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Forum Editorial

Oxidative Protein Folding: From the Test Tube to *In Vivo* Insights

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THE FORMATION of intramolecular disulfide bonds (S-S) has important consequences for protein folding, stability, and biological function. The covalent link of cysteine residues in polypeptides stabilizes their native conformation by decreasing the entropy associated to the unfolded state, increasing thus the thermodynamic gap between the nonstructured and functional states of disulfide-containing proteins. It diminishes at the same time the combinatorial search between different conformational states to attain a unique three-dimensional structure with specific biologic activity. The stability provided by the disulfide covalent linkage also protects polypeptides from oxidant and proteolytic damage, thus increasing their half-life. Finally, although cysteine residues forming the native disulfide bonds of mature proteins have been traditionally considered to be chemically inert, in some cases, their redox state and pairing can be modulated, acting as switches for protein function (11).

The term "oxidative protein folding" describes essentially the composite process by which a reduced, unfolded protein gains both its native disulfide bonds and its native structure. This forum issue reflects the large and diverse research effort devoted to understanding this process and its catalytic control. Its aim is to provide the reader with an overview of the remarkable structural and functional insights obtained in the last few years, which, in addition to expanding our knowledge of protein oxidation, have opened novel intriguing questions, ensuring that the formation of the native disulfide bridges in proteins will continue to focus the interest of many scientists in the years to come.

OXIDATIVE PROTEIN FOLDING MODELS

In recent decades, we have witnessed significant progress in the protein folding field, but unveiling how a string of amino acid residues folds into a biologically active conformation is still a major challenge in biology. This is particularly true for cysteine-containing proteins, in which structural folding is necessarily coupled with the construction of the native disulfide architecture. The apparently straightforward process of oxidation of the free cysteines in a protein into its native set of disulfide bonds is a rather complex route that, in addition to conformational folding, involves a series of covalent reactions such as oxidation (S–S formation), reduction (S–S breaking), and isomerization (S–S rearrangement). The oxidative folding puzzle for disulfide-rich polypeptides is clearly illustrated in the article by Bulaj and Olivera on the folding of conotoxins, in which thousands of distinct amino acid peptide sequences fold to adopt essentially the same general three-dimensional structures (6).

Despite the complexity of their folding mechanisms, from the first experiments of Anfinsen and co-workers (1) on ribonuclease in the 1960s, small disulfide-rich polypeptides frequently have been chosen as models to study, *in vitro,* protein-folding pathways. This is because the oxidative folding technique pioneered by Creighton (9) provides a powerful method for protein-folding analysis in which disulfide(s) can be used as a unique probe of protein conformation. Protein folding commonly proceeds through a series of intermediates that define the folding landscape from the unfolded polypeptide to the native structure. Deciphering the structural properties of the successive intermediates in the folding route would provide the more accurate description of the pathway, helping us to understand the relation between the primary sequence and the functional fold of proteins. Nevertheless, folding intermediates are usually difficult to isolate because of their short half-life and highly flexible conformation. In disulfide-rich proteins, the coupling of the covalent chemistry of disulfide bond formation to the noncovalent folding forces makes it feasible to trap, isolate, and characterize their intermediates (3).

A number of articles in the present issue demonstrate the potency of this approach to investigate in great detail the folding

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of models comprising divergent, physiologically and biotechnologically relevant protein families.

Feng and co-workers review the oxidative folding of the members of the insulin superfamily, which share high sequence homology and similar tertiary structures but not necessarily the folding mechanism, as shown for insulin and insulin-like growth factor-1 cases (10). This exemplifies an unexpected scenario of oxidative-folding diversity for small disulfide-rich proteins. This way, some proteins fold through a few disulfide intermediates that adopt native disulfides pairing and native-like structures, funneling protein conformations toward the native state and preventing the accumulation of nonnative disulfide isomers. This appears to be the case of leech-derived tryptase inhibitor (LDTI), as analyzed here by Ventura and co-workers. In this polypeptide, the same interactions that stabilize the final folded structure seem to guide LDTI toward its native state as well (4). Likewise, the kinetic and structural studies conducted by Craik's group demonstrate that the oxidative folding of cyclic cystine knot proteins, small but topologically complex and extremely stable molecules occurring naturally in plants, is mainly populated by native-like intermediates (7). In the other side of the spectrum, we found proteins like hirudin, the folding of which displays a highly heterogeneous population of intermediates, in which well-populated folding species adopting native disulfides are absent (8). Further to complicate the scenario, when the establishment of native-like contacts is relevant for the folding process, these noncovalent interactions might only funnel folding at certain stages of the reaction, as shown here for conotoxins (6).

A major problem in deriving general laws describing and allowing us to predict the folding of proteins is that, as stated in this forum by Chang, the detailed pathways of protein folding have been derived mainly from the analysis of oxidative folding of disulfide proteins, whereas most protein-folding models have been proposed after studying nondisulfide proteins or disulfide proteins in the presence of intact native disulfide bonds (8). In only few cases have both the folding pathway and the folding model been elucidated, this being certainly an avenue to explore to obtain a consistent view on how proteins gain their functional states from initially unfolded conformations.

Most oxidative protein-folding studies focus on the establishment of intramolecular disulfide bridges, and little is known about the process of formation of intermolecular bonds. Nevertheless, this kind of crosslinking is crucial for the structure and function of many important human proteins. Outstanding examples are the proteins of the extracellular matrix, and specifically collagens, as reviewed here by Moroder and co-workers (5). Understanding how these and other large multimeric polypeptides containing intermolecular disulfides fold by mean of short model peptides is of significant interest, because it might allow the development of a new kind of biomaterials.

OXIDATIVE PROTEIN FOLDING IN THE CELLULAR CONTEXT

Although many proteins, under proper conditions, have been shown to complete the oxidative folding process *in vitro,* it is also now evident that this reaction does not occurs spontaneously in the cell, because the mentioned covalent reactions are dangerous events that can freeze proteins in nonfunctional and even cytotoxic conformations. Therefore, all organisms have evolved mechanisms to regulate oxidative protein folding. Accordingly, an increasing number of enzymes assisting the *in vivo* formation of disulfide bonds are being discovered. Bartolucci and colleagues describe here the machinery for oxidative protein folding evolved in thermophiles and compare it with that in bacteria or eukaryotic cells. The study suggests that eukaryotic thiol-disulfide oxidoreductases of the protein disulfide isomerase (PDI) family might have their origin in a similar enzyme encoded in thermophiles (13).

PDI controls the formation of disulfide bonds in the endoplasmic reticulum (ER) and assists the retrotranslocation of misfolded ER proteins to the cytosol. The redox activity of PDI-like enzymes is determined by the redox state of its active-site cysteines. Although the *in vitro* activity of this polypeptide has been extensively characterized, understanding its *in vivo* redox regulation requires reliable methods to determine quantitatively its redox state in living cells. Here, Appenzeller-Herzog and Ellgaard describe the use of a new approach to demonstrate that *in vivo* human PDI is present in two semioxidized forms, suggesting that neither of the two domains in this enzymes exclusively catalyzes oxidase or isomerase reactions in the cell (2).

Many diseases are characterized by misregulation of folding processes, including formation of disulfides, resulting in the accumulation of aberrant proteins that trigger cell-death–signaling pathways. Therefore, finding compounds able to induce or attenuate the expression of molecular chaperones is a hot topic in biology and pharmacology. In their article (14), Soti and coworkers show that the antioxidant agent resveratrol, a polyphenol from wine, is able to act as an inducer of the heat-shock response. In neurodegenerative disorders, the accumulation of misfolded proteins adversely affects neuronal connectivity and plasticity, thus impairing cognitive processes. Recent studies have suggested that generation of excessive nitric oxide (NO) mediates excitotoxicity, in part by triggering protein misfolding. Nakamura and Lipton present here their recent evidence suggesting that NO contributes to degenerative conditions by covalently modifying the active-site thiols groups of PDI, inhibiting the molecular chaperone and thiol-disulfide oxidoreductase activities of this enzyme in models of Parkinson and Alzheimer diseases, thus providing a link between oxidative protein folding and these highly debilitating disorders (12).

FUTURE DIRECTIONS

The information gathered in this issue undoubtedly reveals that the investigation of oxidative protein folding by using both *in vitro* and *in vivo* approaches is an effervescent and productive research area. Nevertheless, it also evident that, in the past, both kinds of studies have worked a little as sealed compartments, with little information exchange between them. It is very likely that in the next years, we will witness the construction of a more synergic environment in which both kinds of data could be integrated to obtain a clear and uniform description of the mechanism of oxidative protein folding, its regulation, and the role it plays in health and disease.

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