavity lining of GroEL/ES. Rhodanese is an outlier amongst RuBisCo, DapA, MSG and CS in terms of molecular weight, as can be seen in Figure 3. It should be noted that for multimeric proteins (RuBisCo, DapA, and CS) the molecular weights of their independent monomeric subunits were compared as research suggests that individual subunits are folded separately within chaperone cages before complex assembly to form the multimeric protein [27].

Figure 3. Comparison of the molecular weights (in kDa) of the five proteins whose rates of folding were measured in permissive conditions in the presence and absence of GroEL/ES. The presence of an asterisk (*) next to the name of the protein indicates that it is multimeric and that the molecular weight of its polypeptide monomer was used for comparison. The respective Protein Data Bank (PDB) codes of each protein are shown in brackets below its name and the modulus of the Z-value of the molecular weight of each protein (or its subunit) is indicated above its respective bar (as |Z|). The modulus of the Z value indicates the magnitude of the difference (in terms of the number of standard deviations) between the protein's molecular weight and the mean molecular weight of the five proteins.



From the figure above it is evident that rhodanese is the outlier, in terms of molecular weight, amongst the five proteins, as it has the largest absolute z value. This could indicate that its low molecular weight (9.44 kDa) may play a role in GroEL/ES not being able to accelerate the rate of folding of rhodanese (beyond providing permissive folding conditions). If protein folding within the GroEL/ES cavity were to proceed *via* the DCM, an acceleration of the process would require a significant decrease in the value of Q_{pw} for that protein as posited in equation (7). For a substrate protein as small as rhodanese, the absolute number of nonnative protein-water interactions would be significantly smaller compared to a protein with a larger molecular weight, on the basis that there would be fewer micro-domains present (in rhodanese's structure) to form such protein-water interactions. As a result, the number of water molecules sequestered from the cage solution by the hydrophilic cavity lining of GroEL/ES may not be enough to contribute to a decrease in the value of Q_{pw} for rhodanese as there would likely be enough water molecules left in the cage solution to contribute to the formation of rhodanese microstates with nonnative protein-water interactions. The inability of GroEL/ES to accelerate the folding of rhodanese (beyond preventing aggregation) due to an inability to increase the flux of rhodanese micro-domains indicates that protein folding occurs within the GroEL/ES cavity *via* the DCM rather than the ENCM, in the active-cage model of chaperonin function.

The iterative annealing model of chaperonin function suggests that repeated binding and release of partially folded intermediates of the substrate protein accelerates its folding toward the native state [28]. This model posits that along the folding pathway of the substrate protein, there exist intermediates that possess nonnative protein-solvent interactions and nonnative protein-protein tertiary interactions that serve as kinetic traps that do not contribute to productive protein folding [29,30]. In this model, GroEL/ES binds to such misfolded intermediates (in an ATP-dependent manner) and triggers partial unfolding of the intermediate (wherein nonnative interactions are disrupted) before subsequent release into solution to allow the appropriate native interactions to form [31]. The disruption of nonnative interactions in intermediates of the substrate protein by GroEL/ES serves to decrease the height of the potential

energy barrier (U) in equation (3) which would drive an increase in the flux of microdomains [27]. Thus, the iterative annealing model suggests the presence of multiple intermediates of the substrate protein and suggests that the GroEL/ES acts to increase the flux of micro-domains into the correct orientation to drive the formation of native tertiary interactions to stabilize the native state. These are features of the protein folding process that are implied by the DCM which indicates that the iterative annealing model of chaperonin function provides further support for the DCM prevailing as the GroEL/ES-mediated protein folding pathway.

Thus, both the active-cage model and the iterative annealing model of GroEL/ES function support the idea that the DCM, rather than the ENCM is the pathway through which protein folding is accelerated by GroEL/ES [32,33].

CONCLUSION

From this review of the acceleration of protein folding by PPIases and GroEL/ES, using statistical mechanics, it can be concluded that the folding process exhibits kinetic and thermodynamic features that indicate that it proceeds *via* the DCM rather than the ENCM. As has been established, PPIases and GroEL/ES accelerate protein folding essentially through an increase in the flux of microdomains which is consistent with the DCM. Owing to the presence of the vast, intricate network of chaperones that maintain the proteome within cells, these findings imply that the prevalent model of protein folding *in vivo* is the DCM.

However, it must be noted that the DCM and the ENCM are both extremes of a process in which the transition state possesses secondary and tertiary interactions with the difference being the sequence in which those interactions are formed (which shapes the kinetic profile of the process). A better understanding of the kinetics associated with protein folding *in vivo* may be crucial in developing drugs and treatments for diseases associated with protein misfolding and aggregation. An example is Alzheimer's disease wherein PPIases and their cellular roles are being investigated to gain a deeper insight into the mechanism of tau protein aggregation and the development of potential therapeutics.

Beyond the discussion that debates between acceptance of the DCM and the ENCM as the prevalent model of protein folding there persist debates between other ideas, such as the foldon-dependent hypothesis and the energy landscape theory, that present further questions regarding the protein folding pathway. Further research and analysis of such models may perhaps allow for the development of a universal model of protein folding (that may borrow elements from each of these models) and would allow for the accurate prediction of a protein's tertiary structure from its primary structure, based on first principles alone.

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