Minireview

Protein Disulfide Isomerase and Assisted Protein Folding*

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The folding protein (and protein folder) is beset with a number of problems in translating the simple instructions encoded by DNA into the complex, three-dimensional structure of a correctly folded protein. Nature has solved these problems in an adequate, if not elegant, fashion, but we are just beginning to understand the variety of strategies that cells use to ensure efficient protein folding. This minireview will focus on several of the more general principles of assisted folding using the folding catalyst, protein disulfide isomerase (PDI),1 as an example. This remarkable resident of the endoplasmic reticulum (ER) inserts disulfides into folding proteins and provides a mechanism to correct errors in disulfide pairing when they occur. At high concentrations, it functions as an ATP-independent chaperone that inhibits aggregation; yet, at lower concentrations, it can also participate in an unusual interaction with substrate that leads to PDI-facilitated aggregation (anti-chaperone behavior2).

Proteins Fold in Different Environments

Assisted protein folding occurs in at least three intracellular compartments, the cytosol, mitochondria, and the lumen of the ER. PDI is a resident of the endoplasmic reticulum, a specialized folding environment, replete with a variety of folding catalysts and chaperones (1–3). Folding in the ER is specialized to accommodate the structural features of membrane and secreted proteins, including glycosylation and disulfide formation (4–6). As proteins are synthesized and inserted into the ER, the sequence is presented vectorially to the ER lumen so that folding may begin as the protein emerges from the ribosome (7–10). A glutathione redox buffer (a mixture of GSH and GSSG at a ratio of approximately 1:1–3:1) holds the redox state of the ER more oxidizing than that of the cytosol to allow disulfide formation and rearrangement before folding can proceed (36). In other cases such as pancreatic trypsin inhibitor, folding intermediates are dominated by native pairings of cysteines; however, the disulfides are formed in the “wrong” temporal order and must be rearranged before folding can proceed (36). In other cases such as RNase (37, 38) or hirudin (39), disulfides are formed randomly in early folding intermediates. Thus, protein folding is a process of trial and error (40). PDI corrects these mistakes by catalyzing rearrangements to replace wrong disulfides with correct ones, a process that eventually leads to a “native” structure that is resistant to further rearrangement (41, 42).

So far, no evidence has surfaced that PDI or other folding assistants directly guide the folding protein into a specific structure (21, 43). PDI simply accelerates the formation and breaking of disulfides, providing cycles of PDI-catalyzed rearrangements to correct the frequent mistakes that occur. Turnover numbers for PDI-catalyzed disulfide isomerization are

Because of the high reactivity of the active site disulfide of PDI (20), the delay in disulfide formation may be due to protection of certain cysteines against oxidation by folding of the nascent chain or by protection with other ER chaperones (19). However, Ruddon and co-workers (21) have mapped the order of disulfide formation for human hCG-β during folding in the ER and for the same reaction in vitro, catalyzed by PDI. No differences were observed in the identity and order of disulfide formation suggesting, at least for hCG-β, that ER chaperones are not necessary for the inhibition of disulfide formation during folding (21) and that catalyzed and uncatalyzed folding proceeds by similar mechanisms.

PDI Has Unique Structural and Functional Features

During protein folding in the ER, PDI catalyzes disulfide formation and rearrangement by thiol/disulfide exchange (22). A member of the thioredoxin superfamily (14), PDI has two independent (23) but non-equivalent (24) active sites, each with two cysteines (CGHC) that cycle between the dithiol and disulfide oxidation states. One thioredoxin domain is positioned near the N terminus, and another is poised near the C terminus (25). An NMR structure of the N-terminal thioredoxin domain shows that the backbone fold is comparable with that of thioredoxin (26). The active site disulfide is a good oxidant (27, 28) that directly introduces disulfides into protein substrates (29, 33). The dithiol redox state is essential for catalyzing disulfide rearrangements. The necessity of having oxidized and reduced active sites for catalysis of different steps results in a redox optimum (24). The individual thioredoxin domains of PDI have been expressed alone, and they are catalytically active oxidants. However, neither isolated domain exhibits significant isomerase activity despite the fact that the chemical properties (reactivity and redox potential) are near those found for wild-type PDI (30).

PDI is an essential gene in yeast (31, 32), and the isomerase activity is the critical feature (33). Overexpression of thioredoxin itself will not rescue PDI-null strains, but a mutant of thioredoxin in which the active site sequence (CGPC) has been changed to the PDI sequence (CGHC) can support yeast growth (34) even though this mutant thioredoxin (P34H) has only 10% of the isomerase activity of PDI in vitro (35).

Mistakes Occur during Folding

Protein folding is error-prone. For some proteins like bovine pancreatic trypsin inhibitor, folding intermediates are dominated by native pairings of cysteines; however, the disulfides are formed in the “wrong” temporal order and must be rearranged before folding can proceed (36). In other cases such as RNase (37, 38) or hirudin (39), disulfides are formed randomly in early folding intermediates. Thus, protein folding is a process of trial and error (40). PDI corrects these mistakes by catalyzing rearrangements to replace wrong disulfides with correct ones, a process that eventually leads to a “native” structure that is resistant to further rearrangement (41, 42).
isomerization activity drops precipitously (mutating the second active site cysteine to serine, PDI accuracy or reduction. If reductive escape is inhibited by disulfide reduced and released for further attempts at reverse in a timely fashion (Fig. 1). The offending substrate the complex if a substrate–PDI complex fails to rearrange or reverse in a timely fashion (adapted from Ref. 42 with permission). slow by normal enzyme standards (~1 min⁻¹), but given the high local concentration of PDI in the ER (about 0.2 mM) (44), this low turnover number is sufficient to make PDI-catalyzed folding kinetically competent in the cell. The value of \( K_m \) for disulfide isomerization of protein substrates is near 10 \( \mu M \) (43, 44).

Disulfide rearrangements are initiated by the attack of the active site thiol of reduced PDI on a substrate disulfide (45). The choice of which substrate disulfide to attack will depend on the kinetic reactivity and accessibility of the various substrate disulfides that are available. The formation of a PDI-substrate covalent intermediate disrupts one of the substrate disulfides, creating a free sulfhydryl in the substrate. This intermediate can be resolved by three alternative reactions (Fig. 1). If PDI is displaced by reforming the same disulfide that was attacked initially, no disulfide rearrangement results. Rearrangement can occur by an intramolecular reaction of the newly created substrate thiol on another substrate disulfide or by reduction of the original disulfide followed by oxidation to form different disulfides (42). The second cysteine in the active site can serve as a clock to invoke reduction of the disulfide and escape from the complex if a substrate-PDI complex fails to rearrange or reverse in a timely fashion (Fig. 1). The offending substrate disulfide is reduced and released for further attempts at rearrangement or reduction. If reductive escape is inhibited by mutating the second active site cysteine to serine, PDI accumulates in mixed disulfide complexes with substrate, and the isomerization activity drops precipitously (<1% of wild type) (29), suggesting that reduction/reoxidation is the dominant mechanism of disulfide rearrangement for RNase. The initial scanning of the substrate disulfides by PDI and the subsequent reactions of the PDI-substrate intermediate should favor the trial and error replacement of reactive disulfides of the substrate by less reactive ones (42), a process similar to that suggested for repeated cycles of folding and unfolding catalyzed by ATP-dependent chaperones such as GroEL/ES (40).

**Fig. 1. PDI-catalyzed disulfide rearrangements.** Attack of reduced PDI on a substrate disulfide leads to the formation of a covalent PDI-substrate complex. The resolution of this complex may occur through three competing pathways. The red pathway represents reforming the original substrate disulfide. Disulfides that are resistant to attack or which reform very rapidly would avoid rearrangement. The green pathway represents intramolecular rearrangements involving substrate thiols and disulfides. This pathway would have a tendency to replace more reactive substrate disulfides with less reactive ones. The blue pathway represents rearrangement by reduction of one or more disulfides and reoxidation in a new orientation. This pathway also provides PDI with a mechanism to escape from covalent complexes that rearrange or reverse slowly (adapted from Ref. 42 with permission).

**Folding Catalysts Recognize Many Substrates**

In keeping with the lack of obvious sequence cues that specify disulfide connectivity, PDI can catalyze thiol/disulfide exchange reactions involving a wide range of substrates, including proteins, peptides and low molecular weight thiols and disulfides (46). PDI interacts relatively weakly with peptides \( (K_m = 50–1000 \mu M) \) with no obvious correlation between binding and peptide charge, sequence, hydrophobicity (47, 48). A peptide-based photoaffinity probe labels one site near the C terminus and inhibits catalytic activity (49). In contrast to glutaredoxin (50), PDI does not exhibit any special affinity for glutathione as a substrate (27). However, the dominant mechanism of PDI-dependent oxidation of small peptide substrates involves the formation of peptide-glutathione mixed disulfides (51), and protein-glutathione mixed disulfides are good PDI substrates (52, 53). Although mutants of PDI with a single, more N-terminal cysteine might be expected to catalyze oxidation by glutathione-mixed disulfide formation, they do not (29).

In addition, substrate oxidation occurs rapidly in the absence of a redox buffer, where stoichiometric amounts of PDI supply all of the oxidizing equivalents (44). Although glutathione mixed disulfide formation does occur, it is not an obligate oxidation pathway.

**Aggregation Is a Major Complication to Productive Folding**

Folding intermediates with a less organized and more exposed hydrophobic core have an increased tendency to aggregate, sometimes imposing stingy limits on solubility (54). At least for some proteins, aggregation can occur from specific folding intermediates through specific associations (55, 56). Yet hydrophobic aggregation can also be nonspecific, involving the interaction of multiple proteins (57). PDI has two somewhat paradoxical roles in substrate aggregation. It can behave as a chaperone and inhibit aggregation, or under certain conditions, it can behave as an anti-chaperone and facilitate the formation of aggregates.

As a chaperone, PDI inhibits substrate aggregation by two mechanisms, rapid disulfide formation and a chaperone-buffer effect. At concentrations near 10 \( \mu M \), reduced, denatured hen egg white lysozyme tends to aggregate (58), compromising the yield during refolding. If present when lysozyme is diluted into solution, high concentrations (near 100 \( \mu M \)) of catalytically active PDI are required to inhibit aggregation, suggesting that very rapid disulfide formation can divert substrate molecules onto productive folding pathways and decrease the amount of aggregation (59, 60). However, PDI also inhibits the aggregation of proteins that do not form disulfides (61–63). Peptides inhibit the chaperone activity suggesting that the peptide/protein binding site(s) of PDI may interact with the substrate protein and prevent its aggregation by forming soluble, non-covalent complexes that mask aggregation-prone sites (64).

One of the more surprising features of PDI-assisted folding is that PDI can, under certain conditions, actually facilitate aggregation, a behavior that has been called anti-chaperone activity (59, 60, 63). Under reducing conditions, adding low concentrations of PDI to a solution of unfolded, reduced lysozyme causes large, insoluble aggregates to form that contain both lysozyme and PDI (59). PDI at low concentrations accelerates the aggregation of alcohol dehydrogenase during thermal denaturation, but higher concentrations of PDI suppress aggregation (63). This paradoxical change of a folding assistant from anti-chaperone to chaperone has an analogy in the classical immunoprecipitation of a bivalent antigen by a population of bivalent antibodies (Fig. 2) (59). For a protein that has some intrinsic tendency to self-associate into aggregates, multivalent interactions with PDI can provide an additional mechanism for
Aggregation is generally viewed as a folding disaster, and in vitro aggregation so often observed stoichiometric chaperone capacity is exceeded or to protect folding assistants in refolding strategies. However, there are ratios and in competition with PDI (65). Approximately 5–10 unfolded proteins per PDI (59, 63). Another ER aggregate with the unfolded protein in a defined ratio, approximately. The folded protein will bind to a PDI that is not already bound to another unfolded protein, decreasing the extent of cross-link formation, increasing solubility, and favoring chaperone behavior. The interactions that mediate anti-chaperone-facilitated aggregation are relatively specific. PDI is specifically co-precipitated with the unfolded protein in a defined ratio, approximately 5–10 unfolded proteins per PDI (59, 63). Another ER chaperone, BiP, also exhibits chaperone/anti-chaperone behavior, and it too is incorporated into large aggregates at a defined ratio and in competition with PDI (65).

Aggregation is generally viewed as a folding disaster, and facilitated aggregation is obviously a complication to using the folding assistants in refolding strategies. However, there are circumstantial indications that switches between chaperone/anti-chaperone behavior could be useful to the stressed cell as a mechanism to retain unfolded proteins in the ER when the stoichiometric chaperone capacity is exceeded or to protect unfolded proteins from degradation. In contrast to the irreversible aggregation so often observed in vitro, aggregation can be reversible in the cell (66). The inhibition of disulfide formation in cells by including dithiorthiol in the medium results in the accumulation of large, chaperone-associated aggregates that are retained in the ER. When the dithiorthiol is washed out, these aggregates are salvaged, correctly folded, and released from the ER as soluble proteins (67, 68). The chaperone-associated aggregates may involve multiple substrate proteins and multiple ER-chaperones (69) although it is not known whether these are heterogeneous complexes or mixtures of homogeneously complexes. The normal folding of thyroglobulin induced in HepG2 cells by thyroid hormone and the assembly of procollagen (70) proceeds through large, chaperone-associated aggregates (71, 72), and mutants of human chorionic gonadotropin can also be retained in the ER as large, chaperone-associated aggregates that go on to fold productively, albeit at a slower rate (73).

Conclusions
PDI and other folding catalysts and molecular chaperones provide the cell with an effective solution to the protein folding problem. The overall efficiency of folding can be enhanced by increasing the rate of slow chemical steps that limit conformational transitions, by preventing the formation of non-productive aggregates, and perhaps by protecting the folding protein through the formation of transient aggregates. The challenge in the near future will be to understand assisted folding at the molecular and structural levels and to discover how assisted folding “pathways” are orchestrated in the cell.
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