

ATP-Independent Chaperones

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Abstract

The folding of proteins into their native structure is crucial for the functioning of all biological processes. Molecular chaperones are guardians of the proteome that assist in protein folding and prevent the accumulation of aberrant protein conformations that can lead to proteotoxicity. ATP-independent chaperones do not require ATP to regulate their functional cycle. Although these chaperones have been traditionally regarded as passive holdases that merely prevent aggregation, recent work has shown that they can directly affect the folding energy landscape by tuning their affinity to various folding states of the client. This review focuses on emerging paradigms in the mechanism of action of ATP-independent chaperones and on the various modes of regulating client binding and release.

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1. INTRODUCTION

All cellular processes depend on the proper functioning of proteins. Proteins are essential building blocks, vital for the construction of the cellular house of life. However, globular proteins are not like the rigid building blocks that one can buy at home building supply stores. The native states of most globular proteins instead are marginally stable, with folding free energies of typically 5–15 kcal mol⁻¹, a meager amount of energy about the same as that found in a single hydrogen bond (80). Therefore, native proteins can populate alternative misfolded conformations on a biologically relevant timescale. Unfolded proteins are generally nonfunctional, and partially unfolded states like folding intermediates often expose hydrophobic surfaces, which make them prone to self-associate into aggregates (44). The ordered aggregation of proteins into β -sheet-rich structures called amyloid fibrils has been linked to several devastating and frighteningly common age-associated diseases such as Alzheimer's and Parkinson's diseases (54). One of the big challenges that organisms, including humans, face is maintaining function despite the surprisingly unstable nature of the protein building blocks from which they are constructed. This applies under normal conditions but is even more relevant when organisms are subject to protein unfolding stress or during aging (110). Among the tools that nature has evolved to help address these challenges are molecular chaperones. These conserved proteins are often stress induced but are also involved in many aspects of protein quality control under normal conditions; they assist in *de novo* folding, maintain the native state of folded proteins, assemble protein complexes, prevent protein aggregation, disaggregate and unfold misfolded proteins, translocate unfolded polypeptides across membranes, and target misfolded proteins to proteolytic machineries and to autophagy (53). Molecular chaperones can be classified into two broad categories: ATP-independent chaperones and ATP-dependent chaperones that require ATP hydrolysis for their action. A great deal has been learned about how ATP is used to either drive a chaperone's catalytic cycles or regulate allosteric changes and client binding and release (53). ATP-independent chaperones are often regarded as passive holdases that tightly bind onto proteins until conditions allow their release to ATP-dependent chaperones that then do the real work of protein folding. The recent realization that some ATP-independent chaperones allow proteins to fold while bound to the chaperone raises fascinating questions about chaperone–substrate interaction and the delicate balance of forces necessary for folding while

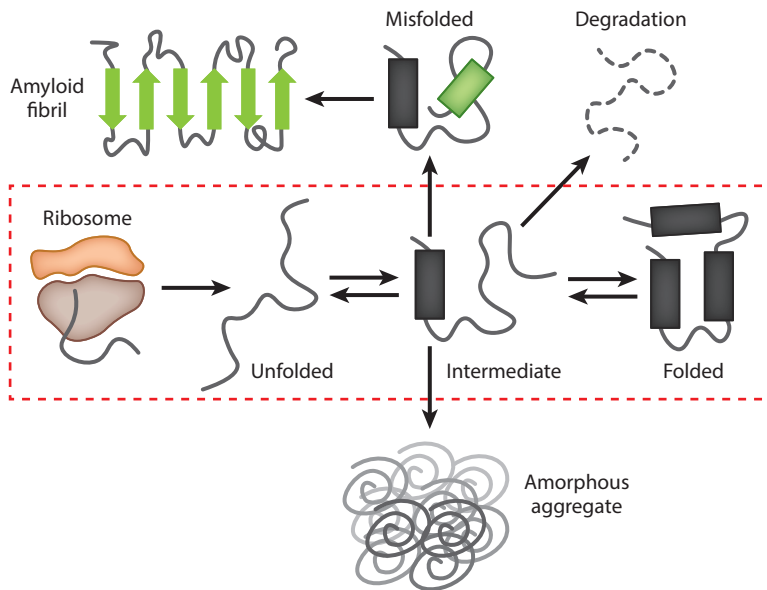


Figure 1

Off-pathway events in protein folding. A nascent polypeptide chain (*gray*) folds into its native state as it emerges from the ribosome (*orange*). Aggregation-prone partially structured intermediates are often populated in the folding pathway of proteins. Owing to their low stability, these intermediates are also prone to proteolytic degradation. Additionally, proteins can encounter kinetic traps in the form of misfolded intermediates that can form amyloid fibrils (*green*). Molecular chaperones prevent these off-pathway processes and assist in the productive folding processes that occur within the dashed red box.

bound to occur. The answers to these questions have the potential to address the fundamental and but still poorly understood issue of exactly how chaperones affect client protein folding.

2. MECHANISMS OF ATP-INDEPENDENT CHAPERONES

Chaperones work by facilitating folding reactions and inhibiting aggregation. Polypeptides come out of the ribosome exit tunnel in a minimally structured form, then undergo folding to the native structure by hydrophobic collapse typically through one or more partially folded intermediates that are sensitive to degradation, aggregation, and misfolding (**Figure 1**). These three processes compete with the folding pathway and render the protein nonfunctional. Chaperones, illustrated in **Figure 1**, are there to keep the proteins in line. They prevent off-pathway events and facilitate proper folding. If we wish to take an informed approach to treating folding diseases, we will clearly need to learn more about the mechanism of host factors like chaperones that affect folding in the cell.

The folding process is usually an equilibrium reaction, but aggregation and proteolysis are generally irreversible and thus need to be avoided by the cell. The action of ATP-independent chaperones can in large part be explained by models that involve kinetic partitioning among the association rate of chaperone binding to an unfolded or misfolded client, the rate of protein aggregation, and the rate of client protein refolding (**Figure 2**). For simplicity, let us consider two chaperone candidates, one that binds tightly to the folded state of proteins and one that binds tightly to the unfolded state. The first candidate will tend to both speed folding by dragging the folding equilibrium toward the folded state and stabilize the folded state thermodynamically,

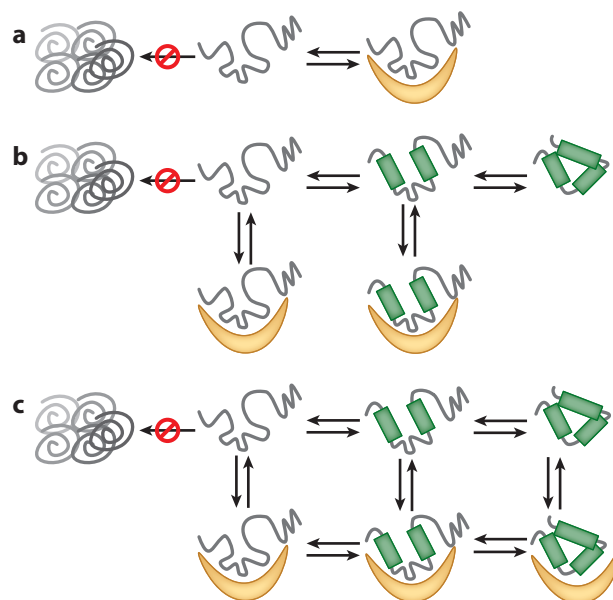


Figure 2

Diverse mechanisms of ATP-independent chaperones. Chaperones are shown as orange crescent-shaped structures, and client proteins are shown in green and gray. (a) ATP-independent chaperones can act as holdase chaperones, which tightly bind to client proteins and sequester proteins in non-native conformations. (b) Some ATP-independent chaperones, like trigger factor, have been shown to promote protein folding by stabilizing productive partially folded intermediates. (c) Other ATP-independent chaperones, like Spy, can allow their client proteins to fold while bound.

making it, one would think, an ideal chaperone (115). However, unless there is a specific mechanism to release this first candidate from the folded substrate, this protein is not a chaperone because chaperones by definition are not part of the final folded structure. These proteins of course exist, but they are not chaperones; instead, they are interacting partners in multisubunit assemblies, and unlike most chaperones that have broad substrate specificity, these subunits are generally very protein substrate-specific, since they often exhibit large and specific binding interfaces.

The second candidate, which binds strongly to the unfolded state, will drag the folding equilibrium in the unfolded direction. If it binds tightly enough to the unfolded state, then it will tend to protect its substrate protein against aggregation and cellular proteases. Such a chaperone is likely most useful under stress conditions, when the cell needs to temporarily protect proteins from an unhealthy folding environment. ATP-independent chaperones will tend to function as holdase chaperones when the client association rate exceeds the client aggregation and refolding rates. Under proteotoxic stress, the cellular concentrations of active chaperones are upregulated at either the transcriptional or posttranslational levels (78). Interactome and gene expression analysis of aging human brains revealed an induction of ATP-independent chaperones (12). With more active chaperones around, the rate at which chaperones bind to their client proteins increases, making binding more likely than refolding and aggregation. If holdases live up to their name, then they will tend to sequester their clients in a non-native state, either slowly releasing their clients or transferring them to ATP-dependent chaperones that then can facilitate folding. Thus, holdase chaperones tend to become kinetic traps that prevent protein aggregation and inhibit protein folding. Tight binding with nanomolar affinity has been reported for many ATP-independent chaperones such

as SecB (82, 117), Skp (76, 87), and the sHsp family member Hsp27 (97). However, this is not a universal feature for ATP-independent chaperones. Many ATP-independent chaperones, such as trigger factor, Spy (spheroplast protein Y), and SurA, have been shown to bind to their client proteins with low affinity, with apparent dissociation constants in the micromolar range. These weak associations are apparently sufficient to allow these chaperones to suppress protein aggregation effectively. While these chaperones may not form a stable complex with unfolded proteins, they employ other mechanisms to protect proteins. One apparently appealing mechanism by which weak-affinity chaperones may facilitate folding is by associating with productive partially folded intermediates. This mechanism has been proposed for the action of trigger factor, which suppresses protein aggregation and promotes correct folding by stabilizing an aggregation-prone intermediate on the folding pathway (70). Association with partially folded proteins has also been observed for two classical holdase chaperones, the small heat shock proteins (sHsps) Hsp26 and Hsp42 (113).

One challenge faced by chaperones, and a central question in the field, is how chaperones can tune their affinity such that they bind with appropriate affinity to a wide range of substrate proteins. This problem is particularly acute for ATP-independent chaperones, as they cannot use ATP to regulate substrate affinity. It is easy for a chaperone to miss the mark; trigger factor, for instance, binds relatively weakly to maltose binding protein (MBP) and has a negligible effect on the folding of MBP, whereas the classic holdase chaperone SecB binds more tightly and strongly inhibits MBP folding (40). Another novel mechanism for the action of ATP-independent chaperones is to allow the protein to fold while bound. This mechanism has been intensively studied using Spy as a model ATP-independent chaperone and is discussed separately in Section 3.1. Together, ATP-independent chaperones display a wide range of client binding affinities, from subnanomolar to micromolar. The diverse binding affinities of ATP-independent chaperones enable them to create a more versatile action than was initially assumed. In the next section, we consider in detail a few representative ATP-independent chaperones selected from those listed in **Table 1**; space considerations prevent us from comprehensively discussing all ATP-independent chaperones known or referencing even the majority of the abundant literature.

3. STRUCTURE AND DYNAMICS OF ATP-INDEPENDENT CHAPERONES

3.1. Spy

Spy is a 16-kDa ATP-independent chaperone discovered in a genetic selection designed to improve protein stability in *Escherichia coli*. Its small size, lack of cofactors, and biophysical amenability have made Spy an excellent model to study the effect of ATP-independent chaperones on protein folding. Spy has a unique fold; it is a thin, α -helical, cradle-shaped homodimer that lacks any globular core. The cradle of Spy is positively charged, with an average thickness of just 9.2 Å, thinner than the width of a single α -helix (88). Spy provides a flexible and amphiphilic surface on its concave side to cradle its bound clients. The binding surface can accommodate multiple conformations of the client, a feature that allows the sampling of various folding states while the substrate remains continuously held by Spy (38) (**Figure 3a**).

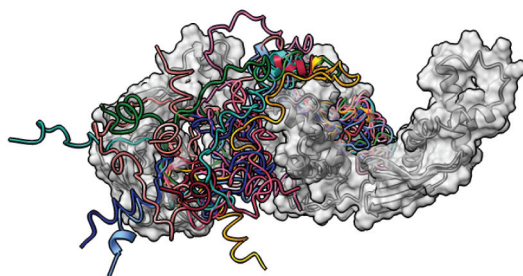
The chaperone cycle for Spy has been elucidated using the α -helical bacterial immunity protein Im7 as a model substrate. Im7 is a very well-studied folding model that populates an on-pathway kinetic intermediate (16). Unlike most chaperones, which are thought to predominantly recognize exposed hydrophobic surfaces on clients, Spy initially recognizes unfolded clients via rapid and long-range electrostatic attractions, with association rates of the order of $10^7 \text{ M}^{-1} \text{ s}^{-1}$. Following the initial encounter, hydrophobic interactions stabilize the Spy-unfolded Im7 complex, thereby shielding hydrophobic surfaces on Spy and Im7 from the solvent.

Table 1 Client affinities of ATP-independent chaperones

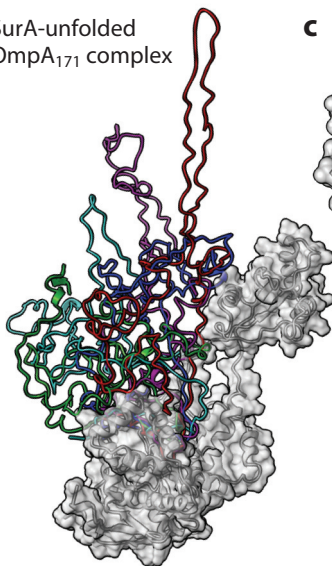
ATP-independent chaperones	Unfolded client proteins	Apparent K_d	References
Trigger factor	$\sigma 32$ peptides	120 μM	85
	Mature PhoA	2 μM	90
	Mature MBP	0.5 μM	40
	Modified LA	1.85 μM	66
	RNC	2–100 nM	11
Spy	Im7	4.7 μM	108
	Fyn SH3	2.9 μM	118
	Apo flavodoxin	0.35 μM	72
SurA	OmpA	1.8 μM	41
	OmpT	9.3 μM	
	OmpX	0.8 μM	15
Skp	OmpLA/OmpW/PagP	10 nM	76
	OmpA/OmpG	20 nM	87
SecB	Modified LA	30 nM	82
	Modified RNase	5 nM	
	Modified BPTI	50 nM	
	Barnase	2 nM	121
	Premature MBP	0.8 nM	117
	Mature MBP	80 nM	
	Mature PhoA	0.5 μM	40
Hsp27	T4 lysozyme	40–200 nM	97
ttHsp40	Mature PhoA	10 μM	48

ATP-independent chaperones have been traditionally thought to only bind partially unfolded clients and prevent off-pathway interactions such as aggregation. This holdase view of ATP-independent chaperones has been recently challenged by the discovery of novel mechanisms whereby Spy allows Im7 to fold while bound (108). Im7 folds via a trapped on-pathway intermediate that forms very rapidly upon denaturant dilution. In addition to the native protein (Im7_N), the intermediate (Im7_I) and unfolded (Im7_U) states of Im7 have been characterized extensively using destabilized mutants that mimic these non-native states (83, 102). Spy binds to all three states of Im7 with comparable affinities in the micromolar range. Kinetic analysis shows that Spy allows protein folding of its substrates while they are bound (108, 118). This folding-while-bound paradigm also holds true for the interaction of Spy with the two-state folder SH3 (118). The thermodynamic requirement for the folding-while-bound mechanism is that the chaperone have weak interactions with various folding states of the client such that the client can explore its folding landscape while being loosely held by the chaperone. Mutants of Spy that bind more tightly slow folding (118). If the client binds tightly enough, then Spy turns into a holdase, as it does, for instance, for the complex folding model apo-flavodoxin. This protein populates an off-pathway intermediate, and Spy kinetically traps this intermediate in a non-native state by tight interaction (72). Probing the backbone dynamics by solution nuclear magnetic resonance (NMR) spectroscopy has shown that the unfolded ensemble of Im7 is spatially compacted by Spy; binding of the client appears to increase the conformational entropy of the chaperone. This transfer of conformational entropy from the client to the chaperone may help explain the ATP independence of the system.

a Trigger factor-PhoA_{365–471} complex



b SurA-unfolded
OmpA₁₇₁ complex



c Spy-bound Im7_{6–45}

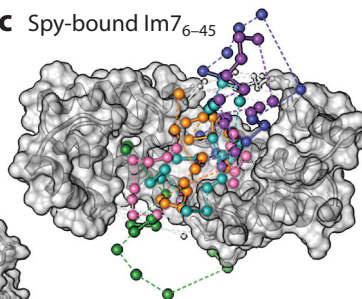


Figure 3

Chaperone–client complexes. (a) Nuclear magnetic resonance spectroscopy–derived ensemble of trigger factor-PhoA_{365–471} complex (90). (b) Sparse ensemble of SurA-unfolded OmpA₁₇₁ complex defined by experimental restraints from SANS and XL-MS (68). (c) Ensemble of Spy-bound Im7_{6–45} derived from residual electron and anomalous density (READ) crystallography (38). In all cases, the chaperones are shown as gray space-filling models, and the client ensemble is shown as a multiple-colored worm (for panels a and b) or ball and stick (for panel c).

In an aqueous solution, proteins generally fold by hydrophobic collapse, where the hydrophobic residues are buried in the protein interior (30). This follows the principle that Anfinsen (4) postulated in 1973, namely, that the amino acid sequence of a protein carries all of the instructions for folding. Koldewey et al. (57) proposed a simple model for Spy's chaperone action where the substrate protein follows similar principles for folding while bound to the chaperone as it does in its absence, namely by burying the hydrophobic residues in its interior while bound to the chaperone. Hydrophobic collapse of the folding client decreases its affinity for Spy and helps favor substrate release in a native or near-native form. One advantage of this mode of chaperone action is that, by providing a folding-friendly surface that loosely cradles the client protein, the chaperone allows the client protein to fold while simultaneously protecting it from aggregation. This is vital because, once a protein is aggregated, it is very difficult for it to recover, especially

in an ATP-deficient compartment like the periplasm. One consequence of this is that the chaperone Spy slows folding somewhat. In general, chaperone binding to either folding intermediates or to the unfolded state will tend to slow folding; however, if this enables the chaperone to inhibit irreversible aggregation, then this seems like a small price for the cell to pay. Importantly, by allowing Anfinsen folding, the chaperone does not need any client-specific folding instructions, perhaps explaining how chaperones can act on so many different proteins. This folding-while-bound mechanism is not limited to Spy; it also has been proposed to occur with SecB and trigger factor and may represent an evolutionarily ancient mechanism, with regulation added later by the inclusion of ATP hydrolysis and cofactors for chaperones like Hsp70, Hsp90, and GroEL (56).

3.2. Trigger Factor

Trigger factor is a 48-kDa ATP-independent chaperone that adopts an evocative dragon-shaped architecture. It consists of three domains: an N-terminal domain that is necessary for ribosome association, a C-terminal domain that engages with nascent polypeptide chains as they emerge from the ribosome, and an internal domain that can facilitate peptidylprolyl isomerization reactions, which are important for proteins to be able to reach their proper folded state (22). The peptidylprolyl isomerase (PPIase) domain can also serve as an auxiliary binding site for nascent chains (65), but curiously, it seems not to be required for trigger factor's chaperone activity (58). A double deletion of the genes encoding trigger factor and DnaK (*E. coli* Hsp70) (9) leads to severe protein aggregation and synthetic lethality (19, 111). This implies a substantial degree of overlap between the substrates and action of trigger factor and those of DnaK and provided an early indication that ATP-independent chaperones can substitute for the action of ATP-dependent ones. Trigger factor has become a paradigm for studying ribosome-associated chaperones and chaperone-mediated cotranslational folding. Trigger factor directly associates with the large ribosomal subunit protein L23 through its N-terminal domain and forms a dome over the ribosomal exit tunnel where the nascent polypeptide chains emerge (8, 22, 71, 96). The trigger factor-ribosome complex has a half-life time of at least 10 seconds, long enough for 200 amino acids to be synthesized by the ribosome while trigger factor remains bound (49, 89). As elongating nascent chains emerge from the ribosome, the N terminus of the polypeptide first encounters the N-terminal domain of trigger factor and subsequently encounters the entire interior cavity of trigger factor's C-terminal domain. The nascent chains may reach the PPIase domain, but only if the chain length is long enough and the association lasts long enough, perhaps helping to explain the nonessential nature of this trigger factor domain (71). However, this trigger factor-mediated cotranslational folding mechanism was challenged by a more recent study that showed that the trigger factor-ribosome complex has a much shorter lifetime of approximately 60 ms, shorter than the >10-s lifetime previously observed (11). This binding mode is hypothesized to allow trigger factor to scan through translating ribosomes rapidly in the cytosol and efficiently recognize aggregation-prone regions within nascent chains (49, 89). In addition, the transient formation of the trigger factor complex may ensure that enzymes that work by modifying the nascent chains' N termini, such as peptide deformylases and methionine aminopeptidases, have adequate access. Indeed, trigger factor only recognizes translating ribosomes that carry a nascent chain of more than 100 amino acids (79). This enables the modifying enzymes to first bind and catalyze nascent chain modifications prior to the binding of trigger factor. As an ATP-independent chaperone, trigger factor has long been thought to solely recognize and stabilize hydrophobic-rich segments of nascent polypeptide chains (49, 85) and improve the refolding yield of its clients by inhibiting protein aggregation and retarding cotranslational folding rates (1). Recent studies, however, have demonstrated that trigger factor may play additional roles in the cotranslational folding and assembly processes. For instance, trigger factor recognizes and unfolds misfolded intermediates, ensuring correct folding

of nascent chains (37). It has also been shown to promote protein assembly by selectively preventing premature interactions of a nascent chain with its binding partners (98).

While ribosome association is a prerequisite for trigger factor to engage with newly synthesized polypeptide chains and to be directly involved in cotranslational folding processes, it is notable that only a small fraction of trigger factor is found to be ribosome bound. The majority of trigger factor instead self-assembles into a dimeric form in the cytoplasm (84). Dimeric trigger factor may serve as a storage form, but free trigger factor is also capable of suppressing protein aggregation both in vitro and in vivo (67). Monomerization of trigger factor exposes the C-terminal domain's hydrophobic substrate-binding pocket by allowing the formation of a complex with non-native substrates (66, 85). NMR studies revealed that trigger factor–substrate complexes are dynamic in nature; trigger factor promiscuously recognizes unfolded peptides mainly through four distinct hydrophobic-rich patches distributed along its C-terminal domain (90) (**Figure 3b**). In addition to recognizing unfolded proteins, trigger factor is also reported to bind to small well-folded proteins through hydrophilic interactions (67). This dynamic binding to substrates and heterogeneous sampling of multiple conformations could allow trigger factor to reshape the folding pathway for its client proteins (70). Most of the molecular and structural details of how trigger factor associates with its clients and how it mediates protein folding have been determined from studies conducted with purified trigger factor in the absence of ribosomes, but it is unclear how accurately the mechanisms derived for free-form trigger factor also apply to ribosome-tethered trigger factor. One notable difference between free-form trigger factor and ribosome-bound trigger factor has been mapped to the N-terminal domain. Upon binding to the ribosome, the N-terminal domain of ribosome-bound trigger factor twists to expose its hydrophobic patch so that this patch faces toward the ribosomal exit tunnel, which may aid in trigger factor recognizing translating polypeptide chains (22, 49, 71). In contrast, no interaction between the N-terminal domain of free-form trigger factor and PhoA peptide has been observed using NMR (90). Thus, it is necessary to further test if the mechanism of the free form of trigger factor is the same as that of ribosome-bound trigger factor.

3.3. Small Heat Shock Proteins

sHsps are widespread in all kingdoms of life. Prokaryotes usually encode one or two sHsps; for example, *E. coli* harbors two sHsps, IbpA and IbpB (32). Unicellular eukaryotes also have relatively few sHsps; for example, *Saccharomyces cerevisiae* has Hsp26 and Hsp42. The number of sHsps increases in multicellular eukaryotes like humans, which have 10 sHsps, and plants like *Arabidopsis thaliana*, which has 19 (32). sHsps are composed of a highly conserved α -crystallin domain flanked by N- and C-terminal regions. Their molecular weight ranges from approximately 12 to approximately 43 kDa. The α -crystallin domain, also found in eye lens α -crystallin proteins, is approximately 90 amino acids long and is a compact β -sandwich structure composed of seven to eight antiparallel β -sheets (7, 39, 45, 50, 86). N-terminal and C-terminal regions are variable in sequence and length and are often structurally disordered. The N-terminal regions of sHsps, ranging from 24 to 247 residues, play a particularly driving role in oligomer formation and in the interaction with substrate proteins (13). The C-terminal regions are <20 amino acids long and, in most cases, they contain the conserved IXI/V motif, which plays a prominent role in sHsp oligomerization (75). As discussed below, the formation and dissociation of oligomers that range in size from 12 to 32 mers are involved in regulating the chaperone activity and substrate binding activity of sHsps (13). The majority of sHsp family members form ensembles of oligomeric structures with varying degrees of polydispersity; a few sHsps only form dimers or tetramers due to the shortened N- and/or C-terminal regions or the lack of the conserved IXI/V motif (14, 55, 63).

sHsps bind misfolded or unfolded proteins and prevent irreversible protein aggregation (39). They are not actively involved in the protein folding process, unlike ATP-dependent folding chaperones like Hsp60, Hsp70, and Hsp90. sHsps are therefore viewed as classic holdase chaperones, which play an essential role in proteostasis under stress conditions (45). Upon sensing stresses, sHsps can rapidly shield non-native proteins; prevent them from aggregating; and keep them in a folding-competent state for poststress refolding or disaggregation, generally performed by other chaperones (27, 33, 47). The N-terminal domains of sHsps are enriched in aromatic residues, which enable them to recognize the solvent-exposed hydrophobic regions of non-native clients (59). sHsp–client complexes can be highly polydisperse, with over 300 different stoichiometries reported in one study (104). However, these assemblies are stable; i.e., they do not spontaneously dissociate. Instead, sHsp–client complexes require the ATP-dependent chaperones to rescue the client and facilitate its refolding (61, 73, 74). Interactions with Hsp70 and J-domain proteins are well conserved, whereas interactions with the hexameric Hsp100 disaggregase only occur in organisms where Hsp100s are found, namely, in bacteria and yeast. The binding of sHsps can reduce the molecular mass of aggregates and increase the surface-to-volume ratio, thereby making more binding sites available for ATP-dependent chaperones. sHsps can also facilitate the disassembly process by diminishing strong interactions between non-native proteins and sequestering proteins in their near-native conformations (103, 113). Zwirowski et al. (123) recently studied the mechanism employed by the Hsp100–Hsp70 system to disassemble sHsp–client assemblies. The architecture of sHsp–client assemblies consists of a dynamic outer shell of sHsps and an immobile inner core composed of sHsps and misfolded client proteins (123). The Hsp70 system displaces the outer surface-exposed sHsps by competitive dissociation. This exposes the misfolded proteins in the stable core that are extracted and refolded by the Hsp100–Hsp70 machinery.

3.4. SurA

SurA is a 45-kDa periplasmic chaperone that is essential for cell survival in the stationary phase. It assists in the folding of several outer membrane proteins (OMPs) (for a review, see 107). *E. coli* SurA is a modular protein with a core domain composed of N- and C- terminal regions. SurA has two parvulin-like PPIase domains (P1 and P2); P1 has no PPIase activity. The core domain of SurA contacts the P1 domain to form a globular region that has an extended substrate-binding crevice, while the P2 domain is separated from the core by two linkers (10). The PPIase domains are nonessential under at least some laboratory conditions, although the independent presence of PPIase-like domains in two unrelated chaperones, SurA and trigger factor, is clear testimony to the importance of proline isomerization in the protein folding process. As with trigger factor, the PPIase domains are not required for chaperone activity, but there is evidence that they can regulate or enhance the chaperone activity of SurA (41). The *in vitro* antiaggregation activity of SurA for its substrate OmpT, for instance, is enhanced by the presence of its PPIase domain(s). Chaperones need to deal with and, in some cases, facilitate large conformational changes within their substrates, so it is not surprising that they too undergo major conformational changes. These conformational changes are often easily isolatable for ATP-dependent chaperones by the use of ATP analogs, and they have been observed for some of the ATP-independent chaperones discussed in this review as well. For SurA, domain-level conformational dynamics appear to be important, as introducing a disulfide bond that tethers the P1 and core domains together results in impaired OMP activity (101). A recent study used an integrative approach employing mass spectrometry–based techniques, single-molecule Förster resonance energy transfer, and molecular dynamics (MD) simulations to show that SurA adopts conformational states in solution that differ greatly from the extended conformation in its static crystal structure, with the P2 domain

lying closer to the core and P1 domains in solution (15). The results also showed that the P1 domain of SurA adopts closed and open states at the core–P1 interface, which could be functionally important (15). However, the relative abundance of the various open and closed conformations is hard to pin down, with another study reporting that the hydrodynamic properties of SurA agree well with the extended arrangement seen in the crystal structure where the P2 domain extends away from the core and P1 domains (68). Regardless, it is still unclear how these conformational changes in SurA are coupled to client binding and delivery to the β -barrel assembly machinery (BAM) complex for folding into the outer membrane.

Multiple lines of evidence have elucidated the dynamics of OMPs bound to SurA (**Figure 3c**). Using single-molecule force spectroscopy, Thoma et al. (112) showed that, upon partial unfolding by mechanical force, the FhuA receptor populates non-native misfolded conformations when allowed to refold in the absence of chaperones. However, when refolding occurs in the presence of SurA, unfolded FhuA inserts into the lipid membrane and folds into native β -hairpins in a stepwise fashion. SurA alters FhuA folding by decreasing the probability of misfolding and increasing the probability of its folding into native β -hairpins. SurA-bound FhuA exists in a dynamic unfolded ensemble with conformational exchange rates in milliseconds. Crosslinking mass spectrometry and SANS data on another client, namely, the barrel domain of unfolded OmpA, supports the idea that SurA expands the unfolded client (68). Collectively, these studies point to the intriguing possibility that ATP-independent chaperones like SurA can not only prevent aggregation, but also alter the folding pathway of their clients, steering them away from misfolded states.

4. REGULATION OF ATP-INDEPENDENT CHAPERONES

Molecular chaperones bind and sequester unfolded proteins to prevent aberrant intermolecular interactions that can lead to cytotoxic protein aggregation. As key players in the proteostasis network, chaperones need to release bound clients in a spatiotemporally regulated manner. Failure to release client proteins could potentially interfere with cellular functions (18, 118). In contrast to ATP-dependent chaperones, which can utilize ATP-based allostery to trigger substrate binding and release, ATP-independent chaperones have evolved diverse strategies to regulate their chaperone activity in the absence of cofactors. In addition to transcriptional control, many ATP-independent chaperones are regulated post-translationally by unfolding stresses like heat shock and low pH. Based on the associated structural changes, regulatory mechanisms in ATP-independent chaperones can be classified into four categories: (a) assembly and disassembly, (b) order-to-disorder transitions, (c) lack of conformational change, and (d) ligand-induced conformational change. We want to point out that these mechanisms are not mutually exclusive, as some chaperones are shown to trigger substrate binding and release by undergoing an order-to-disorder transition coupled to assembly and disassembly. In the following sections, we discuss these mechanisms using the most well-characterized chaperones as representative examples.

4.1. Assembly and Disassembly

Reversible assembly into oligomeric species is a common mode of regulation for ATP-independent chaperones. A notable example is the sHsps, where members assemble into interconverting oligomers that are assembled via weak and dynamic intersubunit interactions (5, 7, 81, 100, 104). The highly variable and intrinsically disordered N-terminal regions of sHsps play a major role in both client binding and oligomer formation. By forming large oligomers, the N-terminal regions of sHsps are buried in the interior, making them inaccessible for client proteins (35, 75). Higher oligomers are, therefore, considered chaperone-inactive storage forms where

the N-terminal region residues are predominantly involved in intersubunit interactions. In response to environmental triggers like changes in temperature (34, 62, 106), acidosis (23) and post-translational modifications (42, 43, 50, 86) the equilibrium shifts toward ensembles of smaller oligomers (often dimers) that expose the N-terminal regions for client recognition (32, 52). Although the rapid shift in equilibrium allows sHsps to efficiently sense environmental changes and protect unfolded and misfolded proteins, client release from the sHsp-client complex does not occur spontaneously. Release and refolding of non-native client proteins require the ATP-dependent chaperone systems.

Trigger factor is another well-characterized ATP-independent chaperone that can regulate its chaperone activity through assembly and disassembly (**Figure 4a**). It can self-associate into a dimeric form at physiological concentrations (91). In the dimeric form, one monomer adopts a head-to-tail orientation in which the N-terminal domain is docked into the cavity created by the C-terminal domain and PPIase domain of another monomer (77, 91). Upon dimerization, both the ribosome-binding motif on the N-terminal domain and the major substrate-binding regions on the C-terminal domain are buried in the dimeric interface (77, 91). Thus, the dimeric form has to disassemble into monomers to bind to its client proteins and attach onto the ribosome (22, 90).

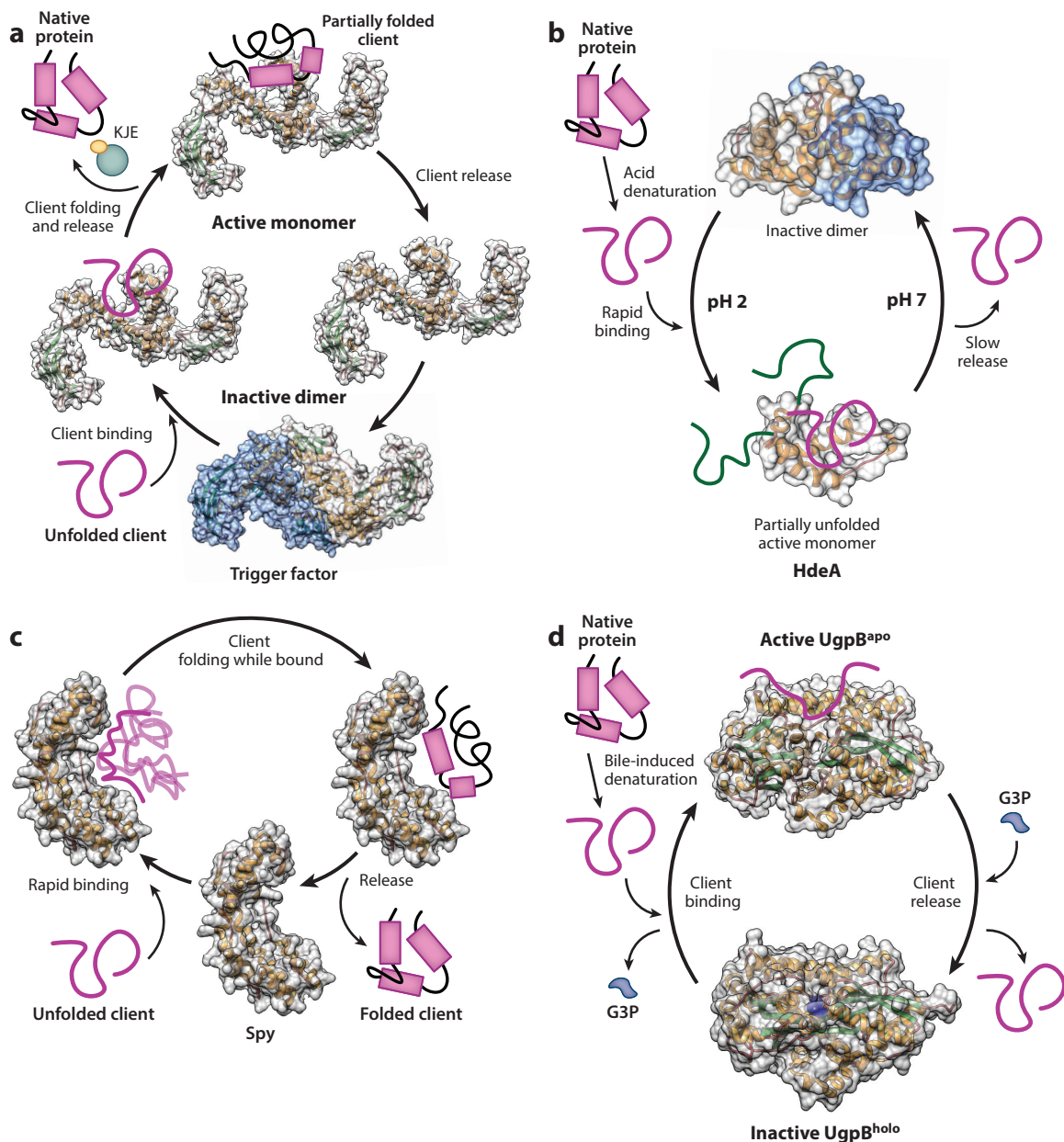
In addition to the chaperones that regain activity by disassembling into smaller oligomers, some ATP-independent chaperones could be activated by assembling into higher oligomeric states. The redox-regulated holdase chaperone Get3 in yeast is a typical example. In the absence of oxidative stress, reduced Get3 exists in a dimeric form and is responsible for the integration of tail-anchored proteins into the membrane of endoplasmic reticulum. Upon oxidation, dimeric Get3 undergoes a massive conformational change involving disulfide bond formation and zinc release, thereby assembling into chaperone-active tetrameric or higher oligomer states (114). Another example is the periplasmic chaperone Skp, which plays an important role in the biogenesis of OMPs in bacteria (94). Skp predominantly exists in the monomeric form at physiological concentrations (93). A recent study showed that monomeric Skp is chaperone inactive and intrinsically disordered (69). Upon binding to its clients, monomeric Skp rapidly folds and assembles into a trimeric form, resulting in encapsulation of the client in the semi-open cavity of the jellyfish-like trimer (69, 95). Upon client release, trimeric Skp disassembles into the monomeric state and completes the chaperone cycle. Another notable example is *E. coli* Hsp31, which enhances its chaperone activity via Zn^{+2} -mediated multimerization (17, 51). The formation of higher oligomers leads to partial unfolding of N- and C-terminal regions, thereby exposing the hydrophobic interior for substrate binding (51). The formation of higher oligomeric forms is reversible, as the oligomers dissociate into a dimer due to the treatment of a chelating agent (51).

4.2. Order-to-Disorder Transitions

Disorder in protein structure plays a vital role in biology. Unsurprisingly, it is often a means to regulate the function of dynamic proteins such as molecular chaperones. Rapid explorations of conformational landscapes can be achieved by order-to-disorder transitions in the absence of ATP-based allostery to regulate binding and release of clients and interaction partners. In this section, we review literature on two chaperones, HdeA and cpSRP43, for which transitions between ordered and disordered conformations are crucial to chaperone action. A common theme for all of these chaperones is that disruption of the order-to-disorder transition by specific mutations dramatically affects chaperone function.

HdeA is a small, pH-responsive chaperone in the periplasm of enteric bacteria. It is highly up-regulated under acid stress and protects several periplasmic proteins, including other chaperones like SurA and DegP, from aggregation due to pH-induced denaturation (28, 122). At neutral pH,

HdeA is chaperone inactive and exists as a well-folded all-helical homodimer. The periplasmic pH rapidly shifts from 7 to 2 when bacteria enter the stomach upon ingestion. Upon the shift to low pH, HdeA rapidly monomerizes, unfolds, and becomes active (26) (**Figure 4b**). Several groups have studied the activation pathway of HdeA using multiple orthogonal approaches like NMR spectroscopy, constant pH MD simulations, and fluorescence-based pH titrations. The activation of HdeA is a complex multistep process (2, 92, 120). Under acidic pH, HdeA populates



(Caption appears on following page)

Figure 4 (Figure appears on preceding page)

Regulation of chaperone cycle. Client proteins are diagramed in purple and black, and chaperones are shown in space-filling crystal structure models. (a) The dimeric storage form of trigger factor monomerizes in order to engage with its client. Trigger factor binds to and stabilizes the unfolded client, which can be transferred to the downstream DnaK-DnaJ-GrpE (KJE) system for subsequent folding. Emerging evidence suggests that trigger factor can also assist in client folding by stabilizing productive partially folded states. Upon substrate release, trigger factor either self-associates into a dimer or engages with another client protein. (b) At neutral pH, HdeA exists as an inactive dimer. Under acidic conditions (pH 2), HdeA is activated due to monomerization and partial unfolding. Active HdeA binds acid-denatured clients and prevents their aggregation. When pH shifts to neutral (pH 7), HdeA releases its client slowly and forms the dimer again. The monomer structure of HdeA is not known; for illustration purposes, a single subunit of the dimer is shown, but the actual structure of the monomer is likely to be expanded and dynamic. (c) Spy rapidly associates with its client proteins through long-range electrostatic interactions and allows the client to fold while bound. Folding into the native state weakens the interaction with Spy due to hydrophobic burial, thereby triggering client release. (d) Chaperone activity of UgpB is regulated by binding to its ligand glycerol-3-phosphate (G3P). In the G3P-bound state, UgpB has greatly reduced chaperone activity. Upon G3P release, UgpB undergoes a conformational change to expose its core hydrophobic cleft and associate with unfolded client proteins.

unfolded states in addition to the folded dimer. Chemical exchange saturation transfer NMR profiles show that the apparent exchange rate constant between folded and unfolded conformations is pH dependent, and the relative population of disordered states increases as pH is lowered from 3.5 to 2.5. Yu et al. (120) identified three pH hotspots for acid-induced conformational transitions. Destabilization of these locks at moderately low pH (3–4) loosens the dimer and exposes client binding sites. At very low pH (1.5–2), HdeA is fully activated and exists as a partially unfolded monomer with highly charged and flexible N- and C- terminal tails and two hydrophobic client binding sites held together by a strictly conserved disulfide bond (120). Although client binding sites were identified in this study using ^{19}F -NMR, obtaining high-resolution NMR structures of the chaperone–client complex of HdeA with the native client MalE is extremely challenging.

Light-harvesting chlorophyll-binding proteins (LHCPs) are found in the thylakoid membrane of plant cells and are the most abundant membrane proteins on earth. Like most membrane proteins, LHCPs are imported into the chloroplast stroma in a translocation-competent unfolded state. LHCPs form a transit complex with the chloroplast signal recognition particle (cpSRP), which is comprised of two subunits, cpSRP43 and cpSRP54 (46). The transit complex interacts with its receptor cpFtsY at the thylakoid membrane and delivers the LHCPs to the Alb3 translocase (20). The cpSRP43 subunit is the chaperone-active component and quantitatively prevents the aggregation of transmembrane domains of LHCP during this process (46). cpSRP43 is a multidomain protein that contains three chromodomains (CD1–CD3), an ankyrin repeat domain (Ank) consisting of four ankyrin repeat motifs, and a C-terminal bridging helix (BH) (105). The substrate-binding domain (SBD) of cpSRP43 is composed of the CD1-Ank-BH fragment (64). It binds LHCPs by specifically recognizing the FDPLGL motif in the L18 region, a conserved 18-amino-acid loop in LHCPs (29). The chaperone activity of cpSRP43 is allosterically regulated by interactions with a motif in the C terminus of the cpSRP54 M-domain (54 M) and the C-terminal stromal domain of Alb3 (64). A recent study has shown that the activation of cpSRP43 involves a disorder-to-order transition in the Ank repeat motifs (99). cpSRP43 populates a chaperone-active closed conformation and an inactive open conformation in slow exchange on NMR timescales (64). NMR spectroscopy revealed that the SBD is partially disordered in the open state, and L18 or 54 M binding shifts the equilibrium to the rigid, well-folded closed state.

4.3. Lack of Conformational Change

While many well-characterized ATP-independent chaperones undergo large conformational changes that are coupled to substrate binding and release, some do not. One notable example is the *E. coli* secretion-specific chaperone SecB, which sequesters premature secretory proteins and keeps

them in a folding-competent expanded state (40). Solution NMR structures of SecB in complex with alkaline phosphatase and maltose-binding protein revealed the structural basis of substrate binding by SecB. SecB uses extraordinarily large hydrophobic grooves (approximately 7,600 Å²) distributed on its surface to associate with unfolded clients (40). Since the major hydrophobic grooves are exposed to the solvent in the apo form of SecB, only a subtle conformational adjustment is observed upon substrate binding. Other ATP-independent chaperones, like HSP40 (48) and Spy (38), that have solvent-exposed binding sites in their apo form do not undergo dramatic conformational changes to bind their substrates.

Given the lack of a regulatory mechanism for substrate binding and release, it is not entirely clear how these chaperones complete their functional cycles. One possibility is competitive release. SecB, for instance, delivers premature clients to either cytoplasmic- or SecYEG translocon-bound SecA, which drives the translocation of secretory proteins across the plasma membrane (21, 31). An asymmetric binding orientation of SecB onto SecA was proposed as a trigger for client release (109). The free energy of binding of OMPs to SurA is generally much smaller than the free energy of OMP folding (24, 25). Therefore, transfer of unfolded OMPs from SurA to the BAM can be driven solely by favorable thermodynamics in the absence of ATP-dependent release mechanisms. Changes in folding state of the chaperone-bound client can also trigger its release. This mechanism has been proposed for the complex of Spy and the model client Im7 (**Figure 4c**). The micromolar affinity of Spy for native Im7 is much weaker than the subnanomolar affinity of Im7 for its natural binding partner, colicin E7. Both Spy and colicin E7 bind to the same locally flexible regions on fully folded Im7. Therefore, release of Im7 from Spy can be triggered by folding to the native state due to its energetically favorable interaction with colicin E7 (36).

4.4. Ligand-Induced Conformational Change

A recent study revealed a new role of the *E. coli* periplasmic protein UgpB as a molecular chaperone that prevents bile-induced protein aggregation (60). Periplasmic substrate-binding proteins are components of ABC-transporter systems involved in the uptake of small molecules like amino acids, peptides, and sugars (3). These proteins typically transfer the bound ligand to an inner membrane-associated complex that transports the substrate to the cytoplasm in an ATP-dependent manner (3). UgpB is a glycerol-3-phosphate (G3P) binding protein in the periplasm (6, 119). The transported G3P can be utilized as a carbon or phosphate source by the bacteria (116). Of note, UgpB exhibits bile-responsive chaperone activity only in the ligand-free state (60). Upon G3P release, the core hydrophobic cleft in UgpB is exposed and binds protein clients (60) (**Figure 4d**). Additionally, some hydrophobic residues in the cleft region are involved in both G3P binding and chaperone activity. UgpB variants that are deficient in G3P binding function as constitutively active chaperones regardless of the presence of G3P (60).

5. CONCLUDING REMARKS

That ATP-independent chaperones are unlikely to be only simple holdases becomes clear when one realizes that, to be effective as chaperones, they need to have a mechanism to release their substrates in a timely fashion. These energy-independent folding factors have a wide variety of mechanisms, ranging from proteins like Spy that loosely cradle proteins, allowing them to fold while bound while also inhibiting aggregation, to more classic holdases like sHSPs that can bind very tightly but can also release proteins via complex oligomerization cycles. Investigation of these chaperones has the potential to lay bare the fundamental biophysics of protein-protein interactions that affect protein folding, often without the distractions of the multiple cofactors and complex reaction cycles that commonly accompany ATP-dependent chaperones.

FUTURE ISSUES

1. The conformation of bound substrate is largely uncharacterized for most ATP-independent chaperones. A multidisciplinary approach combining structural biology, biophysics, and molecular dynamics simulations is required to answer fundamental questions regarding the structure and dynamics of chaperone–client complexes of ATP-independent chaperones.
2. How do ATP-independent chaperones affect the folding pathway of their clients? Efforts to address this question have shown that ATP-independent chaperones like trigger factor and Spy do much more than just preventing protein aggregation. Future work on other chaperone–client complexes should focus on the role of ATP-independent chaperones beyond holdase action.
3. How common is the folding-while-bound mechanism of ATP-independent chaperones, and what are its molecular determinants?
4. Can ATP-independent chaperones allow the bound client to populate near-native conformations that are otherwise inaccessible in a folding trajectory from a fully denatured state?
5. How is a client protein transferred from ATP-independent chaperones to ATP-dependent chaperones?
6. How can we use biophysical models of chaperone action to design in vivo studies of how ATP-independent chaperones prevent proteotoxicity under stress conditions?

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