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The Bonds That Tie: Catalyzed Disulfide Bond Formation

Minireview

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The pioneering studies of Anfinsen on ribonuclease have guided much of the research in the field of protein folding (Anfinsen et al., 1961). Anfinsen showed that, in vitro, reduced and denatured ribonuclease could refold into active enzyme with the formation of the appropriate disulfide bonds. These findings demonstrated that there was sufficient information in the primary amino acid sequence of the protein to guide folding and that, in the appropriate redox environment, the proper disulfide bonds formed. However, the rates and efficiency of protein folding and disulfide bond formation in the test tube are often much worse than those found in the cell. Anfinsen himself subsequently discovered protein disulfide isomerase (PDI), a catalyst of oxidative folding. Despite this indication that auxiliary factors could guide protein folding, the persistent impression remained until recently that the folding process and disulfide bond formation occur in vivo without the need for catalysis.

Two classes of proteins that assist in the protein folding process have been described. Molecular chaperones are thought to act by preventing improper interactions leading to aggregation and other events taking a protein off the productive folding pathway. Chaperones thus do not actually catalyze folding. However, catalysts have been found for two slow steps in protein folding, cis–trans prolyl isomerization and disulfide bond formation. Clear evidence for the function of prolyl isomerases as folding catalysts in vivo is still lacking. The recent isolation of mutants that are severely defective in disulfide bond formation has, however, confirmed that disulfide bond formation is facilitated in vivo.

A substantial body of in vitro evidence had previously implicated PDI in the catalysis of disulfide bond formation and rearrangement (Creighton et al., 1980; Freedman et al., 1989). The description, in this issue of *Cell*, of yeast mutants in PDI that fail to form disulfides in carboxypeptidase Y directly demonstrates the importance of PDI in the process of disulfide bond formation in vivo (LaMantia and Lennarz, 1993 [this issue of *Cell*]). In prokaryotes, mutations in the *dsbA* gene show a dramatic and pleiotropic decrease in the rate of disulfide bond formation in secreted proteins (Bardwell et al., 1991). The proteins affected range from normal Escherichia coli constituents such as alkaline phosphatase and OmpA to the cloned eukaryotic proteins, urokinase and tissue plasminogen activator. It is now clear that the formation of disulfide bonds is a catalyzed process in both eukaryotes and prokaryotes.

Disulfide-linked protein folding can involve both the formation of new protein disulfide bonds and internal isomerization of existing disulfides. Formation of a new disulfide bond usually involves the transfer of a disulfide bond from an external source to the protein. This reaction occurs through a mixed disulfide intermediate. The oxidation of the protein is accompanied by the reduction of the external source. In contrast, disulfide bond isomerization as an intramolecular reaction does not, in principle, require an external electron acceptor.

Properties of the Catalysts

DsbA and PDI have a number of unusual properties appropriate for their role as disulfide bond donors. Disulfide bonds, which generally play a structural role in proteins, are ordinarily stabilizing and nonreactive. The disulfide of DsbA, however, is very reactive, and its presence destabilizes the structure of the protein (Zapun et al., 1993; Wunderlich and Glockshuber, 1993). DsbA thus has strong oxidizing properties, consistent with its ability to form disulfides in proteins. The strain imposed by the disulfide on the structure of DsbA may help explain why its disulfide is so reactive. The disulfide of PDI is also oxidizing, although it may not be as strong an oxidant as that of DsbA (Hawkins et al., 1991; Lundström and Holmgren, 1993). Their similar redox properties make it likely that DsbA and PDI play similar roles in redox reactions with proteins.

Appropriately, PDI and DsbA are present in the cellular compartments where disulfide bond formation takes place; PDI is an endoplasmic reticulum (ER) protein, and DsbA is periplasmic. Both DsbA and PDI can participate in a wide variety of disulfide reactions in vitro, including oxidation, reduction, and isomerization, depending on redox conditions. Using glutathione concentrations similar to those of the ER, PDI speeds the oxidative folding of bovine pancreatic trypsin inhibitor (BPTI) in vitro approximately 40-fold, even though the concentrations of PDI used were considerably lower than those thought to exist in the ER (Creighton et al., 1993; Hwang et al., 1992). DsbA catalyzes the oxidative folding of hirudin, a thrombin inhibitor with three disulfide bonds (Wunderlich et al., 1993). It appears to do so by a rapid initial oxidation reaction followed by disulfide isomerization. The catalytic ability of DsbA is very clear. Rapid folding follows addition of catalytic quantities of DsbA, under conditions in which no spontaneous folding can be detected in its absence.

DsbA and PDI appear to play a similar role in disulfide bond catalysis, and appropriately, they both have domains similar to thioredoxin, a ubiquitous protein with a redox active disulfide capable of catalyzing a wide variety of protein disulfide oxidoreduction reactions (Holmgren, 1989). However, DsbA and PDI differ in other respects. DsbA has one thioredoxin-like domain per monomer and PDI has two. In DsbA, the thioredoxin domain is interrupted by an unusual triple-helical domain (Martin et al., 1993); in PDI, the thioredoxin-like domains are not interrupted. DsbA is a monomer of 21 kd. PDI exists as a homodimer

with a subunit molecular mass of 57 kd in mammals and of 70 kd in yeast. PDI is larger not only because of the duplication of the thioredoxin domains but also because of the presence of several additional domains that are missing in DsbA. PDI is the β subunit of prolyl hydroxylase and is also proposed to be part of the triglyceride transfer complex. The extra domains on PDI may be responsible for these additional roles (Noiva and Lennarz, 1992). If one specifically mutates a cysteine in the active site of yeast PDI, the resulting yeast mutant is viable but fails to form disulfides, similar to null mutations in *dsbA*. Null mutations in PDI are, however, inviable, consistent with the idea that the additional roles of PDI are essential (La-Mantia and Lennarz, 1993).

How do PDI and DsbA interact with proteins? Some evidence suggests that they both may have domains that allow them to bind to polypeptide chains. PDI cross-links to photoexcitable tri- and tetrapeptide probes (Noiva et al., 1993). In addition, peptides of various lengths and sequence act as competitive inhibitors of PDI activity (Morjana and Gilbert, 1991). These observations suggest that PDI has a fairly nonspecific peptide binding activity. This ability to associate with other proteins may help to explain why PDI is found as a subunit of several enzyme complexes. A peptide binding activity for DsbA is implied by the observation that DsbA reacts with reduced proteins much more effectively than it does with the strong reductant dithiothreitol (Wunderlich et al., 1993). The crystal structure of DsbA shows that its active site is surrounded by deep grooves and a hydrophobic patch, features also suggestive of peptide-binding surfaces (Martin et al., 1993).

Both PDI and DsbA act as catalysts (Creighton et al., 1980, 1993), but compared with most enzymes they are not very efficient. In the catalysis of the oxidative folding of RNAase, for example, PDI exhibits an unimpressive K_{cat} of 0.4/min (Lyles and Gilbert, 1991). Perhaps to compensate for this low catalytic efficiency and the need for a wide substrate specificity, PDI and DsbA are present at high concentrations in vivo.

If DsbA and PDI are to act catalytically, they must be reoxidized. An integral membrane protein, DsbB, has been implicated in the reoxidation of DsbA (Bardwell et al., 1993). DsbB has several cysteines in its periplasmic domains, which may act to pass oxidizing potential from some component of the electron transport pathway to DsbA. Candidates for compounds involved in the reoxidation of PDI include oxidized glutathione and selenite.

Isomerization or Oxidation?

Unfortunately, the use of "isomerase" in the name of protein disulfide isomerase gives the impression that reshuffling of incorrect disulfides is its only, or predominant, role. The need for oxidation in vivo is clear: proteins are synthesized in a reduced form. The *dsbA* and PDI mutants are clearly blocked in formation of disulfides. However, since formation must precede isomerization, the properties of these mutants do not help address the issue of a need for isomerase activity. Isomerase activity may be required both for the normal protein folding pathway and for the rescue of proteins that have become misfolded or aggregated. For instance, PDI has been shown to rescue kinetically trapped folding intermediates (Weissman and Kim, 1993). The N and N* intermediates on the BPTI folding pathway appear native by many criteria; however, they only contain 2 of the 3 disulfides found in the final structure. Addition of PDI dramatically accelerates isomerization reactions necessary for the completion of disulfide bond formation. PDI thus is apparently capable of gaining access to buried thiols in a protein that has acquired a substantial percentage of its tertiary structure. If PDI can act late in folding, it could help explain why the correct disulfide bonds are made. The proteins have already folded so as to place those cysteines that are to participate in disulfide bond formation in close proximity. In summary, it seems likely that DsbA and PDI as disulfide oxidoreductases are involved in both formation and isomerization of disulfides in vivo.

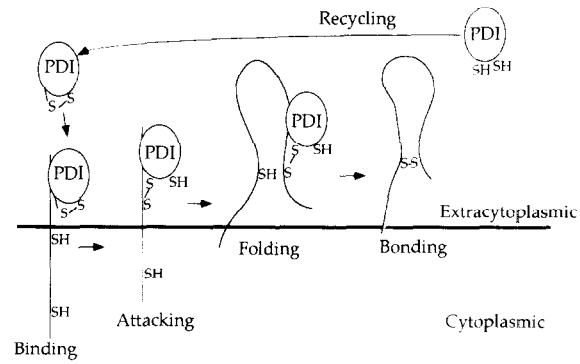

Figure 1. Model for the Catalysis of Disulfide Bond Formation

See text for description of the steps in disulfide bond formation. In E. coli, the catalytic protein is DsbA and the extracytoplasmic space is the periplasm. In eukaryotic cells, catalysis is performed by PDI and the extracytoplasmic compartment is the lumen of the endoplasmic reticulum.

A Model for Catalysis of Disulfide Formation

PDI is directly involved in disulfide catalysis in eukaryotes; DsbA plays the same role in prokaryotes. Proteins are secreted in a reduced and at least partially unfolded state. Upon extrusion into the secretory compartment, the disulfide catalysts recognize the nascent chain. Upon binding, the active site of these oxidoreductases are exposed, bringing a very reactive disulfide next to the protein's free cysteines. This disulfide is attacked by one of the protein's cysteines, forming an unstable mixed disulfide. It is not necessary to postulate that the catalysts direct folding. Rather, information within the polypeptide chain brings the cysteines into the correct fold. As the appropriate free cysteine approaches this very reactive mixed disulfide, a second attack occurs, and the disulfide bond is then transferred to the protein, which is in the process of folding. The disulfide adds further stability to the folded domain in which it lies. The catalyst is released in a reduced form. It is then reoxidized, and the cycle continues. In addition to this role in oxidation of proteins early in folding, these catalysts may also play a role in disulfide isomerization

during later steps of protein folding for proteins with multiple disulfides.

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