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MicroReview

Chaperoning Anfinsen: the steric foldases

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Summary

Some proteins are so much resistant to proteolysis and unfolding that they violate folding rules shared by the vast majority of proteins. These unusual proteins manage to fold into an active native conformation that is thermodynamically at best marginally, but often even less stable than the unfolded state. A huge energetic barrier traps these proteins kinetically in the folded state. The drawback of this situation is the need for a specialized chaperone that adds steric information to the proteins to cross this barrier on the folding pathway. Until now, our knowledge of these intriguing chaperones was restricted to the prodomains of secreted proteases, which function intramolecularly. Recent research has added more examples, which now include the membrane-anchored lipase-specific foldase and the pilus subunit specific chaperone, both acting intermolecularly. The case of the pilin chaperone is somewhat deviant in that steric information is definitely provided, but the pilus subunit adopts a thermodynamically favourable stable conformation.

(Kinetic) traps, violation and salvation: the steric chaperones

It is only 70 years ago that people realized what a protein is about: a polymeric string of a limited number of different amino acids. From then on, the folding from a random polymer into a well-defined three-dimensional structure has attracted scientific interest (Tanford and Reynolds, 2001). Anfinsen (1973) showed that all information needed for a polypeptide to fold into basic secondary structure elements such as α-helices and β-strands and their subsequent collapse into a compact structure is entirely contained within the amino acid sequence. Most proteins adopt a native conformation that has a lower energy than their unfolded state (Fig. 1A). However, it is still fascinating that a protein of, say, 201 amino acids and two possible states for the peptide bond, can theoretically adopt a gargantuan number of 1.6×10^{60} possible conformations, and finally end up in a unique stable low free-energy fold.

Although small single-domain proteins can fold spontaneously and in a reversible way, most multidomain proteins need the help of chaperones to adopt the ultimate native and active conformation. Classical chaperones increase the folding efficiency but normally do not (or only to a small extent) increase the folding rate constants. Folding catalysts, such as peptidyl-prolyl *cis-trans* isomerases and disulphide isomerases, increase the folding rates, but even in the absence of such catalysts, the nascent protein will finally fold into its native conformation, provided inadvertent interactions leading to misfolding and aggregation are prohibited by classical chaperones. These chaperones and folding catalysts are rather promiscuous proteins that assist folding of different substrates. Some proteins, however, fail to fold correctly into their native tertiary structure on a biological relevant timescale. Their most remarkable feature is that their native conformation is less or only marginally more stable than the unfolded state (Fig. 1B). A huge (> 26 kcal-mol^{-1}) unfolding barrier that forms a kinetic trap provides an increased functional existence but simultaneously poses a folding problem (Sohl *et al.*, 1998). Therefore, highly specific steric chaperones have evolved that imprint unique structural (steric) information onto their target proteins while lowering the (un)folding barrier between the native and partially folded state (Fig. 1C). These proteins thus violate Anfinsen's rule. Recently, it was shown that they also perturb folding thermodynamics (Jaswal *et al.*, 2005). This review will cover new members of the bacterial steric chaperone family identified over the last years.

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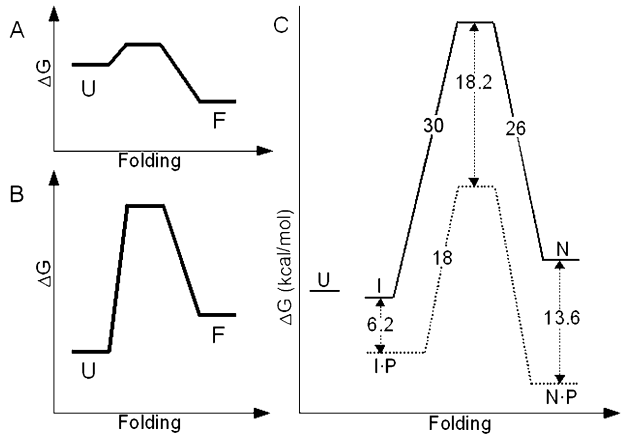

Fig. 1. A. Most unfolded proteins (U) spontaneously fold into their native low energy state (F) on a relevant timescale.
B. Some proteins do not fold spontaneously into their fully folded state or only at an extremely low folding rate. Steric chaperones transiently surmount the high energy barrier thereby accelerating folding. After removal of the steric chaperone, the fully folded protein remains trapped by a high unfolding barrier in its thermodynamically rather unstable native state.
C. The free energy diagram of the α-lytic protease folding reaction in the presence and absence of the propeptide was adapted from Sohl *et al.* (1998).

Protease – prodomains: an internal affair

Most bacterial extracellular proteases are synthesized as precursors with a signal sequence and a propeptide in addition to the mature protease sequence. Whereas the signal sequence is removed after translocation through the cytoplasmic membrane, the propeptide is retained. The propeptide promotes correct folding and also inhibits premature activation of the protease. The best-described examples of such bacterial proteases are the *Lysobacter enzymogenes* α-lytic protease (αLP), the subtilisins produced by *Bacillus subtilis* and *Bacillus amyloliquefaciens*, and the *Pseudomonas aeruginosa* elastase. In the absence of the propeptide, the protease folds into an inactive molten globule-like intermediate that is unable to fold further into the functional native state on a biological timescale (Eder *et al.*, 1993). Sohl *et al.* (1998) measured the initial rate of αLP folding in the absence of the propeptide by an ultrasensitive protease assay. Their measured k_f of 1.18×10^{-11} s^{-1} at 4°C corresponds to a half life of 1800 years whereas the propeptide-catalysed αLP folding reaction occurred with a folding rate of 3.7×10^{-2} s^{-1} ($t_{1/2}$ 18 s). The folding kinetics thus shows that the propeptide accelerates the conversion of the intermediate into the native fold with more than a factor 10^9. Thus, the propeptide functions as a potent folding catalyst and becomes autoproteolysed by the active protease, which in turn becomes kinetically trapped in its native conformation.

The reason why the energetic barrier on the (un)folding pathway of proteases has developed is obvious. Given the harsh and highly proteolytic environment where these proteins have to operate, they evolved to avoid proteolytic attack and extend their lifetime by reducing native state proteolysis and suppressing global and local unfolding events. Indeed, the unfolding kinetics of αLP at 4°C, determined with an inactive mutant (S195A) to avoid self-cleavage, were found to be 1.8×10^{-8} s^{-1} corresponding to a half life of 1.2 years.

In order to restrict conformational fluctuations, these proteases display a characteristic rigidity and a highly co-operative unfolding behaviour. The low crystallographic temperature factors observed together with the results from hydrogen-deuterium exchange experiments and proteolytic survival assays are evidence for these restricted native state dynamics for αLP (Jaswal *et al.*, 2002; Fuhrmann *et al.*, 2004). A high content of glycines is characteristic of αLP and all other propeptide-containing homologues (16–20% vs. 6–12% for non-propeptide-dependent homologues) (Cunningham *et al.*, 1999). The absence of a side chain in glycine residues permits unusual backbone geometries, including sharp turns that enable tight packing and compact loops that are inaccessible to proteases.

The crystal structures of αLP and subtilisin (Fig. 2A and B) with their cognate propeptides, together with mutational and biophysical studies, led to comprehensive mechanistic insights into the energetic and kinetic characteristics of the protease-propeptide systems (Bryan *et al.*, 1995; Sauter *et al.*, 1998; Sohl *et al.*, 1998). The rather large interaction surfaces presumably explain the low dissociation constants between the propeptides and their target proteins (Table 1). The removal of the propeptide, accomplished by autoproteolysis, is needed to liberate active protease in its kinetically trapped state as well as to avoid propeptide-catalysed unfolding.

Although αLP and subtilisin folding share similarities, the structures of the protease-propeptide complexes reveal a mechanistic difference: the propeptide stabilizes two α-helices through N-terminal capping in the case of subtilisin, whereas a β-hairpin that is considered to contribute to the folding barrier is stabilized in αLP through direct interaction with the cognate propeptide (Bryan *et al.*, 1995; Sauter *et al.*, 1998). Moreover, subtilisin exemplifies a striking case of 'protein memory' of the protease and emphasizes that steric information is 'imprinted' upon the protease by the propeptide. In this case, one subtilisin sequence gave rise to two three-dimensional structures with slightly different secondary structures, thermostability and substrate specificity through interaction with either the natural or a genetically altered propeptide (Shinde *et al.*, 1997). An additional role of the propeptide in translocation was demonstrated in the

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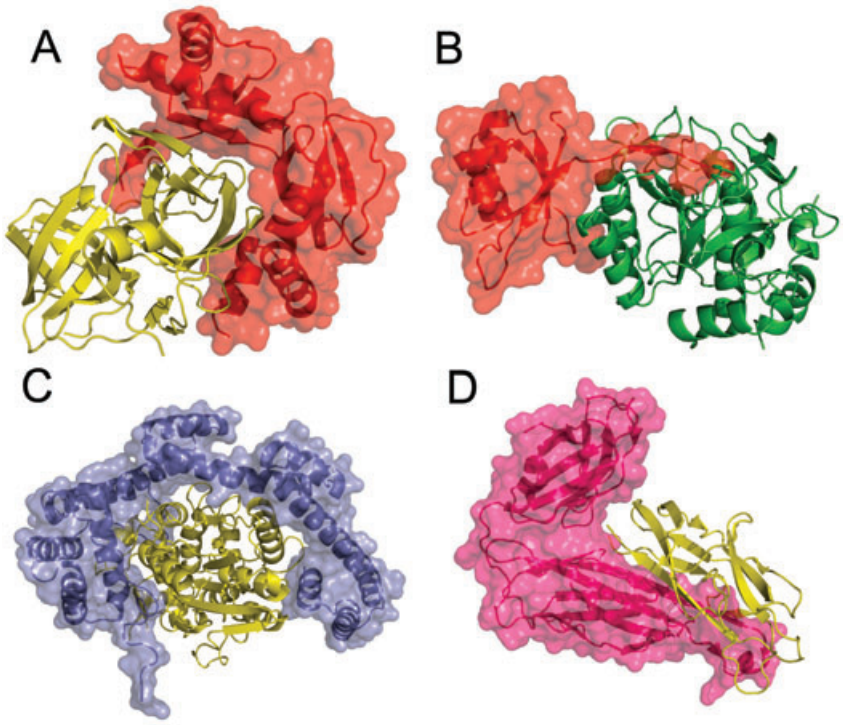

Fig. 2. Crystal structures of steric chaperones in complex with their cognate substrates. The structure models demonstrate the large interaction interface and the clamp-like structures. The chaperones and substrates are shown in surface/ribbon and in ribbon only respectively.
A. *L. enzymogenes* α-lytic protease (yellow) with its propeptide (red) (4PRO.pdb).
B. *B. amyloliquefaciens* BPN′ subtilisin (green) with its propeptide (red) (1SPB.pdb) (C) The *B. glumae* lipase-specific foldase (blue) embracing the lipase (yellow) (2ES4.pdb) (D) *E. coli* FimC (magenta) interacting with the FimH pilin domain (yellow) (1ZE3.pdb) (pdb stands for protein data bank and is proceeded by the entry code).

case of *P. aeruginosa* elastase. The well-coordinated chronology of crucial events including the successive formation of two disulphide bonds and autocatalytic processing, establishes that periplasmic protein folding and secretion into the extracellular medium are tightly interwoven (Braun *et al*., 2001; McIver *et al*., 2004).

Lipase-specific foldase: going intermolecular

Many Gram-negative bacteria, including members of the genera *Pseudomonas* and *Burkholderia*, use the type II secretion pathway (Filloux, 2004) to secrete a variety of toxins and hydrolytic enzymes, including lipases, into the extracellular medium. Because these bacteria also secrete very potent proteases, the lipases must be intrinsically stable to prevent their proteolytic degradation. Indeed, these lipases are highly resistant to proteases and harsh conditions are required for their unfolding (El Khattabi *et al*., 2003), a reason why many of them have been selected for application in household detergents. As with many secreted proteases, the folding of these lipases also requires a steric chaperone. However, because autoproteolytic removal of the chaperone is not an option in this case, the chaperone activity is provided by a separate protein, generically designated Lipase-specific foldase or Lif (Jaeger *et al*., 1994).

In the absence of Lif, and depending on the production levels, *Burkholderia glumae* lipase is either degraded or

Table 1. Interaction between steric chaperones and their substrates.

	complex	K_D	pair-wise interaction surface (Å2)	% of non-polar interface
lipase – Lif	*B. glumae* lipase – Lif	5 nM[a]	5378	63
protease – propeptide	αLP – propeptide	0.3 nM[b]	3173	62.5
	subtilisin – propeptide	5 nM[c]	2232	65
chaperone – pilin	FimC – FimH	< 10 nM[d]	2948	57
Average protein–protein values[e]		NA	1600	56

The pairwise interaction surface and the percentage apolar interaction surface were calculated with NACCESS (Hubbard and Thornton, 1993) based on the atomic co-ordinates in the protein data bank (http://www.rcsb.org/pdb) of the corresponding complexes (resp. 2ES4, 4PRO, 1SPB, 1ZE3).
a. Pauwels *et al.* (2006).
b. Peters *et al.* (1998).
c. Bryan *et al.* (1995).
d. Hermanns *et al.* (2000)
e. Wodak and Janin (2002).
NA, not applicable.

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accumulates as inactive aggregates in the periplasm (Frenken *et al*., 1993). Lif is anchored to the inner membrane via an N-terminal membrane-spanning segment, exposing the active domain in the periplasm. The membrane anchor is not required for function but probably only retains Lif within the cells while lipase is secreted. A variant of Lif without its membrane anchor was secreted together with the active lipase (El Khattabi *et al*., 1999). The membrane anchor is followed by an alanine- and proline-rich linker that also is not required for function, because a variant form of *P. aeruginosa* Lif lacking this region is still active *in vitro* (Shibata *et al*., 1998a). Its function is probably to allow the active domain to protrude sufficiently from the membrane to capture the lipase.

In vitro, the denatured lipase of *B. glumae* refolded into a near-native structure that was very similar to the native lipase in circular dichroism (CD) and tryptophan fluorescence measurements; however, it was enzymatically inactive and sensitive to proteases. Nevertheless, denaturation experiments showed that it was even more resistant to heat than the native form (El Khattabi *et al*., 2000). Thus, lipase folds into a compact fold in the absence of its chaperone and is not trapped in a molten globule, as observed for proteases. The addition of Lif several hours after initiation of lipase refolding immediately activated this folding intermediate, demonstrating that the function of the chaperone is to overcome an energetic barrier late on the folding pathway. Unlike the propeptides of proteases, Lif does not have an additional function as a lipase inhibitor.

How exactly Lif exerts its function is unknown. The crystal structure of the native lipase of *B. glumae* in complex with Lif (Fig. 2C) (Pauwels *et al*., 2006) is very similar to that of the free active lipase. As CD analysis had previously revealed major structural changes upon complex formation (El Khattabi *et al*., 2000), these changes must have occurred primarily in the Lif. Consistently, whereas free Lif is completely degraded by proteases *in vitro*, it is only cleaved down to a 26-kDa fragment within the complex (El Khattabi *et al*., 2000). Within the crystal structure, Lif embraces the lipase molecule such that two minidomains connected by an extended α-helical structure are located at opposite sides of the lipase (Fig. 2C). Three residues previously demonstrated to be required for Lif activity, Tyr99, Ser102 and Arg115 in *P. aeruginosa* Lif (Shibata *et al*., 1998b), are located in one of these minidomains. The interaction interface between the two molecules in the structure is very large and mainly non-polar (Table 1). Surface plasmon resonance experiments confirmed that the affinity of Lif for native lipase is very high (Table 1) (Pauwels *et al*., 2006). Presumably, interaction with the type II secretion machinery dissociates the Lif-lipase complex after folding is accomplished. Interestingly, *Pseudomonas fragi* produces a lipase with high sequence similarity to those of *P. aeruginosa* and *B. glumae*, but it does not appear to require a Lif for correct folding. Molecular modelling showed this lipase to have a much higher content of surface-exposed charged residues, particularly arginines (Alquati *et al*., 2002). Thus, the non-polar solvent-accessible area that interacts with Lif in the case of *B. glumae* lipase is absent in the Lif-independent *P. fragi* enzyme. Similar lipases are encoded in the genomes of *Pseudomonas putida* and *Pseudomonas fluorescens* strains.

A detailed comparison of the folding and unfolding pathways and the structures of Lif-dependent and -independent lipases might yield clues to the working mechanism of Lif. A more detailed characterization of a variant lipase of *Pseudomonas* sp. strain KFCC that is independent of Lif due to a single amino acid substitution, i.e. a proline-to-glutamine substitution located four amino acid residues downstream of the active-site serine in the amino acid sequence (Kim *et al*., 2001), might also be informative. Furthermore, while the solved structure of the Lif-lipase complex provides an excellent platform for further mutational studies, structural characterization of the trapped folding intermediate will be necessary to understand the nature of the folding barrier and the mechanism of foldase action. The recently described approach to display active Lif at the *E. coli* cell surface (Wilhelm *et al*., 2007) may be useful to select for Lif variants that are able to bind the intermediate, but fail to fold it.

Fimbrial chaperones: entering the twilight zone

The assembly of many adhesive fibres on the surface of Gram-negative bacteria requires a soluble periplasmic chaperone and an outer membrane assembly platform (usher). Subunits of adhesive pili share an immunoglobulin-like fold that lacks the last β-strand, thus creating a deep hydrophobic groove on their surface (Sauer *et al*., 1999). The periplasmic chaperone binds to non-native subunits, catalyses subunit folding (Vetsch *et al*., 2004) and remains bound to the native subunits to direct them to the usher (Dodson *et al*., 1993).

The fimbrial periplasmic chaperones have been classified as steric chaperones because they provide a missing β-strand to the folding fimbrial subunit, a mechanism known as donor strand complementation (Sauer *et al*., 1999). In the absence of the chaperone, fimbrial subunits aggregate in the periplasm and are degraded by the DegP protease (Jones *et al*., 1997). *In vitro*, fimbrial subunits can fold in the absence of the chaperone under very specific conditions (low protein concentration and low ionic strength) (Vetsch *et al*., 2002). However, the folded pilus subunits were very unstable [2–2.4 kcal mol^{-1} for the

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pilin domain of the Type 1 adhesin FimH (Vetsch *et al*., 2002)] and the rate constant of folding was quite slow [0.0066 s^{-1} for the pilin domain of the Type 1 adhesin FimH (Vetsch *et al*., 2002) and 0.0049 s^{-1} for the Type 1 pilus subunit FimG (Vetsch *et al*., 2004)]. In the presence of the chaperone, the folding reaction was markedly accelerated [0.59 s^{-1} for FimG (Vetsch *et al*., 2004)]. As *in vivo* fimbrial assembly occurs in the timescale of a few seconds, this acceleration of subunit folding by the chaperone is a prerequisite to overcome the kinetic barrier of subunit folding (Vetsch *et al*., 2004).

In contrast to other steric chaperones, the fimbrial chaperone does not fold its substrates into their final, native fold. Rather, the periplasmic chaperone traps the subunit in a high-energy folding intermediate. In the chaperone-subunit complex, the hydrophobic residues of the chaperone's G_1 strand are deeply incorporated into the hydrophobic subunit core, preventing the subunit from completely folding into the final conformation (Zavialov *et al*., 2003). Vetsch *et al*. (2004) estimated the difference in stability between the Type 1 FimF:FimG subunit:subunit complex and the FimC:FimG chaperone:subunit complex at least –4 kcal mol^{-1}. The large difference in melting temperature between the *Yersinia pestis* Caf1 subunit in its chaperone- and subunit-bound state ($\Delta T_m = 42°C$) also indicates a much higher stability of the subunit bound form (Zavialov *et al*., 2005). The high stability of the Caf1 subunit in its final fold was confirmed using a donor strand complemented construct of Caf1, in which the donor strand was covalently attached to the C-terminus of Caf1 such that the incomplete fold of the subunit was self-complemented (the free energy for the unfolding reaction at 37°C, $\Delta G = 16.7–19.1$ kcal mol^{-1}) (Zavialov *et al*., 2005).

The fact that pilus subunits are more stable in the folded than in the unfolded state does not comply with the hitherto known thermodynamic behaviour for substrates of steric chaperones. In sharp contrast to the propeptides and foldases, which can bind the intermediate folds as well as the mature proteins, folded fimbrial subunits show a reduced ability to interact with the chaperone (Vetsch *et al*., 2002).

Perspectives

A variety of steric chaperones act in a range of pathways from the paradigmatic propeptide-protease system through the intermolecular membrane-based Lif-lipase system to the grey-zone of the fimbrial assembly pathway. It seems unlikely that these steric chaperones have a common activation mechanism. Although there are similarities (see Table 1), they are never shared between all of the different types of chaperones. Probably the best understood mechanism is the fimbrial assembly pathway, which, however, does not activate an enzyme. The subtilisin propeptide and Lif undergo structural changes upon binding to their cognate ligand (El Khattabi *et al*., 2000; Subbian *et al*., 2005). It is tempting to regard propeptide and Lif folding as a 'decoy' such that the entire complex now complies with classical thermodynamic behaviour. However, the fact that amino acid substitutions that stabilize the folded state of the propeptide of subtilisin (Wang *et al*., 1998) accelerate folding rates of the active domain, argues against this idea, because folding of the usually unfolded propeptide is not needed anymore. An important factor in the activation mechanism might be strongly interacting water molecules, which have to be replaced by the snugly embracing chaperone. Interestingly, recent perspectives on structural evolution in chaperones showed that, irrespectively of the nature of the chaperone (ATP-dependent or -independent), they all have a clamp-like structure (Stirling *et al*., 2006). This is clearly the case with Lif, αLP and the fimbrial chaperone (Fig. 2), again demonstrating that these types of chaperones have a similar convergent evolutionary history.

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