Getting a grip on non-native proteins

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It is an underappreciated fact that non-native polypeptides are prevalent in the cellular environment. Native proteins have the folded structure, assembled state and cellular localization required for activity. By contrast, non-native proteins lack function and are particularly prone to aggregation because hydrophobic residues that are normally buried are exposed on their surfaces. These unstable entities include polypeptides that are undergoing synthesis, transport to and translocation across membranes, and those that are unfolded before degradation. Non-native proteins are normal, biologically relevant components of a healthy cell, except in cases in which their misfolding results from disease-causing mutations or adverse extrinsic factors. Here, we explore the nature and occurrence of non-native proteins, and describe the diverse families of molecular chaperones and coordinated cellular responses that have evolved to prevent their misfolding and aggregation, thereby maintaining quality control over these potentially damaging protein species.

EMBO reports 4, 565–570 (2003)

doi:10.1038/sj.embor.embor869

Protein folding and the non-native protein

All proteins are synthesized as linear polypeptide chains that are gradually extruded from the ribosome. To become functional, these nascent proteins must shield their exposed hydrophobic residues and adopt a precise tertiary structure. It has long been known that primary amino-acid sequences dictate the tertiary structures of proteins, but how folding into the native state occurs is still the subject of intense investigation. The folding process is now seen as the downward path that an unstructured polypeptide takes on a funnel-like free-energy surface representing the steadily decreasing number of conformations available to it as it reaches its native state (Dinner *et al.*, 2000).

For small proteins, such as the engrailed homeodomain protein (7.5 kDa), folding is thought to begin with a few native-like contacts that, once formed, promote a rapid transition to the native state (Fig. 1A). In this two-step model, there are no long-lived intermediate states, and the secondary and tertiary structures form virtually simultaneously (Daggett & Fersht, 2003).

The folding of larger proteins, such as lysozyme (Fig. 1B), is more complex, and usually involves transition states and intermediates that are represented as peaks and valleys on the energy landscape. One theory is that on the initiation of folding, secondary-structure elements form autonomously and collide to produce the tertiary structure (Daggett & Fersht, 2003). *De novo*, co-translational protein-folding in the cell probably follows this type of pathway (Hartl & Hayer-Hartl, 2002). Another hypothesis is that the propensity of hydrophobic residues to associate stimulates the collapse of a protein into a 'molten-globule'-like, compact state that has some non-native contacts. In this scenario, the folding bottleneck for proteins is the reorganization of such incorrect associations. A synergistic view of these two mechanisms, called nucleation-collapse, probably explains the folding of most proteins *in vitro* (Daggett & Fersht, 2003).

Folding intermediates, and non-native protein species in general, are usually aggregation-prone, both *in vitro* and in the crowded cellular environment. Therefore, *in vivo*, they must be stabilized and ushered towards their appropriate fate, be it biogenesis (folding, assembly and transport), degradation, or sequestration into aggregated forms if they cannot reach their native state or be disposed of.

Cellular functions of molecular chaperones

A diverse and ubiquitous class of proteins, known as molecular chaperones, has evolved to transiently stabilize exposed hydrophobic residues in non-native proteins. Cellular functions for chaperones include assisting in biogenesis, modulation of protein conformation and activity, disaggregation and refolding of proteins after cellular stress and, perhaps unexpectedly, the disassembly and unfolding of proteins for subsequent degradation (Leroux & Hartl, 2000a). Although they are not historically classified as chaperones, proteins that catalyse the *cis-trans* isomerization of proline residues (peptidyl-prolyl isomerases) and the proper formation of disulphide bonds (protein disulphide isomerases) often bind and stabilize non-native proteins (Leroux, 2001). We now consider some of the best-characterized chaperones, which we classify into three broad functional categories: holding, folding and unfolding.

Holding. Several molecular chaperones seem to have little more than a stabilizing effect on non-native proteins, and usually require the participation of other chaperones to assist with, for example, folding. These chaperones typically lack the ability to undergo ATP-dependent conformational changes.

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Received 25 March 2003; accepted 24 April 2003

For example, heat-shock protein 40 (Hsp40) can prevent protein aggregation *in vitro*, but requires the ATP-hydrolysing chaperone Hsp70 to fold substrates (Mayer *et al.*, 2000). Eukaryotic prefoldin, a hetero-hexameric chaperone complex, interacts with nascent actin and tubulin chains and assists in their biogenesis by docking onto and delivering substrates to the chaperonin-containing TCP1 (CCT), which is a cylindrical 'folding machine' (Martin-Benito *et al.*, 2002). Archaeal prefoldin interacts indiscriminately with non-native proteins *in vitro*, suggesting that it boasts a wider range of substrates than its eukaryotic counterpart (Siebert *et al.*, 2000). Hsp90 is an interesting case of a highly abundant chaperone that requires the cooperation of Hsp70 and other cofactors to facilitate conformational switching between active

and inactive client proteins despite its ability to hydrolyse ATP (Pearl & Prodromou, 2002). Small heat-shock proteins (sHsps) belong to a diverse family of protein complexes that trap denatured proteins on their surfaces (van Montfort *et al.*, 2001). It is controversial as to whether sHsps can use ATP to directly assist the refolding of their substrates, or whether they are strictly reliant on ATP-dependent chaperones, such as Hsp70 or chaperonins, to perform this task (Leroux, 2001). SecB, a bacterial chaperone, recognizes and maintains newly synthesized precursor proteins in forms that are competent for translocation by SecA, a chaperone ATPase that threads proteins into the SecYEG translocon channel (Xu *et al.*, 2000). Last, chaperones such as the periplasmic protein PapD can stabilize non-native proteins—in this case, unassembled pilus subunits—by transiently 'donating' a structural element that is lacking in the substrate's tertiary structure, but that is present (complemented) in the assembled quaternary form (Sauer *et al.*, 2002).

Fig. 1 | Folding pathways of engrailed homeodomain and egg-white lysozyme. (A) The engrailed homeodomain (En-HD) folds rapidly, in nanoseconds to microseconds (Mayor *et al.*, 2003). (B) Lysozyme has two significantly populated intermediates and folds more slowly, with a timescale of milliseconds *in vitro*. The majority (70%) of the lysozyme protein population folds relatively quickly into the α -domain intermediate, but is slow to reach the near-native, short-lived $\alpha\beta$ -intermediate. Another 20% rapidly forms the $\alpha\beta$ -intermediate directly. The α -domain is shown in red, and the β -domain in yellow. Reproduced in part, with permission from Dinner *et al.*, 2000.

Folding. Chaperones that assist in protein folding often couple a holding (or capturing) function with the ability to release the folded substrate in an ATP-dependent manner. Hsp70 has a multitude of functions, two of which are stabilizing nascent polypeptides and promoting the folding of non-native proteins through rounds of ATP-dependent binding and release (Mayer *et al.*, 2000). In bacteria, the function of the Hsp70 homologue, DnaK, partially overlaps with that of Trigger Factor (TF), a chaperone and prolyl isomerase that is located near the ribosomal polypeptide exit tunnel. The chaperonin (Hsp60) family of chaperones assists the folding of newly translated proteins by sequestering aggregation-prone intermediates in a hydrophilic cavity and releasing them after ATP hydrolysis. The bacterial GroEL and eukaryotic cytosolic CCT chaperonins interact with a range of substrates, although the latter probably have specialized functions in folding actin and tubulin (Leroux & Hartl, 2000b).

An emerging concept is that chaperones cooperate extensively, sometimes forming multi-chaperone systems that work sequentially or simultaneously to ensure the efficient biogenesis of cellular proteins in their respective cellular compartments (Leroux & Hartl, 2000a). For example, sequential interactions with cytosolic Hsp70, prefoldin and CCT are probably needed to assist actin and tubulin folding; at least in the case of actin, prefoldin and CCT also cooperate closely to help it reach its native state (Siegers *et al.*, 1999; Martin-Benito *et al.*, 2002). In mitochondria, several newly imported substrates have been shown to interact first with Hsp70, and then with Hsp60, for productive folding (Manning-Krieg *et al.*, 1991). As a final example, several endoplasmic reticulum (ER) chaperones, such as calnexin, calreticulin, Hsp70 and Hsp90 homologues, Erp57 and UDP-glucose:glycoprotein transferase (UGGT) work together or successively to ensure the correct biogenesis of glycosylated proteins (Parodi, 2000).

Some pre-proteins contain sequences that fulfil the criteria for a chaperone, as their cleaved pre-domains assist folding without being part of the final structure. The pro-peptide of the protease subtilisin E encodes such an intramolecular chaperone (Shinde *et al.*, 1993). An autotransporter such as BrkA provides a second example of an intramolecular chaperone. This bacterial protein contains a β -domain that forms a β -barrel channel, which is required for the proper biogenesis (secretion) of its α -domain and is subsequently removed by proteolytic cleavage (Oliver *et al.*, 2003).

Unfolding. Chaperones of the Hsp100/Clp/AAA (ATPase associated with various cellular activities) ATPase family are ubiquitous ring structures that mediate protein unfolding and disassembly. Their activities depend on interactions with several regions within their substrates and on the conversion of the energy gained from ATP hydrolysis into conformational changes that exert a molecular 'crowbar' effect (Horwich *et al.*, 1999; Leroux, 2001). Most AAA ATPases typically cooperate with other chaperones for folding or with proteases for degradation. Yeast Hsp104 and bacterial ClpB, for example, can disassemble aggregated proteins and thus allow their reactivation in conjunction with an Hsp70/DnaK chaperone. When bound to the ends of proteases such as the eukaryotic and archaeal proteasomes, and the structurally related bacterial HslV, the respective AAA ATPase chaperones (Rpt subunits, PAN and HslU) promote the unfolding of their substrates and their subsequent threading into the proteolytic chamber (Leroux, 2001).

Fig. 2 | Chaperone structures, grouped according to how they interact with their non-native substrates. (A) The carboxy-terminal substrate-binding domain of *Escherichia coli* DnaK, comprising a β -sheet cradle and an α -helical lid, bound to a peptide (red; NRLLLTG). (B) Calnexin has a globular lectin domain (yellow) and an extended arm domain (gold). (C,D) Cryoelectron microscopy (cryo-EM) reconstructions of the chaperonin CCT (blue) in complex with (C) actin and (D) tubulin (both in red). (E) HslU AAA ATPase (blue) shown on top of the HslV protease (grey). (F) Archaeal prefoldin (α -subunits shown in yellow; β -subunits shown in gold). (G) Cryo-EM reconstruction of eukaryotic prefoldin (gold) in complex with non-native actin (red). (H) SecB dimer of dimers (the different colours indicate monomers), with the putative substrate-binding groove shown in red. (I) A cofactor-A dimer (yellow and grey) with conserved putative binding residues (red). (J) A small heat-shock protein (Hsp16.5) from *Methanococcus jannaschii* forms a spherical oligomer; each dimer building-block is shown in a different colour. (K) The periplasmic chaperone PapD (grey) supplies a β -strand (red) to the incomplete PapK immunoglobulin fold (yellow). Structures are not shown to scale. The Protein Database identification numbers for these proteins are as follows: DnaK-peptide, 1DKX; calnexin, 1JHN; HslUV, 1G3I; prefoldin, 1FXK; SecB, 1FX3; cofactor A, 1QSD; sHsp, 1SHS; PapD-K, 1PDK.

Molecular strategies for binding non-native proteins

The strategies used by molecular chaperones to stabilize non-native proteins, which have been elucidated mainly by X-ray crystallography and cryoelectron microscopy (cryo-EM) studies, can be grouped broadly into one of four classes: the use of clamps, cavities, specialized surfaces, and structural complementation.

Clamps. Hsp70 contains an amino-terminal ATPase domain (45 kDa) that has an actin-like fold and is connected by a short linker region to a carboxy-terminal polypeptide-binding domain (25 kDa). The latter resembles a clamp, or jaw, which is formed by a β -sheet cradle and a flexible α -helical lid (Fig. 2A). The open, ATP-bound conformation of Hsp70 accepts a peptide segment that is enriched in hydrophobic residues, and ATP hydrolysis, which is induced by a cofactor (for example, Hsp40), locks the substrate in place. For DnaK, three basic features govern its interaction with substrates: hydrogen bonding to the substrate backbone, a hydrophobic pocket that interacts with side-chains, and an arch that sterically restricts bound polypeptides (Mayer *et al.*, 2000). Release of the substrate is enabled by exchanging ADP for ATP through the action of a nucleotide-exchange cofactor, such as GrpE or Bag1 (Leroux & Hartl, 2000a). Like Hsp70, Hsp90 undergoes conformational changes associated with its ATPase activity. In this case, the C-terminal domain mediates dimerization and, on ATP binding, the N-terminal domains of the two Hsp90 proteins also associate, forming a closed, molecular-clamp-like structure that probably confines the substrate (Meyer *et al.*, 2003).

Proteins that undergo maturation in the ER are stabilized by chaperones such as calnexin and calreticulin, which are structurally related and recognize glycosylated, non-native proteins through lectin-like domains. The striking tadpole-like structure of calnexin (Fig. 2B) suggests that it also uses a clamp strategy, stabilizing non-native proteins through its globular lectin domain and recruiting the disulphide-isomerase/chaperone, Erp57, with its extended arm (Leach *et al.*, 2002).

Cavities. Chaperonins consist of two stacked, oligomeric rings that form chambers used to sequester non-native proteins and assist in their folding (Horwich *et al.*, 1999). Individual subunits are composed of a substrate-binding (apical) domain, which lines the opening of the cavity and includes essential hydrophobic residues (mostly leucines, valines and tyrosines), an intermediate domain, and an equatorial ATPase domain. The multivalent binding of substrates to the homo-oligomeric bacterial protein, GroEL, was shown elegantly by sequentially mutating the substrate-binding sites of a single polypeptide that encodes a complete heptameric ring (Farr *et al.*, 2000). Unlike GroEL, the eukaryotic chaperonin CCT has eight unique binding sites per hetero-oligomeric ring that are less hydrophobic in character. These probably evolved to bind a wide spectrum of substrates while retaining some specificity; consistent with these results, cryo-EM images show that actin and tubulin interact with two or more specific subunits of CCT (Fig. 2C,D; Leroux & Hartl, 2000b; Llorca *et al.*, 2000). During chaperonin-assisted folding cycles, the substrates of GroEL and CCT are encapsulated by a cofactor (GroES) and an iris-like structure contained within CCT itself, respectively (Hartl & Hayer-Hartl, 2002).

AAA ATPases form large, hexameric toroids, which probably also bind substrates in a multivalent manner. The exact position and nature of their binding sites is poorly understood, but they are probably adapted to the specific functions of the AAA ATPases. The function of these chaperones in unfolding or disassembly seems to involve coupling substrate interactions with large, nucleotide-dependent conformational changes (Horwich *et al.*, 1999). As shown for the bacterial HslUV chaperone–protease complex (Fig. 2E), many AAA ATPases associate coaxially with proteases, and are poised to untangle and unfold polypeptides and thread them into the central proteolytic chamber.

Prefoldin also forms a cavity and binds substrates multivalently, but in a unique way. The crystal structure of archaeal prefoldin shows a hexamer with coiled-coil ‘tentacles’ protruding from a double β -barrel. Truncation studies show that the combined contributions of its coiled coils are required to stabilize non-native proteins (Siegert *et al.*, 2000; Fig. 2F). Consistent with this observation, a recent cryo-EM reconstruction of eukaryotic prefoldin complexed with non-native actin reveals several contacts near the tips of the tentacles (Martin-Benito *et al.*, 2002; Fig. 2G). As the cavity of archaeal prefoldin is predominantly polar in character, it has been suggested that the distal coiled-coil regions may partially unwind, exposing the inter-helical hydrophobic residues and creating an indiscriminate binding site for unfolded proteins (Siegert *et al.*, 2000).

Surfaces. To prevent inappropriate interactions between non-native proteins and components of the bulk cytosol, many chaperones use a relatively flat or corrugated surface to bind exposed, unstable polypeptide regions. Examples are the general chaperones TF, Hsp40 and SecB, and cofactor A, one of several tubulin-specific chaperones. The crystal structure of bacterial SecB (17 kDa) shows a dimer of dimers that has two hydrophobic grooves on opposite faces of the molecule (Fig. 2H). It has been suggested that a linear polypeptide can wrap around a SecB tetramer, contacting both grooves simultaneously (Randall & Hardy, 2002). Hsp40 is a U-shaped homodimeric chaperone that stabilizes non-native substrates before transferring them to Hsp70. It probably binds its substrates at hydrophobic depressions on the distal tips of its two peptide-binding domains (Lee *et al.*, 2002). TF also contains a conserved hydrophobic pocket, which is the putative substrate-binding site of its peptidyl-prolyl isomerase domain; the

preponderance of phenylalanine residues in this region confers a binding specificity that favours aromatic residues (Patzelt *et al.*, 2001). In contrast to these other chaperones, cofactor A, which is dimeric in yeast (Steinbacher, 1999; Fig. 2I), but apparently monomeric in humans (Guasch *et al.*, 2002), stabilizes its partially folded β -tubulin substrate on a primarily hydrophilic surface.

A unique mechanism for stabilizing non-native proteins is used by sHsps. *Methanococcus jannaschii* Hsp16.5 is a spherical complex that is assembled from 12 dimeric building blocks (Fig. 2J; Kim *et al.*, 1998). van Montfort and colleagues (2001) found that subunits of a wheat sHsp (Hsp16.9) form a smaller, cylindrical, dodecameric complex. They suggested, from the structure and from other studies, that sub-assembled species (dimers) dissociate to partition their exposed hydrophobic residues between substrate binding and higher-order assembly. On oligomerization, it is likely that non-native proteins are not contained within the cavity of the less ordered complexes, but are instead held on the outside surface, awaiting refolding by other chaperone systems.

Structural complementation. Structural complementation is an exceptional case, in which a chaperone contributes specific structural information to stabilize a non-native substrate. The interaction between Pap pilus subunits and the periplasmic chaperone, PapD, from uropathogenic *Escherichia coli* illustrates the use of this strategy. The pilus subunit contains an immunoglobulin fold that lacks a β -strand, which, in the assembled pilus structure, is provided by the neighbouring pilus subunit. Before assembly, PapD complements, and thus stabilizes, a pilus subunit by donating a β -strand in an analogous manner (Fig. 2K; Sauer *et al.*, 2002).

Cellular quality control

Physical or chemical stresses that are brought about by temperature changes, exposure to proteotoxic agents, or other conditions that are conducive to protein misfolding induce a ubiquitous, protective, cellular stress response. Crucial to this response is an increase in chaperone and proteolytic activities, aimed at reducing the presence of damaging non-native protein species. In eukaryotes, the heat-shock transcription factor (HSF) regulates the expression of stress-inducible genes, including all of the well-characterized chaperones (Leroux & Hartl, 2000a).

As a first line of defence against damaging cellular insults, several chaperones, including sHsps and Hsp70s, can stabilize proteins undergoing denaturation and allow their refolding when the stress has subsided. Severe stresses, however, may overwhelm the ability of chaperones to stabilize large pools of non-native proteins, resulting in protein aggregation. Renaturation of these insoluble proteins by members of the Hsp100/Clp family, in conjunction with the Hsp70 system, has been shown (Glover & Lindquist, 1998).

The ER also has its own coordinated unfolded-protein response (UPR). The accumulation of misfolded proteins in this compartment results in the upregulated transcription of numerous quality-control genes, including BiP and Grp94, the ER homologues of Hsp70 and Hsp90 (Hampton, 2000). Interestingly, the sensing mechanism of the UPR relies on the reversible interaction of BiP with the stress-signal-transducing protein IRE1. BiP interacts with and inactivates IRE1 only in the absence of stress, when it is not engaged with misfolded proteins. Similarly, HSF activity is modulated by at least one chaperone (Hsp90) that is able to detect the presence of misfolded proteins (Leroux & Hartl, 2000a).

Fig. 3 | An aggresome formed in HeLa cells by overexpression of a green-fluorescent-protein fusion to the polyglutamine region of huntingtin (green), the protein involved in the neurodegenerative disorder, Huntington's disease. The nucleus is shown in blue, and the microtubules in red.

Perhaps surprisingly, not all cellular strategies for maintaining protein stability involve protein chaperones. Chemical chaperones, such as the disaccharide trehalose, help to stabilize non-native proteins, and are overproduced on heat-shock (Singer & Lindquist, 1998).

Proteins that cannot fold properly because of mutations, errors in translation, faulty biogenesis or damage are normally degraded. It is estimated that ~30% of all nascent proteins fail to fold and are destroyed by the ubiquitin–proteasome system, and under stress conditions, the need for degradation increases greatly (Schubert *et al.*, 2000). An emerging paradigm is that of chaperone systems cooperating with the protein degradation machinery to ensure quality control. The UPR exemplifies this integration because it also regulates ER-associated degradation (ERAD), a process that involves the retrograde transport of misfolded ER proteins into the cytosol for destruction by the proteasome (Hampton, 2000). Mammalian cytosolic Hsp70, for example, uses co-chaperones such as Bag1 and CHIP to assist with the capture and sorting of non-native proteins that are destined for folding or proteasomal degradation (Höhfeld *et al.*, 2001).

Protein misfolding diseases

Most protein misfolding diseases can be directly attributed to mutations in the affected proteins. As might be expected, however, mutations in chaperones can also lead to improper biogenesis or failure to stabilize particular protein substrates, and have been linked to these diseases. Two examples illustrate this point: the sHsp-encoding α -crystallin gene, when modified by a point mutation that results in an R120G amino-acid change, can lead to cataracts, desmin-related myopathy or the neurodegenerative disorder, Alexander's disease (Clark & Muchowski, 2000); alterations in the BBS6 protein, which shows weak homology to the chaperonin CCT, cause the symptoms seen in some patients with Bardet–Biedl syndrome, including blindness, obesity and kidney dysfunction (Katsanis *et al.*, 2000).

In many cases, misfolded proteins associated with disease are sequestered in the form of fibrils or amorphous aggregates. Several of these conditions are collectively called amyloidoses because of the characteristic formation of large fibrils, known as amyloids. A subset of misfolding diseases that cause neurodegenerative disorders, including Huntington's disease, result from the expansion of CAG nucleotide repeats. The severity and onset of these diseases correlates with the length of the aggregation-prone polyglutamine regions (Taylor *et al.*, 2002). Prion disorders, such as Creutzfeldt–Jakob disease, are unique in that the difference between the disease-causing (PrP^{Sc}) and normal (PrP^C) proteins is strictly conformational; misfolded PrP^{Sc} induces PrP^C to change its conformation and polymerize on the growing fibril (Taylor *et al.*, 2002). Interestingly, studies of yeast prion-like proteins, such as Sup35, have led to the intriguing possibility that chaperones could influence the structural permutations of the prion protein (Chernoff *et al.*, 1995).

In protein misfolding disorders, or under severe stress, the machinery needed to fold or degrade a non-native protein may become saturated. Researchers are familiar with inclusion bodies, which are formed in response to the overexpression of some proteins in bacteria. In eukaryotic cells, a dynamic apparatus that involves microtubules and motor proteins assists the transport of at least some misfolded polypeptides to the centrosome, forming a cellular compartment known as the 'aggresome'. This is the fate, for example, of a large proportion of the slow-folding cystic fibrosis transmembrane regulator (CFTR) protein, especially mutants that contain the $\Delta 508$ deletion found in most patients (Johnston *et al.*, 1998). As shown in Fig. 3, a green fluorescent protein (GFP) fused to exon 1 of the huntingtin protein, which contains the long stretch of polyglutamines that is associated with Huntington's disease, forms a typical perinuclear aggresome (Taylor *et al.*, 2002).

Recent findings have led to the suggestion that protein aggregates may sequester chaperones, proteasomes and other proteins, preventing their normal function and thereby leading to the observed pathologies. Artificially increasing the levels of some chaperones has, in some cases, been found to provide a protective effect, and could, therefore, be of possible therapeutic value (Muchowski, 2002).

Perspectives

Ensuring that non-native polypeptides are directed towards their appropriate cellular fates, for example biogenesis or degradation, involves normal cellular activities that are carried out by numerous proteins involved in quality control. However, the number of different molecular chaperones and protein-degradation-associated components that mediate such housekeeping functions may be significantly greater than is known at present. This is supported by the recent finding that erythroid cells have a chaperone that is specifically involved in stabilizing α -haemoglobin, a protein that has been studied for more than a century, before its assembly into a functional $\alpha_2\beta_2$ -heterotetramer (Kihm *et al.*, 2002). At a cellular level, the possibility that unique responses tailored to deal with non-native proteins remain to be identified is highlighted by the recent discovery of a mitochondrial-specific stress response (Zhao *et al.*, 2002). Clearly, the many roles of chaperones and other quality control elements in fundamental cellular activities, as well as in human diseases, emphasize the need for a greater understanding of processes involving non-native proteins.

ACKNOWLEDGEMENTS

We thank V. Daggett, A. Dinner and J. Valpuesta for providing materials to prepare figures, and apologize to those colleagues whose work was not cited due to space restrictions. We acknowledge the Canadian Institutes of Health Research (CIHR) and the National Cancer Institute of Canada for financial support. M.R.L. is the recipient of Michael Smith Foundation for Health Research and CIHR scholar awards, and P.C.S. holds an Natural Sciences and Engineering Research Council of Canada scholarship.

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