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Molecular chaperones: providing a safe place to weather a midlife protein-folding crisis

Patricia L Clark & Adrian H Elcock

Contrary to conventional wisdom that molecular chaperones rely on hydrophobic interactions to bind a wide variety of client proteins in danger of misfolding, three recent studies reveal that the ATP-independent chaperone Spy exploits electrostatic interactions to bind its clients quickly, yet loosely enough to enable folding of the client while it is chaperone bound.

Molecular chaperones are a diverse collection of ubiquitous proteins essential to protein homeostasis *in vivo*. Despite their diversity, the common thread that connects all chaperones is binding to incompletely folded conformations of a substrate ('client') protein and increasing the likelihood that the client will fold correctly instead of forming incorrect intermolecular interactions. This is an important job because misfolding can lead to aggregation and loss of native-protein function (**Fig. 1**). The best-characterized chaperones hydrolyze ATP, in a catalytic cycle that triggers a corresponding cycle of conformational changes within the chaperone that coordinates client binding and release[1–3]. Although under normal growth conditions only ~20% of proteins require a chaperone in order to fold correctly, these clients include many essential proteins, such as tubulin[4]. But chaperones really come into their own during cell stress. Heat shock and chemical stress destabilize the structures of both folded proteins and folding intermediates[5]. In response, many chaperones are massively upregulated to handle the larger client load[6].

Molecular chaperones avert the disaster of protein misfolding and aggregation—but how, exactly? As with all catalytic mechanisms, the crucial interactions, reaction intermediates and transition states are fleeting and thus are challenging to trap and study. For chaperones, a further complication arises from the conformational flexibility of the substrate: in contrast to the classic, well-defined transition state for a chemical reaction, chaperones recognize an ensemble of partially folded states, which presumably lead to a variety of transition states (and products). For this reason, attempts to solve the structure of a client protein bound to a chaperone have heretofore led to only low-resolution cryo-EM structures[7]. Equally puzzling is how chaperones recognize such a

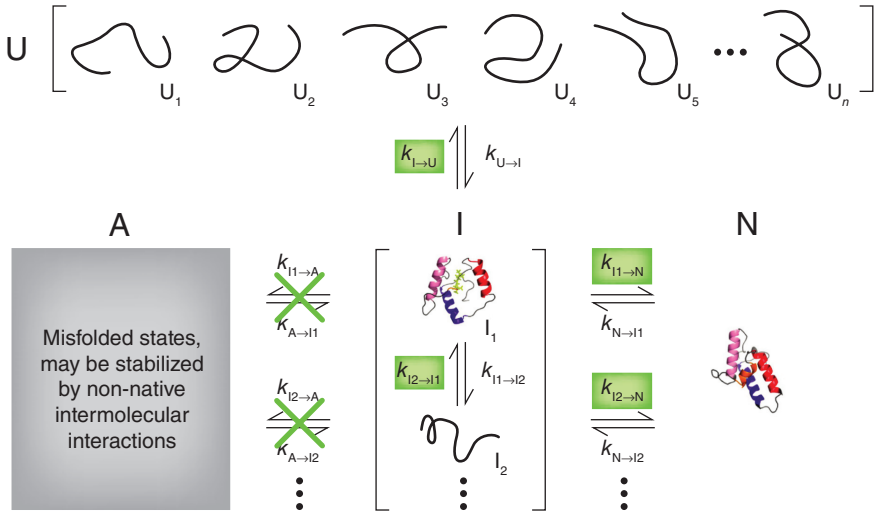

Figure 1 Protein folding involves the selective stabilization of the functional native conformation (N) of a protein, versus globally unfolded conformations (U), partially folded intermediates (I) and misfolded states (A). Both N and A are stable states, and hence the likelihood that a protein will fold successfully to N depends on the apparent rate constants for interconversions between various conformations (for simplicity, only a single rate constant is shown for the conversion of U to I). For example, a population of I_1 will occur if $k_{U\to I1} >> k_{I1\to N}$, $k_{I1\to I2}$ and $k_{I1\to A}$. Once I_1 has formed, it will partition to N if $k_{I1\to N} >> k_{I1\to A}$, or to A if $k_{I1\to A} >> k_{I1\to N}$. Cell stress can change the stabilities of these states and the rate constants for their interconversion. Molecular chaperones, many of which are upregulated during cell stress, can block aggregation by altering the stabilities of, or the access to, various conformations (green highlights). For example, the well-studied heat-shock-induced *E. coli* molecular chaperone GroEL has been shown to accelerate or decelerate the folding of different substrates. In all cases, however, GroEL sterically shields its substrate, thereby preventing the formation of intermolecular interactions (green Xs).

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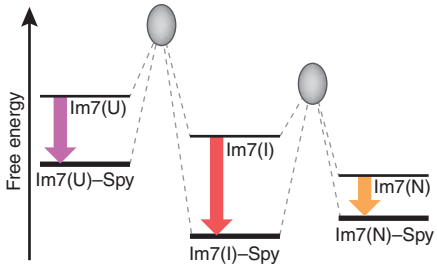

Figure 2 Effects of Spy binding on the folding-energy landscape of Im7, a model *in vivo* substrate. Equilibrium and kinetic results[12] demonstrate that Spy forms a stable complex with all three major Im7 conformational forms: unfolded (U), a well-populated on-pathway intermediate (I) and the final native structure (N). Colored arrows represent changes in free energy between Im7 unbound and in complex with Spy (purple, U; red, I; yellow, N). Although the binding constants for these complexes are similar, tighter binding to the intermediate stabilizes its Spy complex (Im7(I)–Spy) more than the others (Im7(U)–Spy and Im7(N)–Spy). Energy barriers for the transitions between these states (gray ovals) were not measured directly, but the slower folding of chemically denatured Im7 in the presence of Spy indicates that one or both transitions have higher free energy than that of the folding of Im7 alone.

wide variety of client proteins—this breadth is in direct contrast to the scope of conventional enzymes, which tend to have exquisite substrate specificity. The conventional model is that chaperones must recognize a common feature present in the partially folded states of all proteins, such as an exposed hydrophobic surface area, a general feature of polypeptide chains that have undergone hydrophobic collapse but still present hydrophobic groups not buried within the native protein interior[8–10]. Early support for this model came from structures and mutational analyses of the *Escherichia coli* Hsp60 chaperonin GroEL: a hydrophobic patch implicated in client binding is exposed in the unbound chaperone but becomes buried after a client is bound and the ATP hydrolysis cycle has begun[11].

Three new reports from Bardwell and colleagues, including one in this issue, shed new light on these conundrums, specifically on how a chaperone recognizes its substrate and promotes its proper folding versus aggregation[12–14]. For each of these studies, Bardwell and colleagues use Spy, a small, dimeric, cradle-shaped, ATP-independent chaperone from the *E. coli* periplasm. Whereas chaperones that hydrolyze ATP are typically considered to be 'foldases' because they can catalyze client-protein folding and unfolding, ATP-independent chaperones such as Spy have classically been considered to be 'holdases', which are confined to stably binding partially folded structures until the stressful condition is resolved. After stress resolution, the native structure returns to a state more stable than the chaperone-client interactions, thus leading to client release. In agreement with this model, the level of Spy shifts from nearly zero in unstressed cells to 20–50% of *E. coli* periplasmic protein under stress conditions[15]; thus, there should be a copy of Spy available to bind each non-Spy protein in the periplasm.

In a study published in *NSMB* in January, Bardwell and colleagues have shown that, contrary to conventional wisdom regarding an ATP-independent holdase, a client protein can fold while it is bound to Spy[12]. Indeed, Spy binds with similar affinity to the native state of Im7, a model client, as it does to Im7 mutants designed by Radford and colleagues to mimic unfolded and on-pathway kinetic intermediate conformations. Nevertheless, Spy binds the Im7 intermediate most tightly, thereby leading to partial unfolding of native Im7 and perhaps explaining why the concentration of Spy is near zero under unstressed conditions[15]. Notably, the Im7 folding rate is slower in the presence of Spy, thus suggesting that Spy lowers the energy of the unfolded and intermediate states more than the energies of the transition states between them and native Im7 (**Fig. 2**). Overall, these results paint a picture of Spy functioning as a sanctuary for stress-induced partially folded states—a safe place for Im7 to retreat to when it is partially folded, yet also an environment in which Im7 can fold before its release back into the periplasmic milieu. What is still not clear, however, is the timing for launching this Spy sanctuary. Although Spy binds Im7 quite rapidly (on a submillisecond timescale) *in vitro*[12], the concentration of Spy at the onset of stress is near zero. Hence, Spy function first requires Spy transcription, translation and secretion into the periplasm, and each of these steps is orders of magnitude slower than the rate of Spy-client binding. A related complication is the need for Spy itself to fold in the periplasm before it can function, and this folding must be achieved under conditions of cell stress. In the future, it will be important to measure the kinetics of Spy appearance in the periplasm and the consequences of altering this rate on the ability of a client protein to withstand cell stress.

Another important open question from the study above is what specific forces enable Spy to bind its substrates so quickly. In a new study in *Cell*, Bardwell and colleagues show that, in contrast to hydrophobicity-centric models for chaperone-client binding, electrostatic interactions greatly increase the rate of Spy-Im7 binding *in vitro*[13]. Whereas hydrophobic interactions are strong only over very short distances, the distance dependence for electrostatic interactions is much longer[16], thus explaining the faster binding rate[17]. As expected, after the initial electrostatically driven Spy–Im7 complex forms, it is further stabilized by hydrophobic interactions. Also, as expected, upon Im7 folding, these Spy-Im7 hydrophobic interactions are broken as client hydrophobic surface area becomes buried within the interior of Im7. To what extent is this Spy-Im7 binding mechanism common for the binding of Spy to other clients? Intriguingly, most periplasmic proteins have a net negative charge[18] and would therefore be candidates for similar interactions with the positively charged binding face of Spy. It will be interesting to determine the extent to which net charge shapes the binding of other chaperones to their clients. Is Spy's positively charged binding surface a feature shared by other periplasmic chaperones? Is a similar mechanism used by cytoplasmic chaperones to bind their client proteins, which also tend to have a net negative charge[19]?

Although the kinetic studies described above provide a nuanced understanding of Spy's effects on Im7 folding, they do not resolve one of the most fundamental questions surrounding chaperone-client binding: what is the ensemble of structures adopted by the client when it is bound to the chaperone? To resolve this, Bardwell collaborated with the Brooks group to develop a new technique based on molecular dynamics simulations of partially folded conformations of the client protein that are compatible with residual electron and anomalous density (READ) from X-ray diffraction of Spy cocrystallized with a fragment of Im7 ($Im7_{6–45}$)[14]. In this study, eight individual positions within $Im7_{6–45}$ were labeled with the strong anomalous scatterer iodine, and the residual electron density from the disordered client and the anomalous scattering from each iodine label were used to select compatible conformations of $Im7_{6–45}$ from a pool of 'energetically reasonable' $Im7_{6–45}$ conformations generated by coarse-grained molecular dynamics simulations. These simulations used a one-bead-per-residue model, and, as is typical for such a simplified model, interresidue interactions were described through statistical potential functions[20]. Six $Im7_{6–45}$ conformations were sufficient to recapitulate the experimental data. Each of these conformations deviates from the native structure to a different extent, and the authors argue that the more folded conformers of this ensemble have fewer hydrophobic contacts with Spy. It will be interesting to determine whether this possibility can be corroborated through READ—can the Im7 ensemble be shifted more toward the folded or unfolded states, for example

by introducing destabilizing mutants in the client, and, if so, are the predicted changes observed in the fitted ensembles? An obvious next step, given the importance of Spy-client electrostatic interactions, would be to use a somewhat higher-resolution model (multiple beads per residue), which would allow favorable and unfavorable electrostatic interactions between the client and chaperone to be modeled explicitly rather than implicitly via the statistical potential functions.

Molecular chaperones have long been regarded to be crucial contributors to successful protein folding *in vivo*, particularly under conditions of cell stress[21,22]. Yet despite three decades of active investigation, many details on exactly how these proteins function are still lacking. By focusing on an ATP-independent chaperone, these three new studies strip away some of the inherent complexity of chaperone mechanisms, highlighting the (still quite complex) crucial steps of client-protein recognition, binding and folding. Not surprisingly, results from these studies raise as many new questions as they resolve. The rapidity with which Spy binds its client brings up the issue of how the cell coordinates the much slower upstream steps necessary to position Spy for action. The striking contributions of electrostatics to this rapid binding lead to queries about the generality of this mechanism: is it used by Spy to recognize other clients? To what extent do other chaperones exploit electrostatics? Teasing apart the subset of partially folded conformations bound to Spy highlights the potential for using the new READ approach to test longstanding hypotheses regarding the specific hydrophobic interactions formed between these client conformations and the chaperone-binding surface. The answers may have important implications for developing a general understanding of chaperone-client interactions. Finally, it is important to bear in mind that these studies were performed under conditions in which Im7 can fold autonomously, and hence it remains to be determined how the crucial interactions are affected when client folding intermediates become prone to self-association (**Fig. 1**).

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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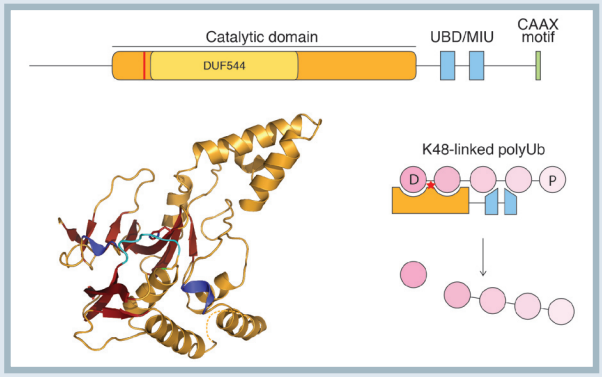
New DUBs on the block



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Ubiquitination is a post-translational modification in which the ubiquitin protein (Ub) is attached to an acceptor lysine residue in the substrate. Ub itself has seven lysine residues and an N-terminal methionine that can serve as acceptors for another Ub; thus, chains with different linkages and different functional outcomes may form. In particular, K48-linked chains target their substrates for proteasomal degradation. Ub conjugates can be removed by deubiquitinating enzymes (DUBs), of which five families have been identified in the human genome.

Kulathu and colleagues have now identified a new DUB family, named MINDY (for MIU-containing novel DUB family), that has selectivity toward long K48 Ub chains (*Mol. Cell* doi:10.1016/j.molcel.2016.05.009).

The authors started by investigating a previously uncharacterized protein, FAM63A, which bears tandem motifs interacting with Ub (MIUs; blue boxes in the domain-architecture schematics at top). A fragment containing the MIU motifs specifically binds K48 chains. FAM63A also contains a domain of unknown function, DUF544 (yellow box), and a conserved cysteine (red line), which together are part of a catalytic domain that displays cysteine protease activity with specificity toward K48 linkages. Other members of the family that were identified in humans and other eukaryotes on the basis of sequence similarity showed comparable properties *in vitro*.

To understand how FAM63A (renamed MINDY-1) recognizes and cleaves K48 Ub chains, the authors solved the crystal structure of its catalytic domain, both alone (model, bottom left) and in a covalent complex with a modified Ub moiety. The structures reveal a new cysteine protease folding variant that has no structural similarity to other DUB families; they also indicate how substrate binding activates the enzyme.

Furthermore, detailed biochemical analysis showed that MINDY-1 trims K48-linked Ub chains from the distal end. This activity requires chains with at least four Ub moieties, because the tandem MIU motifs bind a K48 di-Ub, and the catalytic domain binds another K48 di-Ub and cleaves the intervening linkage (bottom right). Importantly, coimmunoprecipitation assays on cell lysates demonstrated that MINDY-1 recognizes K48-linked polyubiquitinated proteins in a manner that requires the MIU motifs.

The identification of a new family of DUBs paves the way for functional studies to bring their cellular roles to the fore.

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