Do Chaperonins Boost Protein Yields by Accelerating Folding or Preventing Aggregation?

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ABSTRACT The GroEL chaperonin has the ability to behave as an unfoldase, repeatedly denaturing proteins upon binding, which in turn can free them from kinetic traps and increase their folding rates. The complex formed by GroEL+GroES+ATP can also act as an infinite dilution cage, enclosing proteins within a protective container where they can fold without danger of aggregation. Controversy remains over which of these two properties is more critical to the GroEL/ES chaperonin's function. We probe the importance of the unfoldase nature of GroEL under conditions where aggregation is the predominant protein degradation pathway. We consider the effect of a hypothetical mutation to GroEL which increases the cycle frequency of GroEL/ES by increasing the rate of hydrolysis of GroEL-bound ATP. Using a simple kinetic model, we show that this modified chaperonin would be self-defeating: any potential reduction in folding time would be negated by an increase in time spent in the bulk, causing an increase in aggregation and a net decrease in protein folding yields.

INTRODUCTION

Aggregation is the formation and growth of clusters of proteins, typically with no biological function, whose size may potentially grow without bound. It is one of the principle degradation processes that competes with protein folding in the cell (1–3). In addition to depleting the concentration of available proteins, the aggregates themselves can be toxic (4,5).

Chaperones are large biomolecules that help proteins fold in the cell under conditions in which spontaneous folding is not possible. Chaperonins are a class of chaperones which form a cylindrical cage large enough to surround and enclose their protein substrates. In general, proteins which need chaperones to fold tend to be both aggregate prone and fold via a complicated process with on and off-pathway intermediates (6–9).

The GroEL/GroES chaperonin system is almost a prototypical example of a promiscuous chaperonin. It assists in the folding of 10% of the proteins in *Escherichia coli* (10), a set which spans a wide range of folds and functions. Much effort has been invested to uncover the mechanism used by GroEL/ES to help proteins fold. There is an abundance of data on GroEL structure, substrates, co-chaperones, and kinetics. The task of interpreting this data is important. Surprisingly, serious disagreements regarding the basic mechanism used by GroEL/ES remain.

Fig. 1 shows a simplified overview of the sequence of events that occur during a typical cycle of binding and release to the GroEL chaperonin (11–15). GroEL is composed of 14 subunits, 57 kDa each, arranged into two cylindrical rings stacked end-to-end. Under typical circumstances, one of them is bound to its co-chaperone GroES. A group of concentrated hydrophobic residues near the opening of the *trans* ring (*red*) enables GroEL to target and bind to exposed hydrophobic residues which are likely to be found on the surface of misfolded proteins (Fig. 1, *top*). Less than 1 s later (11,12), substrate and ATP binding is followed by the binding of the co-chaperone GroES, which seals the protein inside (Fig. 1, *bottom*), and releases GroES, ADP from the opposite ring, and (possibly) the protein contained inside (16). The process of binding to ATP and GroES also triggers a conformational change in GroEL that buries these hydrophobic residues (*red*), increasing the cavity volume, freeing the protein, allowing it to move inside the chaperonin (17–19). The protein remains sequestered within the cavity until (~8–20 s later (11,12)) the hydrolysis of ATP (Fig. 1, *upper-left*) allows the opposite ring to bind to protein and ATP, beginning a new cycle, and triggering the release of the currently held protein. The hydrolysis of ATP constitutes the rate-limiting step in the GroEL/ES cycle. Once released, if the protein remains misfolded, it will quickly bind to another chaperone and the process will repeat itself (typically on the order of 20 times) until the protein either folds to its biologically active native state or aggregates (11,20,21).

Several mechanisms have been used to explain how GroEL/ES assists protein folding in the cell:

- The traditional Anfinsen cage model (ACM) proposes that chaperonins help proteins fold by sealing them within a cage where they can fold safely without risk of aggregating. (Even chaperones that bind to, but do not enclose, their substrates may act as a holdase, preventing their substrates from aggregating while bound (1,2,22–24)).
- The iterative annealing model (IAM) says that periodic cycles of forced unfolding upon binding and release from a chaperone can free proteins from long-lived off-pathway intermediates, and accelerate folding. For many chaperones including GroEL/ES, the kinetics of protein binding and release is regulated by ATP (21,25–33).

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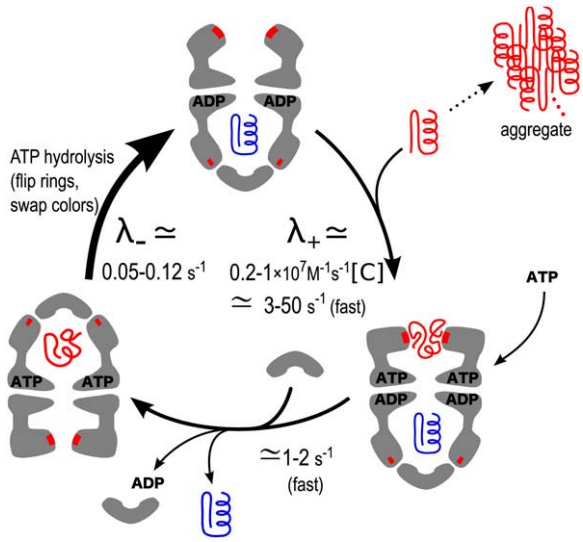

FIGURE 1 The GroEL/GroES binding and release cycle. GroEL/ES is composed of two cylindrical cavities, one of which is typically open, the other closed. Step 1 (*upper-right*): Nonnative protein (*red*) bind to the apical domain of the open *trans* ring of the GroEL chaperonin, along with ATP, possibly denaturing the protein, and changing the shape of the *trans* ring so that it is ready to bind to the GroES co-chaperone. Step 2 (*bottom*): GroES functions like a lid sealing the protein inside the container. Its binding also triggers the release of GroES and ADP from the opposite ring, and possibly the protein contained inside (*blue*). Step 3 (*upper-left*): Eventually, hydrolysis of the ATP bound to the closed ring (the rate-limiting step in the cycle) induces a conformational shift in the opposite ring which enables it to bind to other (nonnative) proteins. Thus, the cycle begins again, starting with the opposite ring. Kinetics data are taken from the literature (11,12). Typically, the concentration of GroEL, denoted here by [C], is in the μM range in vivo (16).

In addition, the environment inside the GroEL+ES cavity is different from the bulk, and this may assist protein folding by truncating, smoothing, or otherwise modifying the protein's energy landscape (20,29,30,32,34–41). This can lead to increased folding rates inside the cavity for some, but not all proteins (20). The GroEL chaperonin even has the limited ability to break apart small aggregation clusters (7).

In this article, we examine the relative roles of the increased folding rates suggested by the IAM and the protective environment offered by the cage (ACM) in enhancing protein folding yields. Our analysis will take into account some of the kinetics data from the Horwich and Clarke labs (11,12).

We focus on conditions where aggregation is the dominant degradation pathway for the protein (the majority of known stringent GroEL substrates are indeed highly aggregation-prone). To probe the predictions of IAM in such a situation, we consider a hypothetical mutation that would accelerate the cycling frequency of the chaperonin. Earlier theoretical work (28,29,42) has suggested that such a mutation would optimize chaperonin function and increase folding yields. However, in these studies, yield was defined as the percentage of proteins which fold after some arbitrarily determined time. While folding rates and yields may be correlated if folding occurs under dilute conditions, this may no longer be the case under conditions where aggregation is the chief process that prevents protein folding on biologically relevant timescales. A mutation that increases cycling frequency may accelerate folding; however, it also increases the percentage of time that proteins would spend in the bulk. Using a simplified kinetics model for the protein and chaperonin, we prove that the latter would override any potential acceleration of folding that could come as a result of faster cycling (all other conditions being equal), and would decrease the true yield.

RESULTS AND DISCUSSION

Unlike some of the smaller heat shock proteins that are only present under conditions of stress, chaperonins like GroEL and its homologs are always present in the cell. (43). Consequently, we focus on the behavior of GroEL/ES under ordinary (nonstress) conditions. We assume that under such conditions, the concentration of most nonnative proteins does not fluctuate significantly, at least on a timescale commensurate with folding in vivo. When applicable, we refer to these conditions as steady state. While beyond the scope of this work, it is important to mention that the conclusions we will reach are independent of this assumption (unpublished).

Aggregation prevention under steady-state conditions

Under nonstress conditions, it is reasonable to assume that nonnative proteins in a cell tend to be in a state of dynamic equilibrium, where the processes of folding and degradation are offset by the constant production of new protein. Some of this protein will aggregate and some of it will fold (or be destroyed through proteolysis). To keep the concentration static, the rate at which nonnative protein is introduced (v_0) must equal the rate at which it is removed through folding. (Although some proteins may degrade by other means, they are in the minority (43,45), and we do not consider this effect here.) It can be shown that

$$[\mathrm{X}] = v_0\langle t_\mathrm{F}\rangle. \quad (1)$$

See Appendix 1 and Fig. 2 *a*, where [X] denotes the ambient concentration of a given species of nonnative protein monomers which can participate in aggregation, $\langle t_\mathrm{F}\rangle$ denotes the average time before folding occurs, and v_0 denotes the velocity at which a given species of nonnative protein is introduced into the cytosol, in units of moles $\times$ volume^{-1} $\times$ time^{-1}. This is either due to the expression of new proteins, and/or the gradual denaturation of existing protein. Aggregation will proceed more rapidly if the concentration [X] is higher.

How can chaperones reduce this concentration of nonnative protein in the bulk?

When chaperones are present (Fig. 2 *b*), they can prevent or discourage aggregation either by binding to the same

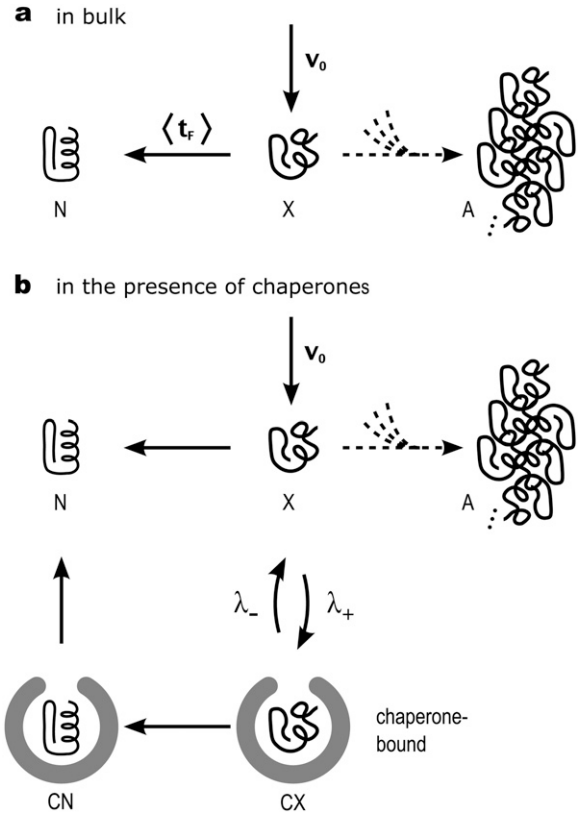

FIGURE 2 A simple kinetics diagram showing the competition between folding and aggregation (*a*) in the absence and (*b*) in the presence of chaperones. *N*, *X*, and *A* denote the protein in a native, nonnative, and aggregated states, respectively, and *CN* and *C*X denote the protein bound to a chaperone. Notation: v_0 denotes the velocity at which new nonnative proteins are introduced into the system. The value $\langle t_F \rangle$ denotes the average folding time, and λ_+ and λ_- denote the probability-per-unit-time that a nonnative protein will bind to, and release, from a chaperone. Note that λ_+ depends on the chaperone concentration.

exposed hydrophobic residues also participating in aggregation, or in the case of GroEL/ES, sealing proteins inside a cavity where they can fold safely. The above formula must be slightly modified,

$$[\mathrm{X}] = v_0 \langle t_{\mathrm{bulk}} \rangle, \tag{2}$$

where $\langle t_{\mathrm{bulk}} \rangle$ denotes the average time a protein spends in the bulk (i.e., not bound to chaperones) before folding. We can study how effective a chaperone would be at reducing aggregation simply by studying how much a chaperone can reduce $\langle t_{\mathrm{bulk}} \rangle$. Estimating $\langle t_{\mathrm{bulk}} \rangle$ is particularly easy for proteins that depend on GroEL/ES, because they typically experience many cycles of binding and release from GroEL/ES before folding (11,16,20,21). In this case, as pointed out by Ranson et al. (11), the fraction of the time that the protein spends bound to the chaperone can be estimated in terms of the ratio of the binding and unbinding times.

Let $\langle \tau_{\mathrm{unbound}} \rangle$ and $\langle \tau_{\mathrm{bound}} \rangle$ denote the average time for binding and unbinding, and λ_+ and λ_- denote the binding and unbinding rates ($\langle \tau_{\mathrm{unbound}} \rangle = 1/\lambda_+$ and $\langle \tau_{\mathrm{bound}} \rangle = 1/\lambda_-$. See Fig. 2 *b*). The fraction of time proteins spend in the bulk (i.e., unbound) is

$$f_{\mathrm{bulk}} \approx \frac{\langle \tau_{\mathrm{unbound}} \rangle}{\langle \tau_{\mathrm{unbound}} \rangle + \langle \tau_{\mathrm{bound}} \rangle} = \frac{\lambda_-}{\lambda_+ + \lambda_-}, \tag{3}$$

which typically amounts to <2% for wild-type GroEL/ES (11). (The time before binding, $\langle \tau_{\mathrm{unbound}} \rangle$, could be even less if the interaction with other chaperones is considered as well (3,46).)

In summary, the rate of aggregation increases with the concentration of available monomers, [X], which under steady-state conditions is proportional to both f_{bulk} (the fraction of time that the protein spends in the bulk) and the folding time $\langle t_F \rangle$:

$$[\mathrm{X}] = v_0 \times f_{\mathrm{bulk}} \times \langle t_F \rangle, \tag{4}$$

where $f_{\mathrm{bulk}} \times \langle t_F \rangle = \langle t_{\mathrm{bulk}} \rangle$. Chaperones can reduce aggregation by reducing f_{bulk}, or $\langle t_F \rangle$, or both.

The effect of iterative denaturation on protein folding kinetics

We now turn to an examination of the effects of chaperonins on protein folding times. Since the ACM does not mandate any changes in folding rate, we focus on the prediction of the effect of the IAM on the folding time, $\langle t_F \rangle$.

Let us consider the optimal realization of the IAM, in which binding to GroEL unfolds the protein completely every time it binds to the chaperone, causing it to forget all memory of its former structure. (We note that, in reality, chaperones like GroEL have been observed to partially distort their substrate's conformation during binding (26,47). By considering a full denaturation, we are able to assess the maximal benefit that can be obtained from IAM.)

How might cycles of full denaturation affect the kinetics of folding?

Let t = the time that has elapsed since the protein was first introduced into the cytosol in its unfolded state, and $P(t)$ = the probability that the polymer in its folding environment (usually the chaperonin cavity) has not yet reached a folding-committed conformation after time t.

Typical GroEL/ES substrates spend 98% or more of their time bound to GroEL/ES, of which at least 90% of this time is spent inside the closed cavity formed by GroEL+GroES+ATP (11). Consequently, $P(t)$ usually reflects the protein's folding kinetics under these conditions, inside the GroEL/ES cavity. We note that the kinetics of protein folding in this new environment can differ from the kinetics of folding in the bulk for certain proteins (20,29,34,36–41).) We will not consider this effect here as we are only interested in

studying how the average folding time $\langle t_F \rangle$ can be altered by changing the ATP-regulated unbinding rate, λ_-.

It is not difficult to find the average folding time of a protein folding under the influence of cycles of repetitive denaturation. We assume that denaturation events are separated by Poisson-distributed randomly-spaced intervals which mimic the frequency of binding to GroEL/ES.

Let λ_D denote the probability-per-unit-time that a nonnative protein will bind to a chaperone and be forcibly unfolded. The average time interval between binding events is $\langle \tau_{unbound} \rangle + \langle \tau_{bound} \rangle$, which is

$$1/\lambda_+ + 1/\lambda_- \approx 1/\lambda_D. \quad (5)$$

In the specific case of GroEL (11,12), $\lambda_D \approx 0.05 - 0.12\ s^{-1}$.

In Appendix 2, we show that

$$\langle t_F \rangle = \frac{1}{\lambda_D}\left[\left(\lambda_D \int_0^\infty P(t) e^{-\lambda_D t} dt\right)^{-1} - 1\right]^{-1}. \quad (6)$$

This equation has the property that, for any protein, the folding time is, at most, inversely proportional to λ_D (see Appendix 3). Doubling the denaturation frequency can reduce the folding time by a factor that approaches at most two. We note that denaturation can only accelerate the folding of proteins with multiexponential kinetics (25,28). (The specific case of proteins with multiexponential kinetics is discussed in Appendix 3.)

The effect of increased cycling rates on protein folding yields

How would a mutation that increases the cycle frequency affect the yield of folded protein? Combining Eqs. 3–6, we see that the concentration of nonnative protein in the bulk, [X], is an increasing function of cycle frequency, λ_D (and also of the ATP hydrolysis rate, λ_-, since they increase together):

$$[X] = \frac{v_0}{\lambda_+}\left[\left(\lambda_D \int_0^\infty P(t) e^{-\lambda_D t} dt\right)^{-1} - 1\right]^{-1}. \quad (7)$$

One can verify this is an increasing function of λ_D by taking the derivative with respect to λ_D, integrating by parts, and observing that $(d/dt)P(t) \leq 0$.

Recall that folding competes with aggregation, and that aggregation increases with the monomer concentration [X]. This suggests that the final yield decreases as the rate of ATP hydrolysis, λ_-, increases.

Physically, we see a competition between two effects. Recall that [X] is proportional to $f_{bulk} \times \langle t_F \rangle$. Increasing λ_- may reduce $\langle t_F \rangle$ but it increases f_{bulk} by a larger proportion. To see why, consider a mutation to GroEL which stimulates the rate of ATP hydrolysis in the *cis* ring (λ_-) by a factor of two. For typical GroEL substrates, λ_- is the rate-limiting step in the cycle ($\lambda_- \ll \lambda_+$). Consequently, this mutation would effectively double the cycle frequency λ_D. Such a mutation could free proteins from off-pathway kinetic traps twice as frequently, potentially boosting the folding rate by a factor of, at most, two. (But this is only true in the best-case scenario, when the protein's folding is entirely rate-limited by escape from long-lived kinetic traps.) On the other hand, such a mutation would also release proteins into the bulk earlier, effectively doubling the fraction of time, f_{bulk}, that the protein spends in the bulk where it can aggregate. The second effect dominates, and, if anything, increases the average time the protein spends in the bulk before folding (all other circumstances being equal). Such a mutation would not help increase the yield of aggregate-prone proteins.

One effect we have ignored is the fact that chaperones may alter their substrate proteins' folding kinetics during the time they are bound. For example, some proteins fold more rapidly when trapped inside the GroEL/ES cavity than they do in the bulk (20). A mutation which doubles the rate of ATP-hydrolysis-driven protein-release, λ_-, would reduce the fraction of time proteins spend in this favorable environment, perhaps further slowing folding, and increasing aggregation. Why have we ignored this effect? Unless λ_- were increased above λ_+ (a two orders-of-magnitude increase), this effect would be difficult to observe, only reducing the fraction of time spent in the cavity from $\approx$98% to $\sim$96%, in this particular example. (Instead, we have attempted to absorb this effect of the cavity into the definition of $P(t)$ above.) Either way, for GroEL/ES, this effect does not change our results qualitatively. However, it could be important for other chaperones which occupy a smaller fraction of their substrates' time.

In future work, we will extend our model to other promiscuous chaperones which allow their substrates to fold while bound. This may include some type II chaperonins/cochaperones (48,49).

CONCLUSION

The cage formed by GroEL/ES appears to be indispensable to its function, protecting proteins from aggregation while they fold. GroEL also has the ability to unfold its substrates upon binding. Whether this feature is important can be measured by considering the effect that a mutation has which increases the number of times a protein will bind to GroEL before folding. We have shown that, because of the chaperonin cage, yield is maximized when the cycling rate is reduced, not increased. Taking our conclusion to its logical extreme, the optimal yield would be reached if the chaperonin binds to its protein substrate only once and does not release it until folding. Clearly, this is outside the range of validity (the chaperonin must eventually release its protein substrate); however, this illustrates the importance of the protective cage over a chaperonin's potential as an iterative annealing machine.

When protein aggregation is the predominant degradation pathway, the original iterative annealing model as typically stated (21,25–28,30,50), does not fully describe the

chaperonin-assisted folding of proteins. The existence of the cage (17), the rapid rate of binding, the slow rate of unbinding (11,12), and the ability of noncycling GroEL/ES mutants to promote folding (20,22,23), are all further evidence that GroEL/ES is more than just an unfoldase. GroEL is unlikely to be further optimized by increasing the ATP-regulated rate of cycling. The widespread use of GroEL/ES (and their homologs) among some of the oldest organisms on earth, suggests that these chaperones are already highly optimized. The rate of cycling for chaperonins may be determined by other constraints, including efficiency, chaperonin availability (11), and the competing demands of a diverse set of substrates.

The IAM continues to provide a logical explanation for how chaperonins may be able to assist the folding of proteins which are not prone to aggregation. The iterative annealing model also remains an invaluable concept that can be applied to other chaperones and similar problems. For instance, a variant of the IAM, one in which denaturation is caused by thermal fluctuations rather than ATP-driven, can explain accelerated folding inside the cage of a chaperonin (29,36,41,51) and at the surface of a mini-chaperone (the transient-binding-release mechanism) (51,52).

APPENDIX 1: CONTINUITY RELATION FOR [X]

Here we show that the steady-state concentration of a given species of nonnative protein in a cell (denoted [X]) should be the product of its average lifetime (folding time, denoted $\langle t_F \rangle$), multiplied by the rate at which new nonnative proteins are introduced into the cytosol (denoted v_0, which has units of moles $\times$ (volume)$^{-1}$ $\times$ (time)$^{-1}$).

Let $p(t)\,\Delta t$ = the population of nonnative proteins which were introduced into the bulk during a time interval between t and $t + \Delta t$ seconds earlier (units: moles $\times$ volume^{-1}), and $P(t)$ = the probability that a nonnative protein introduced into the bulk t seconds earlier has not yet folded, which equals $p(t)/p(0)$ under steady-state conditions, and $\rho(t)\Delta t$ = the probability that a protein folds between time t and $t + \Delta t$.

$$\rho(t) = -\frac{\mathrm{d}}{\mathrm{d}t}P(t). \tag{8}$$

The average folding time is $\langle t_F \rangle = \int_0^\infty t\rho(t)\mathrm{d}t$. Substituting Eq. 8 yields $\langle t_F \rangle = \int_0^\infty p(t)\mathrm{d}t/p(0)$, which is [X]/$p$(0). By continuity, $p(0) = v_0$, hence [X] = $v_0\,\langle t_F \rangle$.

APPENDIX 2: THE EFFECT OF PERIODIC DENATURATION ON A PROTEIN'S RATE OF FOLDING

We consider a single protein interacting with a single type of chaperone. Although the sequence of events that occur during each cycle of binding, denaturation, and release may be complicated, at this crude level, we ignore such details. In our crude model, we will use λ_D to represent probability-per-unit-time that a nonnative protein will be forcibly denatured by a chaperone. In real life, this probability will vary depending upon the state of the chaperone; for example, depending upon whether or not the chaperone is in a high-affinity or low-affinity state. This probability will also depend on the chaperone concentration. For simplicity, we have assumed that this probability is independent of time, and also independent of the conformation that the protein is in (other than the native conformation).

It is helpful to define the following notation. Let $P_0(t)$ = the probability that neither folding nor denaturation has taken place by time t.

$$P_0(t) = e^{-\lambda_D t}P(t), \tag{9}$$

Let $P_i(t)$ = the probability that the protein has been forcibly denatured i times before time t and yet remains unfolded.

$$\begin{aligned} P_i(t) &= \int_0^t P_{i-1}(\tau)\,(\lambda_D\,\mathrm{d}\tau)\,P_0(t-\tau) \\ &= \lambda_D(P_{i-1}\circ P_0)(t) \\ &= \lambda_D^i\left(\underbrace{P_0\circ P_0\circ\ldots\circ P_0}_{\times(i+1)}\right)(t). \end{aligned} \tag{10}$$

$P_D(t)$ = The probability that the protein has not yet folded after time t under dilute (folding-permissive) conditions in the presence of chaperones that cause repetitive denaturation.

$$P_D(t) = P_0(t) + P_1(t) + P_2(t) + P_3(t) + \cdots. \tag{11}$$

Let $\rho_D(t)$ = the probability-per-unit-time that a protein folds in the presence of such a chaperone.

$$\rho_D(t) = -\frac{\mathrm{d}}{\mathrm{d}t}P_D(t).$$

Let the Fourier-transform of $P(t)$, $P_D(t)$, and $P_i(t)$ be denoted $\tilde{P}(\omega)$, $\tilde{P}_D(\omega)$, and $\tilde{P}_i(\omega)$, and defined according to the convention $\tilde{P}(\omega) = \int_{-\infty}^{\infty} e^{-i\omega t}P(t)\mathrm{d}t$. According to Eqs. 10 and 11,

$$\begin{aligned} \tilde{P}_D(\omega) &= \textstyle\sum_{i=0}^{\infty}\tilde{P}_i(\omega) \\ &= \tilde{P}_0(\omega)\textstyle\sum_{i=0}^{\infty}(\lambda_D\tilde{P}_0(\omega))^i \\ &= \tilde{P}_0(\omega)[1-\lambda_D\tilde{P}_0(\omega)]^{-1}. \end{aligned} \tag{12}$$

Convergence of the series follows from $\tilde{P}_0(\omega) < 1/\lambda_D$. We calculate

$$\langle t_F \rangle = \int_0^\infty t\rho_D(t)\mathrm{d}t = \int_0^\infty P_D(t)\mathrm{d}t.$$

Since $P(t)$, and $P_D(t)$ and $P_0(t)$ are undefined for $t < 0$, for convenience, we set them to zero for negative t. With this convention,

$$\begin{aligned} \langle t_F \rangle = \tilde{P}_D(0) &= \frac{1}{\lambda_D}\left[(\lambda_D\tilde{P}_0(0))^{-1} - 1\right]^{-1} \\ &= \frac{1}{\lambda_D}\left[\left(\lambda_D\int_0^\infty P(t)e^{-\lambda_D t}\mathrm{d}t\right)^{-1} - 1\right]^{-1}. \end{aligned} \tag{13}$$

APPENDIX 3: MULTIEXPONENTIAL FOLDING KINETICS

So far, we have not said anything specific about the properties of $P(t)$. Throughout this article, we have assumed that the proteins of interest evolve ergodically; i.e., we assume these proteins fold in a finite time, with or without the help of chaperones. Our goal was to consider how simple chaperones can reduce aggregation. Again, we have focused on proteins for whom aggregation is the dominant degradation pathway. Without loss of generality, we assert that $P(t)$ can be represented by one or possibly the sum of many independent decaying exponentials,

$$P(t) = \Theta(t)\int_0^\infty P(\lambda)e^{-\lambda t}\mathrm{d}\lambda. \tag{14}$$

Here $\Theta(t)$ is the Heaviside-step function, and equals 1 if $t \geq 0$, and 0 otherwise. This type of relaxation kinetics is common to all physical systems which satisfy detailed balance at equilibrium (53). Substituting Eqs. 14 and 9 into Eq. 13 yields

$$\langle t_F \rangle = \frac{1}{\lambda_D}\left[\left(\int_0^\infty \frac{P(\lambda)}{\lambda/\lambda_D + 1}\,d\lambda\right)^{-1} - 1\right]^{-1}. \qquad (15)$$

In this form, it is easier to see that, for any protein, the average folding time is, at most, inversely proportional to λ_D. This formula derived there is qualitatively similar to, but more general than, Eq. 3 from Sfatos et al. (28).

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