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The Folding of Disulfide-Rich Proteins

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Abstract

The articles in this forum issue describe various aspects of the folding of disulfide-rich proteins. They include review articles using proteins such as bovine pancreatic trypsin inhibitor as models to highlight the range of folding pathways seen in disulfide-rich proteins, along with a detailed analysis of the methods used to study them. Following two comprehensive reviews on the methods and applications of protein folding, three original articles in this issue focus on two specific classes of disulfide-rich proteins that have applications in drug design and development, namely cyclotides and conotoxins. Cyclotides are head-to-tail cyclic and disulfide-rich proteins from plants and function as a defense against insect attack. Conotoxins are the disulfide-rich components of the venom of marine cone snails that is used to capture prey. These research articles report on factors that modulate protein folding pathways in these molecules and determine the outcomes of protein folding, that is, yield and heterogeneity of products. Finally, the issue concludes with a comprehensive review on a different type of disulfide bond, namely those that have a functional rather than structural role in proteins, with a particular focus on allosteric disulfide bonds that modify protein function. *Antioxid. Redox Signal*. 14, 61–64.

Introduction

Disulfide bonds play a unique and important role in stabilizing the structures of a diverse range of proteins. This forum issue presents a series of articles that examine how the final folded structures of disulfide-rich proteins are formed. The main emphasis of these articles is on the folding pathways, rather than on the final structures themselves. The general area of protein folding is of course a huge field (5, 15, 16, 21, 22) that has blossomed since the pioneering work of Anfinsen (3), which first suggested that the information necessary to define the final folded shape of a protein was encoded within its primary sequence. Our focus here is on disulfide-rich proteins, which, in addition to being subject to the conformational folding processes that occur in all proteins, are subject to the additional process of oxidative folding to correctly link pairs of cystine residues to form specific disulfide bonds. In a broad sense, conformational folding and oxidative folding can be regarded as analog and digital processes, respectively, with the former involving, in many cases, a continuum of intermediate states, whereas the latter is punctuated by more specifically definable intermediates, where individual disulfide bonds are either "on" or "off." The articles in this issue describe approaches to define these digital states and structures of the corresponding intermediates.

Principles and Methods

The issue commences with two forum review articles that provide an overview of the folding pathways of disulfide-rich proteins. In the first article by Arolas and Ventura (6), the protease inhibitor class of proteins is used to illustrate the principles of folding of disulfide-rich proteins. Protease inhibitors are an excellent choice to illustrate these principles, because these inhibitory proteins have a wide range of important biological functions, are generally small and hence tractable to study, are typically cross-linked by multiple disulfide bonds, and indeed are among the most widely studied classes of proteins from a folding perspective. Their natural functions range from regulatory roles for a variety of physiological processes, to host defense roles. The authors describe the great diversity of folding pathways that are observed in this family of proteins, with the pathways differing in the heterogeneity and native disulfide content of their intermediates. This diversity is nicely illustrated by the study of two extreme examples of the folding process, for bovine pancreatic trypsin inhibitor and hirudin, which fold *via* few native intermediates and through heterogeneous scrambled isomers, respectively. The authors also describe other protease inhibitors that fold through intermediate types of pathways, such as the leech carboxypeptidase inhibitor. The lessons learnt in these single-domain proteins is then extended to studies of

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two-domain protease inhibitors, with examples including cases in which the two domains fold autonomously, or other cases in which the folding is synergistic, with one domain assisting in the folding of the other.

The authors also describe several recent studies in which, for the first time, the structures of actual intermediates on the folding pathway have been determined. In most early studies of the folding of disulfide-rich proteins, there was little quantitative information available on the structures of these intermediates. Their structures were typically deduced from artificially mutated constructs, or predicted, rather than being experimentally determined to high resolution, but with experimental structures of actual intermediates now becoming available, folding pathways can be described much more precisely.

In the second article in this issue, Chang (9) uses two example proteins, bovine pancreatic trypsin inhibitor and tick anticoagulant protein, to describe methodological aspects of the study of oxidative protein folding. These methods are placed in context by first providing an overview of protein folding in general, including a description of conformational folding and oxidative folding. There are currently two favored models of conformational folding. The framework model proposes that secondary structures form first during the early stages of folding and this is followed by the packing of these preformed secondary structure motifs to form the native tertiary structure. In contrast, the hydrophobic collapse model involves a rapid hydrophobic collapse as the major driving force for folding, which is followed by conformational searching within a confined volume to reach the native structure. The article focuses mainly on methods to study disulfide-rich proteins and describes two distinct approaches: one in which a denaturant is used together with a reducing agent to provide the starting material for oxidative folding, and another involving a denaturant and thiol catalyst. The latter, referred to as the scrambled folding method, leads to a mixture of oxidized scrambled isomers that are amenable to fractionation, isolation, and further structural analysis. The folding pathways measured by either method are defined by the heterogeneity, structures, and kinetic properties of the intermediates identified. One of the main conclusions of the article is that one of the underlying causes of the diversity of folding pathways is the relative stability of protein subdomains.

New Experimental Results in the Folding of Cyclic Disulfide-Rich Proteins

Following these two introductory overview articles are a series of original research articles that examine various aspects of the folding of disulfide-rich proteins. The focus is on two specific classes of naturally occurring peptides in these studies, namely cyclotides and conotoxins. Cyclotides are plant-derived disulfide-rich miniproteins of about 30 amino acids in size that were discovered more than a decade ago (13) and have the distinguishing feature of a head-to-tail cyclized backbone and a cystine knot arrangement of their three conserved disulfide bonds. This makes them exceptionally stable (11), a feature that has led to suggestions of their use as templates in peptide-based drug design (12, 23, 24). In the cystine knot motif, two disulfide bonds and their connecting backbone segment form an embedded ring in the structure that is penetrated by the third disulfide bond. Such a topologically compact and complex motif makes them particularly interesting molecules that can be used to study oxidative folding to answer the question—how does the knot form (17)?

Cyclotides occur in a wide range of plants, including species from the Rubiaceae (coffee) and Violaceae (violet) families and are thought to play a role in plant defense, a suggestion consistent with the finding that they are potent insecticidal agents (8). However, our focus in this issue is not on their natural functions but on the way in which they achieve their final folded state. The reason for this focus is there is a great deal of interest in developing ways to chemically synthesize cyclotides so that they can be optimized for pharmaceutical or agricultural applications (14). To optimize their chemical synthesis, it is important to understand the folding pathways that lead to the cystine knot motif.

The team led by Ulf Göransson at Uppsala University describe in their article (1) new approaches to understand and optimize the oxidative folding of cyclotides. They report that the folding of selected cyclotides is heavily influenced by the concentration of redox reagents, with the folding rate and final yield of the native isomer greatly enhanced by high concentrations of glutathione. The addition of a hydrophobic solvent such as isopropanol to the buffer also accelerated the folding rates and, in some cases, altered the folding pathway. The hydrophobic solvent appears to help because cyclotides tend to have a patch of surface-exposed hydrophobic residues in their native structures. The elucidation of the factors that influence the folding of cyclotides should greatly facilitate chemical methods for producing native cyclotides or modified derivatives as stable peptide-based pharmaceutical or agricultural agents.

Conotoxins are disulfide-rich peptides from the venom of marine snails of the genus *Conus* (29). These snails use their venom for the capture of prey, which includes fish, worms, and other snails. Conotoxins typically target a wide range of receptors, including ion channels and transporters, and aside from their use in prey capture they have attracted the interest of pharmaceutical chemists as leads in drug design (2, 20, 25, 27). As with the cyclotides, there is interest in synthetically modifying conotoxins to exploit their potential in drug design. As they are disulfide-rich, the challenge has been to discover ways of properly folding them. Further, there has been interest in attempting to stabilize their structures, to improve their biopharmaceutical properties. Recent efforts have focused on developing methods for cyclizing conotoxins to make them more cyclotide-like, thus engendering them with high stabilities (10).

Two original research articles in this issue focus on the folding of cyclized derivatives of one particular conotoxin, namely AuIB. This molecule targets specific subtypes of the nicotinic acetylcholine receptor. Lovelace *et al.* (26) describe the role of linkers of four to seven amino acid residues in joining the N and C termini of AuIB to make cyclized derivatives. Peptide cyclization was achieved using an intramolecular version of a native chemical ligation reaction (19), as had earlier been applied to the synthesis of cyclotides (18, 28). The linker length strongly influenced the relative proportions of different disulfide isomers produced in the oxidative folding reaction. In a complementary study, Armishaw and colleagues (4) examined a range of cyclized AuIB derivatives that included shorter linkers and were again fo-

cused on folding outcomes. They found that the linker had an important influence on folding outcome, and in particular, shorter linkers favored the formation of a nonnative "ribbon" disulfide connectivity (Cys^{I}-Cys^{IV} and Cys^{II}-Cys^{III}), compared with the native, globular form (Cys^{I}-Cys^{III} and Cys^{II}-Cys^{IV}). Overall, these studies have provided new insights into the factors controlling the oxidative folding of cyclized conotoxin derivatives and new knowledge underpinning their applications in drug design.

Another Side of the Coin—A Functional Role for Disulfide Bonds

In the final article of this issue, the focus turns from the structural role of disulfide bonds to the functional role played by certain disulfide bonds. The two types of functional roles identified for disulfide bonds are as catalytic moieties or allosteric disulfides. Catalytic disulfides/dithiols transfer electrons between proteins, whereas allosteric disulfide bonds modulate the functions of the proteins in which they reside when they undergo a redox change. Azimi *et al.* (7) describe the five known examples of allosteric disulfide bonds and speculate on the existence of several others. They describe how these disulfide bonds control the activity of the respective proteins. A common feature of all of the allosteric bonds identified so far is that they link β-strands or β-loops.

Future Trends

Some of the important findings that have become apparent from the articles in this issue include an understanding of the factors affecting the diversity of folding pathways, a trend toward quantitative analysis of the structures of folding intermediates, the ability to modulate folding outcomes *via* cyclization reactions, and an increasing recognition of the importance of allosteric disulfide bonds in modulating protein function. The capability to determine the structures of individual disulfide-bonded intermediates has arisen because of improved biophysical modalities, including, in particular, nuclear magnetic resonance and mass spectrometry. It seems clear that such studies will continue to provide a stimulus for more precisely defining protein folding pathways. With this increased knowledge of folding pathways will come improved ways of designing disulfide-rich peptides for pharmaceutical applications. Cyclic disulfide-rich peptides appear to be particularly suitable targets for such studies, as illustrated by the examples of cyclotides and cyclized conotoxins reported in this issue. I thank all of the contributing authors for their timely and authoritative articles and hope that readers will find the issue informative and useful.

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