



Research review paper

Formation of disulfide bonds in proteins and peptides

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Available online 12 October 2004

Abstract

For many proteins and peptides, disulfide bridges are prerequisite for their proper biological function. Many commercialized proteins are crosslinked by disulfide bridges that increase their resistance to destructive effects of extreme environment used in industrial processes or protect protein-based therapeutics from rapid proteolytic degradation. Manufacturing of these products must take into account oxidative refolding—a formation of native disulfide bonds by specific pairs of cysteines located throughout a sequence of linear protein. This review describes basic and practical aspects of oxidative folding that should be considered while designing and optimizing manufacturing of proteins using chemical synthesis, semi-synthesis and a recombinant expression.

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Keywords: Oxidative folding; Disulfide bond

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1. Introduction

Proteins share a respectable, and expanding, marketplace in industrial processes and as pharmaceutical products. A vast majority of protein-based products, ranging from the smallest peptide-based therapeutics to large industrial enzymes, contain disulfide bridges (Table 1). The formation of protein disulfide bonds and their effects on protein stabilization encompasses basic scientific questions, such as “protein folding problem”, along with practical challenges of protein engineering, such as *de novo* introduction of disulfide bonds. In particular, these challenges include stabilizing proteins used for industrial applications and large-scale manufacturing of protein-based therapeutics. Understanding factors that determine formation of the native disulfide bonds (those found in a properly folded protein) will help in optimization of manufacturing processes.

2. Disulfide bonds and protein stability

Many proteins and peptides, such as enzymes, growth factors, hormones or toxins must function outside cells from which they were secreted. To be protected from often hostile extracellular environments, proteins evolved several “defense” mechanisms preventing them from denaturation and proteolytic degradation. Disulfide bonds and/or glycosylation serve this protective function. Disulfide bonds introduce conformational constraints to a polypeptide backbone, improving their thermodynamic stability. The improved stability can be translated in practical terms as a better resistance to environmental extremes such as high temperatures, very acidic or basic pH, high concentrations of organic solvents, or to increased half-life of protein therapeutics.

It is generally accepted that native disulfide bonds stabilize the properly folded conformation of proteins and/or destabilize denatured conformations by decreasing conformational entropy. However, despite many studies on engineering disulfide bonds in proteins (T4 lysozyme, RNases, subtilisin, dihydrofolate reductase), the mechanism by which disulfides affect stability is not well understood. The challenge of *de novo* introduction of disulfide bonds that stabilize proteins is reflected only by a handful of successful reports. For example, a significant thermal stabilization of thermolysin-like

Table 1
Examples of commercialized proteins and peptides containing disulfide bridges

Product	Structure (# amino acids, # disulfide bridges)	Application
Oxytocin	9 AA, 1 S-S	therapeutics: initiate labor
Insulin	51 AA, 3 S-S	therapeutics: diabetes
Papain	212 AA, 3 S-S	brewing and food processing
Pepsin	326 AA, 3 S-S	cheese production
Pectinase	335 AA, 4 S-S	fruit processing
α -Amylase	496 AA, 5 S-S	laundry detergents, alcohol production
Tissue plasminogen activator	527 AA, 17 S-S	therapeutics: neurodegeneration in stroke, cardioprotection

protease was achieved by engineering a new disulfide bond that reduced local partial unfolding and minimized autolysis (Mansfeld et al., 1997).

3. Mechanism of disulfide bond formation

The mechanism of disulfide bond formation in proteins involves a series of thiol/disulfide exchange reactions between cysteine thiols and an oxidizing disulfide. The chemistry of disulfide bond formation is identical whether the reaction occurs in small peptides or in larger proteins. Oxidation of protein cysteine thiols (deprotonated thiol –SH groups) can be mediated by either specialized enzymes, such as protein disulfide isomerase (PDI), low molecular weight disulfide reagents, such as cystine, cystamine, oxidized glutathione or dithiothreitol, dithiobis(2-nitrobenzoic acid), or by other electron-accepting reagents such as oxygen (air), iodine, dimethyl sulfoxide, diamide and potassium ferricyanide. Whereas thiol/disulfide redox buffers are more applicable to larger polypeptides, cysteine-rich peptides can be oxidized by all types of organic and inorganic reagents.

There are two important aspects of forming native disulfide bonds in proteins: (1) chemistry of thiol/sulfide exchange and (2) the kinetics and thermodynamics of oxidative folding (Creighton, 1997). Thiol/disulfide exchange is a two electron oxidation reaction, where oxidizing equivalents are transferred from a disulfide reagent to cysteine thiols. As illustrated in Fig. 1, formation of a single protein disulfide bond is a two-step, reversible process and involves two consecutive thiol/disulfide exchange reactions. In the first step, a low molecular weight disulfide is directly attacked by a nucleophilic protein thiolate, resulting in the formation of a mixed disulfide species. The first step is primarily determined by a reactivity of the cysteine thiolate. In the second step, the mixed disulfide

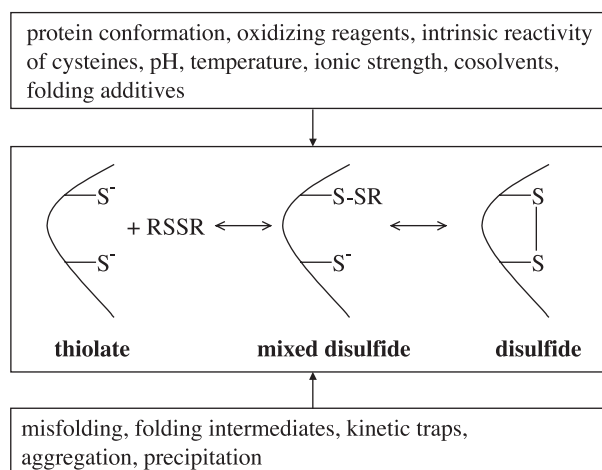


Fig. 1. Mechanism of protein disulfide bond formation. Key factors affecting this reaction and potential by-products are summarized.

is broken by another nucleophilic attack from a second protein thiolate. As a result, a low-molecular weight thiol (leaving group) is replaced by a cysteine thiolate and a protein disulfide bond is formed. The second step is thermodynamically coupled to the conformational properties of a protein. By judicious application of appropriate redox buffers, oxidative folding can be under kinetic control of either the reactivity of the Cys thiols (formation of the mixed disulfide) or by the conformational properties of a polypeptide (formation of the protein disulfide bond).

Thermodynamic stability and conformational tendencies of a polypeptide to form native disulfide bonds determine how much of the properly folded species accumulate in the equilibrium relative to the “non-native” folding products. For a protein containing four cysteines, there are three possible oxidation products with different pairing of disulfides. For six-cysteine polypeptide, the number of possible connectivities increases to 15. There are many documented instances where the correctly folded species is a major oxidation product, but many reports also describe very poor folding yields for other proteins and peptides. As summarized in Fig. 1, poor folding yields may result not only from accumulation of non-native (misfolded) folding species, but may also occur due to accumulation of folding intermediates or kinetic traps. Since these by-products often have an increased non-polar surface area exposed to solvent, they exhibit a higher tendency for aggregation and precipitation.

During formation of disulfide bonds in proteins, the second nucleophilic attack may come from the intramolecular thiolates (within the same molecule), or from another molecule (intermolecular thiolates). This competition between formation of the intramolecular bond (ultimately leading to the native disulfide) and intermolecular disulfide bond (resulting in formation of oligomeric by-products) is governed by “effective” concentrations of the cysteine pairs and total protein concentration in the folding solution. Since effective concentrations of cysteines in unfolded, reduced proteins typically range from 0.001 to 0.1 M, this imposes a requirement of using very dilute concentrations of proteins during the oxidative folding reaction.

3.1. Practical considerations for optimizing oxidative folding

There are two alternative strategies in oxidative folding, regioselective formation of disulfide bonds and direct oxidation (Moroder et al., 1996). The first strategy is more appropriate for smaller peptides, typically containing up to three disulfide bonds, since it requires orthogonal protection of cysteines commonly used in chemical peptide synthesis (Annis et al., 1997). The second strategy, simultaneous oxidation of all cysteines (called direct oxidative folding), is applicable to both smaller peptides and larger proteins. There are many intrinsic and external factors that determine formation of native disulfides and thus influence oxidative folding yields (Fig. 1). Conformational tendency of a polypeptide chain to form native disulfide bonds is arguably the most important factor determining folding yields. Oxidative folding of marginally stable proteins and peptides will almost certainly result in poor folding yields. Thus, all factors that may stabilize the native conformation will likely translate to improved oxidative folding yields.

Optimization of oxidative folding is very empirical, since each protein and peptide has unique optimal folding conditions. The matrix screening approach (simultaneous

screening of variations of two or three factors) for individual proteins is likely to result in improved folding yields. Several key factors should be considered while screening folding conditions:

- kinetics (sometimes continuing folding reaction until reaching equilibrium is not advantageous, since a partially folded protein may precipitate at a faster rate than the rate of forming the native protein);
- redox system (testing a broad range of ratios of the reduced and oxidized equivalents, as well as different redox buffers with varying chemical properties);
- temperature, pH, ionic strength (varying these conditions allows for “tuning-in” folding reaction);
- folding additives (these should be screened “randomly” from an assortment of detergents, osmolytes, small molecules such as chaotropic agents and organic cosolvents, using fixed concentrations and upon identification of the most effective folding enhancer, concentrations should be optimized).

Reactivity of individual cysteine thiols can influence folding kinetics and thermodynamics and may be used to manipulate oxidative folding reactions. Key external factors affecting cysteine reactivity are pH and ionic strength. Interestingly, the presence of charged residues adjacent to cysteine residues in the protein primary sequence may significantly change their reactivity due to electrostatic effects that affect the ionization and nucleophilic properties of thiolates (Bulaj et al., 1998). This phenomenon was suggested as a mechanism of manipulating thiol/disulfide exchange kinetics in proteins produced in recombinant systems, where manipulation of the oxidizing environment is somewhat limited.

4. Technological advances

Despite a relatively slow technological progress in improving oxidative folding, some novel successful approaches are worth exploring while designing and optimizing manufacturing of disulfide-rich proteins and peptides. First, use of less common oxidation strategies, such as polymer-supported disulfide reagents (e.g. Clear-Ox™ or tris(2-mercaptoacetamidoethyl)amine on styrene-methacrylate microspheres), fast-acting trans-dichlorotetracyanoplatinate (IV) or protein-disulfide isomerase mimetic *trans*-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC) may prove superior in improving folding yields, but more comparative studies must be performed to prove their effectiveness over traditional methods (Woycechowsky and Raines, 2000). Second, oxidative refolding chromatography with immobilized key cellular components was found very effective in producing a 66-residue scorpion toxin with four disulfide bridges that previously could not be folded using standard approaches. Cell-free protein synthesis appears advantageous for toxic proteins that cannot be expressed in host cells and was also shown to be applicable to proteins containing disulfide bonds. However, more technological progress is needed to scale-up production capabilities beyond a laboratory scale. With respect to recombinant expression, significant progress was reported in overexpression of folding catalysts, such

as the protein disulfide isomerase DsbC (Bessette et al., 1999). Overexpression of Ero1P, an enzyme that regenerates PDI, and manipulation of the *in vivo* redox state should be further explored to find out if cellular engineering can additionally boost oxidative folding in host cells (Tu and Weissman, 2004). In summary, technological improvements in recombinant and chemical production of commercialized proteins and peptides are recognized as key factors in reduction of manufacturing costs. There is a continuing need for new oxidative folding technologies that will rapidly produce disulfide-rich proteins in high yields.

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