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Research review paper

Disulfide bond formation and its impact on the biological activity and stability of recombinant therapeutic proteins produced by *Escherichia coli* expression system

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ABSTRACT

Therapeutic proteins require correct disulfide bond formation for biological activity and stability. This makes their manufacturing and storage inherently challenging since disulfide bonds can be aberrantly formed and/or undergo significant structural changes. In this paper the mechanisms of disulfide bond formation and scrambling are reviewed, with a focus on their impact on the biological activity and storage stability of recombinant proteins. After assessing the research progress in detecting disulfide bond scrambling, strategies for preventing this phenomenon are proposed.

Contents

1. Introduction

Prior to the advent of modern biotechnology, medical applications of protein biopharmaceuticals were restricted due to their limited availability from natural sources such as live animal tissues. With the development of recombinant DNA technology, therapeutic proteins have entered a new era with Eli Lilly's recombinant human insulin (Humulin, 1982) being the first officially approved recombinant pharmaceutical by the Food and Drug Administration, USA. Currently about 150 therapeutic proteins have been approved in the USA and EU, demonstrating their importance regarding the diagnosis and treatment of various diseases such as cancers, autoimmune diseases, and metabolic disorders (Walsh, 2003). Considering the number of projects in the pipelines as well as continued research advances, the global biopharmaceutical market is growing at an average annual rate of 10.3% and the revenues are projected to reach $92 billion in 2011 (www.pharmtech.com). Currently, more than 500 protein drug candidates are being evaluated in clinical trials, and advanced biotechnologies such as genomics, proteomics and high-throughput screening are facilitating new drug discovery (Walsh, 2006).

For many of these therapeutic proteins, the formation of correct disulfide bond is essential for their biologically active three-

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dimensional (3D) conformation (Guo et al., 2004; Hua et al., 2006). The formation of incorrect disulfide bonds can result in protein misfolding, aggregation and malfunction (Dangoria et al., 2002; Niwa et al., 2007; Wang et al., 2009). Therefore, the biological activity of therapeutic proteins can be significantly affected by formation of incorrect disulfide bonds. This issue is particularly significant for the *Escherichia coli* expression system, since it solely possesses the intracellular machinery for this post-translational modification of the expressed proteins in the periplasm. Although this problem is much less worried when yeast and mammalian cells are used as the production systems since they routinely modulate the disulfide bond formation in their endoplasmic reticulum (ER) during gene expression, incorrect disulfide bond formation might still occur and worsen with the increased number of cysteines in therapeutic proteins. On the other hand, the in vivo expressed protein intermediates or final products may undergo aberrant disulfide bond formation and/or disulfide bond scrambling during downstream processing and storage via a series of chemical reactions (Trivedi et al., 2009). In the ensuing sections the mechanisms of disulfide bond formation and scrambling are reviewed and strategies for facilitating the correct formation of a disulfide bond and preventing its scrambling are suggested.

2. Disulfide bond chemistry

2.1. Mechanism of disulfide bond formation

In the past two decades, the mechanism of disulfide bond formation in vivo has been intensively studied in both prokaryotic and eukaryotic systems. In common, the formation of protein disulfide bond is the result of a series of thiol/disulfide exchange reactions between cysteine thiolate (S^-) and oxidizing disulfide bond (S–S) through the nucleophilic attack mechanism that are catalyzed by cellular enzymes. There are three kinds of chemical reactions involved: oxidation, reduction, and isomerization, which are illustrated in Fig. 1. By reacting with the oxidase (Fig. 1A), a disulfide bond is introduced into the protein substrate. Similarly, a disulfide bond formed in the protein can be reduced by the reductase (Fig. 1B). The rearrangement of disulfide bond within a protein molecule can occur through two different mechanisms: a two-step process with a reduction reaction first (Fig. 1B) followed by an oxidation reaction (Fig. 1A) or a single-step isomerization process (Fig. 1C). The two-step mechanism involves two oxidoreductase catalyzed reactions: a reduction of a disulfide bond to two thiols and a catalyzed oxidation of thiols to form new disulfide bonds. The single-step mechanism involves only an isomerase, which isomerizes a disulfide bond to form the mixed disulfide bond. Depending on the conformation of the protein molecule, the mixed disulfide bond may react with an adjacent cysteine residue, resulting in the formation of a new disulfide bond.

Particular cellular enzymes have developed in *E. coli* and eukaryotes for catalyzing the reactions depicted above. In the periplasm of *E. coli*, a set of Dsb proteins (DsbA, DsbB, DsbC, DsbD, and DsbG) synergistically catalyze disulfide bond formation in translocated proteins. DsbA is the primary oxidase that introduces disulfide bonds to protein substrates. DsbC and DsbG function as reductase and isomerase that reduce/unscramble incorrectly formed disulfide bonds in substrates. Their functions are determined and efficiently maintained by their respective redox partners, cytoplasmic membrane proteins DsbB and DsbD (DsbA/DsbB, DsbC/DsbD, and DsbG/DsbD) through elaborate electron transfer pathways (Messens and Collet, 2006). In the ER of eukaryotic cells, the best characterized disulfide promoting enzyme is the protein disulfide isomerase (PDI), a versatile enzyme that has oxidase, reductase and isomerase activity. As is the case of DsbA/DsbB in *E. coli*, a conserved ER membrane associated protein Ero1p (ER-oxidoreductin 1) specifically oxidizes PDI through a flavin adenine dinucleotide (FAD)-dependent pathway with molecular oxygen as the terminal electron acceptor. Therefore,

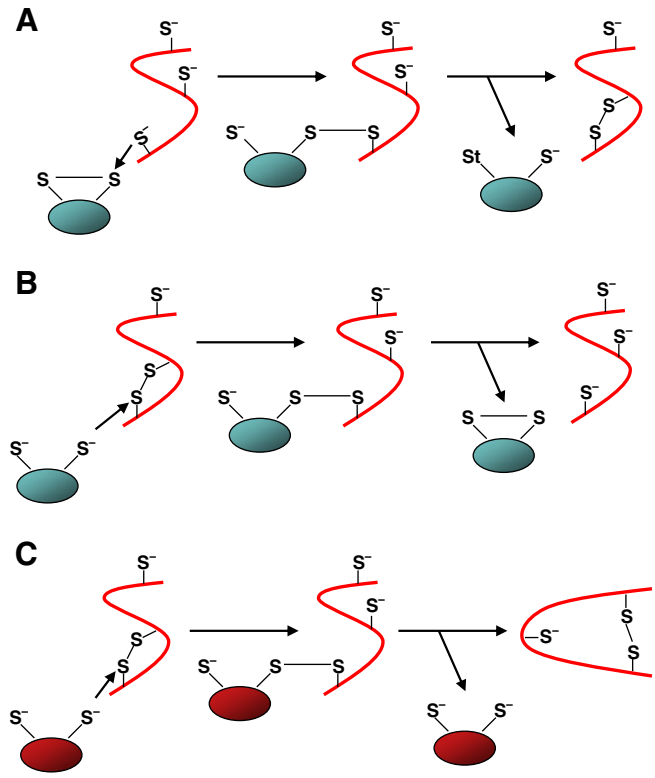

Fig. 1. Disulfide bond reactions in vivo catalyzed by oxidoreductase and isomerase. (A) Oxidation, (B) Reduction, and (C) Isomerization. The blue and red ovals represent the oxidoreductase and isomerase enzymes, respectively.

PDI is found predominantly in an oxidized form in the ER. In parallel, reduction of oxidized PDIs can be achieved by the major ER redox buffer glutathione. Consequently, PDI in a reduced form functions as reductase and isomerase. In addition, numerous PDI homologues have been identified that might modulate those disulfide bond formation reactions. They are probably kept in reduced state due to the high specificity of Ero1p for PDI, and thus catalyze reduction and isomerization of disulfide bond (Tu and Weissman, 2004).

In vitro, disulfide bond can form simply via oxidation in the presence of electron-accepting reagents, such as atmospheric oxygen, iodine, dimethyl sulfoxide, diamide and potassium ferricyanide (Bulaj, 2005). To make this process controllable, redox buffers containing low molecular weight thiol/disulfide chemical reagents are introduced. Conventionally, reduced and oxidized forms of glutathione (GSH/GSSG) (Shioi et al., 2004), dithiothreitol (DTTre/DTTox) (Wang et al., 2010), and cysteine (cysteine/cystamine) (Bouvier et al., 2003) are used to adjust the redox potential. In the meantime, novel redox reagents have been developed with advantageous properties. For example, ortho, para, and meta-substituted aromatic thiols accelerate disulfide-coupled protein folding (Gough et al., 2006). Selenoglutathione (GSeSeG), an analog of glutathione that contains a diselenide bond in place of the natural disulfide, increases the rate of folding and extends the working pH to more acidic conditions (e.g., pH 5) for oxidative folding of protein in vitro (Beld et al., 2008).

A critical factor in the thiol/disulfide exchange reactions is the pH of solutions. Thiols are reactive only in their thiolate anion form (S^-) but not in their corresponding protonated form (SH). Owing to their pKa values (pKa = 8 to 9), they are most reactive under alkaline conditions. However, a decrease of pKa of thiols in proteins can occur under the influence of the surrounding amino acid residues.

Therefore, thiol/disulfide exchange reactions can occur in vivo at physiological or even lower pH values. Thus, protein products may undergo progressively slow thiol/disulfide exchange reactions during storage. The rate-limiting step of thiol/disulfide exchange reactions is usually the nucleophilic attack of thiolate anion on the sulfur atom of the disulfide bond, since it takes place only when, depending on the protein conformation, a thiolate and a disulfide bond come in a close proximity. Since the burial of reactive groups in the protective tertiary structure of a protein can inhibit thiol/disulfide exchange reactions, protein destabilizing conditions will aggravate these reactions. Turning these observations into practice, a methodology for the efficient rearrangement of disulfide bonds in proteins needs to be developed in alkaline buffers in the presence of chaotropic reagents.

2.2. Alteration of disulfide bonds

The disulfide bond exchange within proteins may even occur in the absence of an exogenous thiol/disulfide reagent, since the free cysteine thiols of proteins are able to alter existing disulfide bonds, resulting in disulfide bond exchange (Wedemeyer et al., 2000). This phenomenon is known as disulfide scrambling that can take place intra- and intermolecularly, leading to protein conformational changes, which are illustrated in Fig. 2. Scrambling tends to be exacerbated under protein destabilizing conditions, resulting in the misinterpretation of analysis that are performed under denaturing conditions, like sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It has been reported that in monoclonal antibodies IgG1 and IgG4, lower molecular weight artifacts in their non-reducing SDS-PAGEs were caused by the breakage of inter-chain disulfide bonds during SDS-PAGE sample preparation through disulfide scrambling (Liu et al., 2007; Taylor et al., 2006). Recently, in the structural monitoring study of recombinant extracellular domain of human CD83 (hCD83ext), a potential protein drug under pre-clinical trial to treat autoimmune disorders, disulfide scrambling was demonstrated to occur during SDS-PAGE sample preparation (Zhang et al., 2010a). As a result, not only the disulfide bridged dimers disappeared, but also the intramolecular disulfide bond pattern was altered.

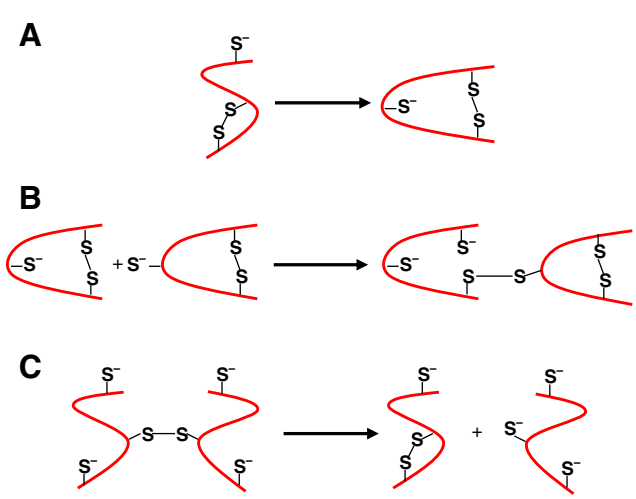

Fig. 2. Disulfide bond scrambling. (A) thiol/disulfide exchange reaction occurs intramolecularly, resulting in a different intramolecular disulfide bond. (B) thiol/disulfide exchange reaction occurring intermolecularly, resulting in the formation of an intermolecular disulfide bond, (C) an intermolecular disulfide bond being broken by a thiol group via thiol/disulfide exchange reaction.

In alkaline solutions, disulfide bond may undergo irreversible β-elimination reactions as shown in Eqs. (1) and (2) (Kosen, 1992):

$$R_1CH_2SSCH_2R_2 + OH^- \rightleftharpoons R_1CH_2SS^- + CH_2{=}CR_2 + H_2O \quad (1)$$

$$R_1CH_2SS^- + OH^- \rightleftharpoons R_1CH_2SO^- + HS^- \quad (2)$$

As a result, the disulfide bond is cleaved concomitantly with the formation of dehydroalanine and persulfide. The reaction is known as β-elimination. Following electrophilic addition reaction, the ε-amino group of lysine or the thiol of cysteine can be inserted into the double bond of dehydroalanine, yielding lysinoalanyl or lanthionyl cross-links, respectively. Persulfide may further react with hydroxide to release hydrosulfide that opens the chance for disulfide bond scrambling. The β-elimination is recognized as a major disulfide associated degradation of protein. It develops very rapidly at 90–100 °C in the pH range from 6 to 8, and at ambient temperature in extremely alkaline solutions (Kosen, 1992). In addition, disulfide bond scrambling can occur under strongly acidic conditions, where a sulfur atom in a disulfide bond is protonated to become a sulfenium cation, which subsequently carries out an electrophilic attack on a sulfur atom of a different disulfide bond, resulting in disulfide bond exchange (Benesch and Benesch, 1958).

3. Effects of disulfide bonds on the biological activity of proteins

Formation of correct disulfide bonds is indispensable for the biological activity in many proteins; for example, human insulin requires correct formation of both intra- and interchain disulfide bonds to function properly (Qiao et al., 2003). There are a number of roles that disulfide bond may play in a biologically active conformation. First, disulfide bond may be the part of active sites, which is typical for oxidoreductases, such as Dsb proteins and PDI that catalyze reduction, oxidation or isomerization in proteins. Secondly, disulfide bond regulates protein function in an allosteric manner through its breakage and/or formation, which, in turn, can cause protein conformational change. For example, the formation of an intermolecular disulfide bond linked dimer must be induced to activate the natriuretic peptide receptor-A (Labrecque et al., 1999). Statistically, 7% of all structurally defined disulfide bonds in Protein Data Bank (PDB) are possibly allosteric bonds (Schmidt et al., 2006). Thirdly, certain proteins do not fold into their native conformations until nearly all of their native disulfide bonds have been formed (Mamathambika and Bardwell, 2008) A few examples comprise bovine pancreatic trypsin inhibitor (BPTI) (Kibria and Lees, 2008), bovine pancreatic ribonuclease A (RNase A) (Scheraga et al., 2001), hirudin (Thannhauser et al., 1997), porcine insulin (Qiao et al., 2001), human proinsulin (Qiao et al., 2003) and leech carboxypeptidase inhibitor (Arolas et al., 2009). Therefore, the folding pathway of these proteins can be studied in detail by employing the disulfide bond as a probe, by which their folding is coupled with disulfide bond formation and reshuffling, and substantial disulfide-containing folding intermediates can be harvested for structural analysis. Fourthly, disulfide bond can prevent or alleviate unfolding, degradation or aggregation of a biologically active conformation. The interchain disulfide bond in IgG1 antibodies, for example, significantly reduces their propensity for aggregation in comparison to IgG2 antibodies that share 95% sequence identity but lack disulfide bond formation (Franey et al., 2010). Apparently, the scrambling of disulfide bonds of IgG1 antibodies and other proteins leads to the loss of biological activity.

4. Effects of disulfide bonds on the stability of proteins

Owing to their covalent nature, disulfide bonds are generally believed to stabilize proteins by lowering their conformational entropy compared with their unfolded state, which makes de novo introduction

of disulfide bond a rational approach to engineer additional conformational stability into proteins (Bulaj, 2005). However, success is limited due to the lack of fine rules for selecting appropriate places for disulfide bonds. To date, only a few correlations between disulfide bond and protein stability have been explored. For example, disulfide bonds that close long loops are reportedly more efficient for stabilizing proteins than those closing short loops (Abkevich and Shakhnovich, 2000). In addition to loops (Schirra et al., 2010) they are known to stabilize β-sheets (Venkatraman et al., 2002) and α-helices (Kibria and Lees, 2008) in the correct conformation of protein. But their effect on turn structures (e.g. β-turn or hairpins) depends on the amino acid content of the turn (Santiveri et al., 2008). While the introduction of intermolecular or interchain disulfide bonds often leads to an enhanced stability, the impact of the introduction of intramolecular disulfide bonds is rather unpredictable, either stabilizing, no effect, or destabilizing (Chakraborty et al., 2005; Janssen et al., 1999; Marshall et al., 2003; Pecher and Arnold, 2009). The underlying reason might be associated with general observations that intermolecular disulfide bonds mainly strengthen conformational rigidity, whereas intramolecular ones could disturb the native disulfide bond formation via thiol/disulfide exchange reactions, which results in scrambled disulfide bonding and non-native conformations (Hsu et al., 2006). Currently, manipulation of protein disulfide bonds can be facilitated by bioinformatics tools, such as molecular modeling and dynamic simulation, which can predict alterations on protein structure and energetics and help the determination of manipulation targets (Lu and Liu, 2008; Wei et al., 2010).

Mutating cysteine residues to prevent the formation of unwanted disulfide bonds has been practiced less often. Part of the reason is the lack of knowledge on correct disulfide bond pattern and the effects of disulfide bond and free cysteine residue on protein structure, folding, stability and function. Therefore, the critical impact of the pattern (or position) of disulfide bond on the protein stability is highlighted, suggesting that correct disulfide bond may stabilize proteins, whereas its scrambling may result in adverse effects. This provides an interpretation of the observations made in several recombinant therapeutic proteins, where the storage stability is significantly enhanced by mutating one cysteine residue, for example, in human CD83 (Zhang et al., 2011), two cysteines in keratinocyte growth factor (Hsu et al., 2006), and three cysteines in human fibroblast growth factor 1 (Culajay et al., 2000) since these cysteines possibly trigger disulfide bond scrambling.

Usually the stability of proteins has been studied by characterizing their thermodynamic properties, such as the Gibbs free energy of the unfolding process and melting temperature, which might not be enough for therapeutic protein products. Since these products often undergo extensive storage before administration, consequently their storage stability or shelf life, which might differ from their thermostability, needs to be clearly addressed. Hence, the investigation of the effect of scrambled disulfide bond on the storage stability of therapeutic proteins is proposed. Accelerated storage stability study can be established by incubating protein products at a physiological temperature, during which samples taken at different time intervals are subjected to biological activity assays and structural analyses for disulfide bond scrambling.

5. Detection of disulfide bond formation and scrambling

The analytical advances regarding disulfide bond detection mostly arise from the study of disulfide-coupled oxidative protein folding, which is usually initiated by introducing thiol/disulfide reagents to partially or fully reduced and denatured protein samples. Mixed disulfide bonds are then formed between protein molecules and reagents. Followed by numerous thiol/disulfide exchange reactions, substantial disulfide bonded folding intermediates are trapped, isolated and characterized to elucidate disulfide-coupled folding pathways. Thiol blocking reagents, iodoacetic acid (IAA), iodoacetamide (IAM), N-ethylmaleimide (NEM), and 4-vinylpyridine (4-VP), are conventionally used to trap disulfide-containing intermediates. For the separation of trapped intermediates, reversed-phase high performance liquid chromatography (HPLC) is most frequently employed due to its high sensitivity to protein structural changes and acidic running conditions that minimize disulfide bond scrambling. However, not all intermediates can be separated effectively and the separation becomes problematic as the number of disulfide bonds increases. This could be solved by manipulating thiol blocking reagents. Intermediates blocked with high-molecular-weight reagents such as polyethylene glycol (PEG)-maleimide (molecular weight ranging from 2000 to 50,000 Da), biotinylated PEG-maleimide and aminomethylthiosulfonate which derivatize free protein thiols to positively charged 2-aminoethanethiol groups can be separated by SDS-PAGE, avidin–biotin affinity columns and ion exchange chromatography, respectively. In addition, the other analytical techniques, such as 2-D gel electrophoresis and capillary electrophoresis, can also be employed to separate the trapped intermediates.

The characterization of folding intermediates usually starts with measurement of the number of free thiol groups. A conventional method, known as the Ellman assay, utilizes the absorbance of 5-thio-2nitrobenzoic anion (TNB^{2-}) at 412 nm produced by the reaction of 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) and protein thiolate anions. A derivative of DTNB, n-octyl-5-dithio-2-nitrobenzoic acid (ODTNB) is developed with improved accuracy of the Ellman assay, thanks to its increased reactivity with buried thiols (Faulstich et al., 1993). Meanwhile, more reagents have been derived for the quantification of free thiols. Biotin-tagged IAM (BIAM) and NEM (Bt-NEM) directly label thiols and the loss of the biotin signal, measured by standard immunoblotting-type protocols using streptavidin-conjugated horseradish peroxidase (HRP), is proportional to the degree of thiol modification (Hill et al., 2009). Fluorescence-based reagents are appreciated due to their much higher sensitivity. ThilGlo® reagents, maleimide derivatives of naphthopyranone fluorophores (Trivedi et al., 2009) and N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl (BODIPY) labeled thiol blocking reagents (BODIPY-labeled IAM and NEM) are employed to quantify reactive thiols by fluorescence intensity measurement and imaging, respectively (Hill et al., 2009).

Disulfide linkages of the intermediates are usually explored by peptide mapping relying on chemical/enzymatic digestion followed by HPLC fractionation. Traditionally disulfide assignment for each fraction is achieved by Edman sequencing. Obviously, this approach is very time-consuming and a large protein sample is needed. With the advent of desorption/ionization techniques, it is possible to analyze large biomolecules using mass spectrometry (MS), such as fast atom bombardment (FAB), electrospray (ESI), and matrix-assisted laser desorption ionization (MALDI), which provide a rapid and sensitive approach for determination of disulfide linkages. A number of proteases are commercially available for protein digestion for MS analysis and mass values of fragments can be easily calculated using commercially available programs. However, incomplete digestion is often the result of inaccessible cleavage sites which are buried in partially folded conformations of proteins. Therefore, ambiguous results of disulfide linkages are likely to arise. Furthermore, cyanylation (CN), a specific chemical reaction at thiol groups has been employed to simplify the assignment. CN-induced cleavage has been developed by using cyanylating reagents, such as 2-nitro-5-thiocyanobenzoic acid (NTCB) and 1-cyano-4-dimethylamino-pyridinium (CDAP), to selectively cyanylate free cysteine thiol groups. Cyanylation is followed by specific chemical cleavage at the N-terminal of modified cysteine residues under alkaline conditions (Gallegos-Perez et al., 2005). If thiols are engaged in disulfide bonds, partial reduction by Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) has been employed under acidic conditions prior to cyanylation (Wu and Watson, 1997). In this method, more unfolded protein conformations can be obtained than that in proteolysis by increasing the concentration of denaturants for more complete cleavage.

Apparently, the aforementioned disulfide mapping methodologies result in irreversible destruction of protein samples without obtaining 3D structural information surrounding the disulfide bonds. The specific spatial position of a disulfide bond can be identified in high-resolution 3D protein structure by X-ray crystallography and nuclear magnetic resonance (NMR). However, these processes are time-consuming and resource intensive. Recently, two non-invasive NMR methods have been specifically advanced for disulfide assignment without the need to solve the high-resolution structure or destruct protein conformation (Mobli and King, 2010). One is based on the detection of the nuclear overhauser effect (NOE) between the side chain β-methylene protons of two cysteine residues (i.e. inter-residue ^{1}H–^{1}H) using 2D ^{1}H–^{1}H NOE spectroscopy (NOESY) experiment, because NOE can be observed between two nuclei at a distance of ~5.5 Å that well covers the average distance between two C_{β} atoms of two cysteines in a disulfide bond (~4 Å) (Dombkowski and Crippen, 2000). In case the inter- and intra-residue NOEs are difficult to differentiate, then a 3D ^{13}C-edited HSQC-NOESY experiment that additionally measures the resonance frequency of $^{13}C_{\beta}$ needs to be performed. However, the accuracy of this method can be severely affected by the intervention of close cysteine residues. Another method is to measure a scalar coupling across a disulfide bond, which is an unambiguous way to determine disulfide linkages. Replacing cysteine residues with selenocysteines (Sec) significantly facilitates this measurement as the selenium isotope (^{77}Se) has more favorable NMR properties than the sulfur isotope (^{33}S) (Mobli and King, 2010). The connectivity between two ^{77}Se residues can be directly identified in correlation spectroscopy (COSY) experiment based on the large scalar coupling of the diselenide bond, and the assignment of ^{77}Se can be further achieved through a 2D ^{1}H-^{77}Se heteronuclear multiple quantum coherence (HMQC) experiment.

6. Strategies for correct disulfide bond formation and prevention of its scrambling

6.1. Correct disulfide bond formation

The periplasm of *E. coli* is a favorable intracellular compartment to express proteins with disulfide bonds because it is an oxidative environment and possesses the Dsb proteins for catalyzing the in vivo formation of disulfide bonds (Messens and Collet, 2006). This performance appears to be further enhanced by the overexpression of the Dsb proteins, through which several disulfide-containing therapeutic proteins, such as t-PA (Qiu et al., 1998), leptin (Jeong and Lee, 2000) and single-chain Fv antibody fragment (Sandee et al., 2005), are successfully expressed in a soluble and functional form. Extracellular secretion provides an alternative means for disulfide bond formation with extra advantage in simplifying downstream product purification. However, its production yield is much lower than that by cytoplasmic expression due to the inefficient cellular machinery for translocation and export, even though a multitude of strategies have been developed to alleviate this intracellular limitation (Choi and Lee, 2004). Consequently, several *E. coli* expression systems enabling the formation of disulfide bonds in the cytoplasm have been developed (Bessette et al., 1999) and several strains, including Origami (Novagen; with two mutations of the trxB gene encoding thioredoxin reductase and the gor gene encoding glutathione reductase) and SHuffle (New England Biolab; similar to Origami but with an overexpression of DsbC), are commercially available. While functional expression has been achieved in Fab antibody fragment (Levy et al., 2001), tissue plasminogen activator (t-PA) (Bessette et al., 1999), and lipase B (Larsen et al., 2008), negative results have been demonstrated while expressing 11-β-hydrooxysteroid dehydrogenase (Walker et al., 2001) and hCD83ext (Zhang et al., 2010b) because of aberrant disulfide bond formation. Therefore, the success of this approach can be severely limited by the lack of knowledge on disulfide bond formation and pattern in proteins.

Owing to the relatively high yield and streamlined product recovery, cytoplasmic inclusion body formation followed by refolding is still the most popular industrial process when *E. coli* is used as the host for expression (Schmidt, 2004). During refolding, rearrangement of disulfide bond in vitro is frequently entailed to achieve a desirable disulfide bond pattern. It starts with denatured and reduced proteins, followed by exposing proteins to redox reagents. Theoretically, mixed disulfide bonds are first formed between proteins and the redox reagents. Then the ionized thiols present in proteins will initiate a series of thiol/disulfide exchange reactions with the mixed disulfide bonds to attain correct disulfide bond patterns. Using this strategy, a number of disulfide-containing therapeutic proteins have been successfully renatured. Optimization of this process is based on experimentally testing key factors, including the redox system, temperature, pH, ionic strength and folding additives (Bulaj, 2005). For the redox system, the redox potential of the protein solution can be precisely controlled through the concentrations of the reduced and oxidized forms of the redox reagent together with their ratio to the protein thiols. Therefore, evaluation of a broad range of redox reagents, their concentrations, and their ratios to free thiols in protein need to be established. In addition, Dsb proteins and PDIs from different sources have been exploited, either in a free or an immobilized form (Altamirano et al., 1999), to promote the correct formation of disulfide bonds in vitro (Vinci et al., 2000; Winter et al., 2002), particularly in cell-free systems for protein synthesis (Yin and Swartz, 2004). Also, the inclusion of redox reagents in downstream processing is anticipated to be helpful (Chadwick et al., 1995).

6.2. Prevention of disulfide bond scrambling

Disulfide bonds are susceptible to scrambling in the presence of free cysteine thiols even under mild conditions. As a consequence, the conformation and biological activity of proteins might be altered. Based on the aforementioned mechanism of thiol/disulfide exchange reactions, the pH values of buffers used in the downstream processing are preferred to be slightly acidic or neutral, if disulfide bond rearrangement is suspected and undesirable. Likewise, slightly acidic formulation (e.g., ~pH 6.5) would be beneficial for the stability of proteins. In case the free thiols are inessential for structure and function, more aggressive strategies may be applied to prevent disulfide bonds from scrambling. Derivatization of free thiols to unreactive alkylate forms with alkylating reagents, such as IAA, IAM, NEM, PEG-malemide, PEG-IAM, PEG-acrylamide, PEG-acrylate, PEG-vinyl sulfone and PEG-epoxide, is one of them, through which the protein stability can be maintained (Trivedi et al., 2009). Most importantly, the specific reaction of PEG derivatives with cysteine thiols provides a site-specific PEGylation approach to effectively enhance the half-lives and biological activities of therapeutic proteins and decrease their immunogenicity without loss of specific activities (Hao et al., 2006; Slavica et al., 2007). In addition to chemical derivatization, mutation of cysteines to alternative amino acid residues is frequently practiced. However, the two aforementioned strategies are often limited by insufficient information on protein structure to determine legitimate targets of manipulation, particularly for proteins with unknown 3D structure and disulfide bond pattern. For example, the recombinant hCD83ext has five cysteine residues with unidentified disulfide bond pattern. The comparison with its homologous protein, mouse CD83ext, provides no sign of structural role of any of those five cysteine residues since they share identical cysteine pattern (Lechmann et al., 2005). Therefore, a broadened consensus sequence driven strategy, conserved domain search, was adopted, in which non-conserved cysteines are identified as legitimate targets for mutagenesis (Zhang et al., 2011). As a result, the stability and biological activity of hCD83ext was improved (Zhang et al., 2010a, 2011).

7. Perspectives

As a vigorously growing industry, biopharmaceutical manufacturing has mushroomed from laboratory-scale to industrial bioprocesses. *E. coli* is the earliest host expression system and is still attractive for the production of small and simple proteins, mainly due to the cost effectiveness of the bioprocess and ease of genetic manipulation. A critical technical issue associated with the *E. coli* host expression system is disulfide bond formation and scrambling. In this regard, the following recommendations are provided to warrant the safety and efficacy of therapeutic protein products:

1. Intensive efforts should be made to elucidate disulfide bond patterns and their impact on the biological activities and storage stability of recombinant proteins, which will provide insights for developing protocols for their manufacturing and storage.
2. Monitoring the formation and scrambling of disulfide bonds should be applied for therapeutic protein products during and post manufacturing.
3. Regulation of disulfide bond formation during all stages of bioprocessing, including host/vector construction, cultivation, and downstream purification needs to be implemented.
4. Prevention or minimization of thiol/disulfide exchange reactions should be considered in the formulation of recombinant protein drugs.

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