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Disulfide Bond Formation in Prokaryotes: History, Diversity and Design

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Abstract

The formation of structural disulfide bonds is essential for the function and stability of a great number of proteins, particularly those that are secreted. There exists a variety of dedicated cellular catalysts and pathways from Archaea to humans that ensure the formation of native disulfide bonds. In this review we describe the initial discoveries of these pathways and report progress in recent years in our understanding of the diversity of these pathways in prokaryotes, including those newly discovered in some Archaea. We will also discuss the various successful efforts to achieve laboratory-based evolution and design of synthetic disulfide bond formation machineries in the bacterium *E. coli*. These latter studies have also led to new more general insights into the redox environment of the cytoplasm and bacterial cell envelope.

Keywords

Disulfide bond formation; Bacteria; Archaea; Hyperthermophiles; DsbA; DsbB; VKOR; PDI; Oxidative protein folding

Introduction

Covalent linking of amino acids side chains within a polypeptide adds to the stability and function of a great number of proteins. Among the small number of such known covalent modifications, disulfide bridges between two cysteines are the most commonly observed and studied. In the 1950s and 1960s Anfinsen and coworkers showed that when the disulfide bonds of bovine pancreatic ribonuclease were reduced *in vitro* after denaturation, subsequent incubation of the protein in the presence of oxygen or small molecule oxidants allowed renaturation and reformation of the disulfide bonds [1]. Bovine pancreatic ribonuclease is a secreted protein containing 8 cysteine residues forming 4 disulfide bonds. The staggering number of possible cysteine connectivities, over 700, and the requirement for a dithiol

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reducing agent for renaturation suggested that the rate-limiting step in the assembly of ribonuclease might be the formation of the correct array of disulfide bonds. The time required for protein folding *in vitro* was much longer than one would have expected for efficient growth of a cell. This proposal led to the discovery of a microsomal component that significantly increases the rate of refolding, named protein disulfide isomerase (or PDI) [2, 3]. By mediating efficient rearrangement of incorrectly formed disulfide bonds via thiol-disulfide exchange reactions, PDI was the first protein folding catalyst identified [4]. The reshuffling of disulfides was not the only observed rate-limiting step in the renaturation of chemically reduced proteins; at physiological pH the sluggish oxidation of thiol groups to disulfides was thought to be limited by the chemical process of air oxidation. Neither PDI nor small molecule oxidants such as oxidized glutathione (GSSG) were regarded as a source for the generation of disulfide bridges [5]. Since these early experiments, there have been multiple attempts to identify the physiological oxidant of thiols, including the isolation of microsomal flavoprotein amine oxidase, the use of sulfhydryl oxidases and metalloproteins such as Transferrin and Lactoferrin [5–8].

In bacteria, the need for a catalyst of disulfide bond formation in the cell envelope was anticipated because of studies on the kinetics of disulfide bond formation. In particular, pulse-chase studies showed that disulfide bond formation was concurrent with protein translocation across the cytoplasmic membrane, exhibiting much faster kinetics than was seen in the *in vitro* [9]. Nevertheless, it was not until thirty years after Anfinsen's findings that studies in bacterial genetics presented the first *in vivo* evidence for the requirement of a disulfide bond formation catalyst by the serendipitous discovery of DsbA [10]. Ironically, subsequent to identification of DsbA, PDI was shown to play the same role in eukaryotes as DsbA as well as being a disulfide isomerase [11].

Both PDI and DsbA generate disulfide bonds in protein substrates by performing efficient thiol-disulfide exchange reactions utilizing their thioredoxin-like domains [12]. This exchange reaction is in essence a transfer of two shared electrons from sulfur atoms in two cysteines in the substrate to a pair of thiols in a second pair of cysteines (disulfide-bonded) in PDI or DsbA. The transfer of electrons results in the chemical reduction of the former pair of cysteines and the oxidation of the latter. The consequence of this reaction is that, while the substrate protein is released oxidized, the electron receiver, e.g. PDI, must transfer electrons to another electron receiver before it can participate as an oxidant in a subsequent thiol-disulfide reaction cycle. This cascade of electron exchange must continue to pass on electrons until they reach an ultimate electron acceptor. The flow of electrons from cysteine thiols can be directly shuttled to molecular oxygen by the action of the flavin-dependent family of sulfhydryl oxidases, such as Ero1p and Erv1, giving rise to a *de novo* disulfide bond formation [13, 14]. In the bacterial cell envelope, however, such a family of oxidases has not been found, and instead DsbA oxidation is linked to the membrane respiratory chain via the quinone reductases DsbB and VKOR [15–18].

In this review, we will describe the pathways and mechanisms of native as well as laboratory-evolved disulfide bond formation in prokaryotes. We will also discuss how knowledge of a native pathway can be utilized to design a mechanism tailored for a particular purpose, e.g. cytoplasmic production of disulfide-containing proteins, and how the

elucidation of a laboratory-evolved pathway can potentially enhance the discovery of alternative native mechanisms. We will also draw some parallels between the pathways in bacteria and the native pathways of the eukaryotic cell.

Disulfide Bond Formation in the Bacterial Cell Envelope

The cell envelope of the prokaryotic cell is a major line of defense against environmental challenges. It is also a major site for the maturation of proteins exported from the cytoplasm to the periplasm, outer membrane and to the external environment. In some bacterial species, many of these proteins are stabilized by one or more disulfide bonds (approximately 300 predicted proteins in *E. coli* [18]). While disulfide bond formation is essential for the viability of eukaryotes examined so far [13, 19], members of the prokaryotic kingdom show diversity in their capacity for the formation of such covalent bonds. Some prokaryotes make many disulfide-bonded proteins, others make few and some appear not to make any as far as can be assessed. There are at least three different approaches that can be used to assess whether an organism makes proteins with disulfide bonds. First, protein structural studies enable the measuring of the distance between sulfur atoms of two cysteines. A distance that is less than 2.5 Å in a protein structure with a high enough resolution can be a good indicator for the presence of a disulfide bond [20, 21]. Structural homology modeling of a protein sequence with a homologue with a known 3-dimensional structure can also be used to deduce the presence of a disulfide bond. Second, perhaps since cysteine is the most reactive amino acid, cysteines that do not directly participate in catalytic processes nor form important structural features such as disulfide bonds, may have been selected against during the course of evolution [22]. This may explain the observation that compartments of organisms containing disulfide-bonded proteins tend to be enriched for proteins with even numbers of cysteines, thus eliminating most proteins of free and exposed cysteines [23, 24]. This enrichment is common, for instance, in the periplasmic space of many Gram-negative bacteria. Third, the presence of predicted obvious orthologs of known disulfide bond formation catalysts, e.g. DsbA or DsbB (Figure 1), would seem to be a reasonable indicator that an organism contains proteins with disulfide bonds. Generally, this approach is successful, although some caution must be taken in concluding the existence of a DsbA from homology searches alone, as this protein is part of a very large family of thioredoxin homologues. Ultimately, *in vivo* studies demonstrating the function of the protein in disulfide bond formation are necessary. The cysteine counting approach and the identification of DsbA and DsbB homologues amongst prokaryotes suggests that protein disulfide bond formation and the DsbAB pathway are found in α , β , γ and ϵ -Proteobacteria, members of the phylum Deinococcus-Thermus, the Euryarchaeal family Halobacteriaceae and some Bacillales [18, 25]. The DsbAB pathway is also found in Chlamydia which may use it for a subset of exported proteins during one stage of its lifecycle. Other organisms predicted to generally oxidize exported proteins include Cyanobacteria and chloroplasts, most Actinobacteria, aerobic δ -Proteobacteria, Leptospira and some aerobic Bacteroidetes and some Crenarchaea use the DsbA-VKOR pathway. Some Chloroflexi which are predicted to make disulfide bonds in exported proteins have both pathways. Furthermore, while bacteria making a large number of proteins with disulfide bonds exhibit *dsbB* and *dsbA* genes that are not genetically linked, many bacteria contain an operon containing *dsbA*

and *dsbB* homologues that appear to be very specific in their substrates, sometimes promoting disulfide bond formation in only one or a few substrates [26]. For example, the genomes of *Geobacter metallireducens*, several pathogenic *E. coli* (UTI89, APEC 01, 536, CFT073) and some *Salmonella* species encode an arylsulfate sulfotransferase adjacent to DsbL and DsbI, the two being paralogous to DsbA and DsbB, respectively.

The First Prokaryotic System for the Formation of Disulfide Bonds Identified

The discovery of DsbA arose out of studies designed to investigate protein insertion into the cytoplasmic membrane of *E. coli* [10]. For this purpose, a gene fusion was constructed in which the gene for the cytoplasmic enzyme β -galactosidase and the gene for a cytoplasmic membrane protein MalF, a component of the maltose transport system [27], were fused. The β -galactosidase, when fused to the second periplasmic domain of MalF, was exported to the periplasm where it was no longer enzymatically active. Thus, a strain carrying the MalF- β -galactosidase fusion (MalF- β -Gal), and deleted for its normal *lac* region exhibited a Lac⁻ phenotype. Based on this property, it was anticipated that a selection for suppressor mutants restoring a Lac⁺ phenotype to this strain would contain mutations in genes that interfered with assembly of the MalF protein in the membrane, thus returning β -galactosidase to its normal cytoplasmic location. Instead, characterization of such suppressor mutations revealed that the mutations were in genes encoding proteins required for protein disulfide bond formation. The explanation for the inactivity of the β -galactosidase expressed from the MalF- β -Gal fusion construct in wild-type *E. coli* was that disulfide bonds were formed in the protein during or after its translocation to the periplasm, thus disrupting its structure. (β -galactosidase has 16 cysteines in the reduced form when expressed in the cytoplasm).

DsbA is a soluble periplasmic protein (21 kDa) structurally belonging to the thioredoxin-superfamily [28]. This family of proteins is named after the abundant cytoplasmic protein thioredoxin 1 (TrxA), which has a characteristic fold functioning as a scaffold for a variety of enzymes, many of which are involved in reduction-oxidation (redox) reactions. These redox reactions are commonly mediated by one or more catalytic CXXC motif (where X can be any amino acid except cysteine). The two cysteines of thioredoxin family members alternate between the oxidized and the reduced states, the thermodynamics of such alteration being defined largely by the reduction potential (E_0') of the CXXC motif. The reduction potential of the *E. coli* DsbA has been measured *in vitro* to be -120 mV, which makes it much more oxidizing than the cytoplasmic *E. coli* TrxA ($E_0' = -270$ mV) [29]. Oxidized DsbA can engage with nascent chain substrates in a cotranslational and co-translocational manner as they appear in the periplasm out of the inner membrane translocon [30]. The chemistry by which DsbA couples substrate thiols takes place via an S_N2 nucleophilic substitution reaction. Nucleophilic thiols of a substrate attack DsbA's oxidized CXXC motif, in particular DsbA Cys30, displacing Cys33 and thereby forming a mixed disulfide bond between the catalyst and the substrate [12, 30, 31]. The mixed disulfide is eventually resolved by another round of nucleophilic attack of substrate thiols, resulting in the release of a reduced DsbA and an oxidized (disulfide-bonded) substrate.

DsbA is Recycled by DsbB

A more extensive genetic selection in several labs of other suppressors of the Lac⁻ phenotype of the MalF-LacZ fusion, suppressors of DTT sensitivity or of mutants that affect *E. coli* motility yielded mutations in the gene for another enzyme important for disulfide bond formation DsbB [15, 16, 32]. This inner cytoplasmic membrane protein has four transmembrane helical segments, and two periplasmic loops each containing a pair of essential catalytic cysteines (Cys41–Cys44, and Cys104–Cys130) and is essential for the reoxidation of reduced DsbA [33–35]. The helical bundle of DsbB engulfs a quinone molecule, ubiquinone or menaquinone, which lies in the vicinity of the CXXC motif [33, 36]. The oxidation of this motif by oxidized quinone represents the first step in the genesis of disulfide bonds in the periplasm via DsbA [17, 35, 37].

Mechanistic aspects of electron transfer in disulfide bond formation have been revealed by mutants. In particular, searching for a *dsbA* mutant that displays a dominant negative effect, Guilhot and co-worker obtained a *dsbA* in which a mutation had resulted in the substitution of cysteine 33 by tyrosine thus decreasing disulfide bond formation capacity in both a cis- and trans-fashion [38]. At the same time Kishigami and Ito obtained a cysteine 33 to serine mutant that had the same dominant properties [39]. The dominant phenotype of these mutants was due to the accumulation of DsbA in a mixed-disulfide complex with DsbB. This complex represents an intermediate species of DsbA oxidation by DsbB which is the result of a thiol-disulfide exchange reaction. The explanation for the appearance of the trapped complex is that the DsbA Cysteine 33 nucleophilic thiol ordinarily plays an important role in the resolution of the intermediate complex, by attacking the Cys30 (DsbA)-Cys104 (DsbB) mixed disulfide bond, thus releasing oxidized DsbA and reduced DsbB. Interestingly, the purified complex exhibited a characteristic pink color coming from a thiolate-quinone charge transfer complex [40]. This intermediate species is thought to be a precursor for a cysteinylquinone covalent adduct formed during the reduction of quinone to quinol [41, 42]. Through intra-domain thiol-disulfide exchange reactions, disulfides are passed over from the CXXC motif to the second pair of cysteines residing in the periplasmic loop between helix 3 and 4. Hence, the passage of electrons takes the following route: from reduced cysteines of nascent polypeptides substrates to DsbA's Cys30–Cys33, then to DsbB redox pairs, Cys104–Cys130 and Cys41–Cys44, respectively, and finally to oxidized quinone (depicted in Fig 2). This catalytic recycling, or re-oxidation, of DsbA by DsbB is necessary for efficient oxidative protein folding of many exported and periplasmic proteins. Mutants of DsbB that has a reduced affinity towards ubiquinone display deficiency in the oxidation of DsbA and in disulfide bond formation [43].

VKOR is a Functional Homologue of DsbB in Prokaryotes

In a study aimed at finding alternative pathways for disulfide bond formation among bacterial species, it was noted that some species known to have proteins with disulfide bonds, contain a DsbA homologue, but lack a DsbB homologue [18]. Instead, these species utilize a member of the Vitamin K epoxide reductase family (VKOR) to reoxidize DsbA. The VKOR family is widespread across the domains of life including prokaryotes, plants, vertebrates and some insects such as drosophila, but not in fungi [44]. In vertebrates,

this enzyme is central to blood coagulation and is the target of the blood thinner warfarin [45, 46].

The gene for vertebrate VKOR, while its microsomal activity was well documented, had been elusive for decades before it was identified in 2004 by Stafford and colleagues [47]. DsbB and VKORs from bacteria, archaea and vertebrates share in common their overall mechanism of action as well as their architecture (Figure 2) [48–50]. These similarities include the presence of two pairs of redox-active cysteines, one of them the redox active motif CXXC. In some species, VKOR is fused to a soluble domain of the thioredoxin family, presumed to be a DsbA-like enzyme [51]. Moreover, bacterial and plant members of the VKOR family are able to efficiently complement DsbB in *E. coli* [49]. These observations strongly suggested a role of this enzyme in disulfide bond formation in bacterial species that lack DsbB. It is not clear whether any of the vertebrate VKORs play a role in disulfide bond formation. Human VKOR, known as VKORC1 localizes to the membranes of the endoplasmic reticulum (ER), where it recycles vitamin K 2,3-epoxide to its reduced form which is necessary for the γ -carboxylation of glutamic acids in various proteins, including blood coagulation enzymes [52].

While no direct involvement of VKORC1 in disulfide bond formation within secretory proteins has been shown, some evidence suggests its contribution to the maturation of some secreted proteins when other components of the ER oxidative protein folding machinery are compromised [53]. One issue in understanding whether VKORC1 plays a role in oxidative protein folding in the ER is disagreement about the controversial membrane topology of the enzyme. Human VKORC1 has been shown experimentally by various groups to have either 3 or 4 transmembrane helices or even that it can alternate between both by mutations [48, 54, 55]. The 4 transmembrane helices model, placing the pair of catalytic cysteines in the ER lumen, is consistent with what we know about the mechanism of action and structures of DsbB and the bacterial VKOR. In this model, an interdomain thiol-disulfide exchange reaction takes place to shuttle electrons from a thioredoxin-like protein (or domain) to the menaquinone co-factor vitamin K1. In contrast, a three transmembrane-helix arrangement would place the two pairs of conserved catalytic cysteines far apart and isolated by the ER membrane. While this model has the potential of linking vitamin K reduction to the cytoplasm, it raises questions about the mechanism by which the membraneous and lumen proximal catalytic motif (CXXC) reduction can take place. Several of the experimental approaches aimed at probing the activity of VKORC1 in microsomal preparations use the dithiol reducing agent DTT [56]. This membrane diffusible chemical can directly reduce the CXXC motif, bypassing thereby the need for the second pair of active cysteines to provide reducing equivalents [50]. It is worth mentioning that in some prokaryotes (such as *Natronomonas pharaonis*, *Halobiforma nitratireducens*, *Bacillus cellulosilyticus* and *Bacillus pseudofirmus*), homologues of both DsbB and VKOR can be identified [18]. However, the VKOR homologues in these organisms only retain the conserved CXXC redox active site and not the other two catalytic cysteines that are necessary for receiving electrons from a DsbA-like redox partner. Similarly, some DsbB homologues in ϵ -proteobacteria (e.g. *Campylobacter jejuni* and *Helicobacter pylori*) have only the CXXC motif [57]. These

observations suggest that DsbB and VKOR in some prokaryotic organisms might have functions in addition to the generation of disulfide bonds [58].

Disulfide Bonds Isomerization by the DsbC/DsbD System

When a disulfide containing protein has more than two cysteines, the possibility of forming the incorrect disulfide bonds is quite real. This problem is due to the fact that DsbA, since it promotes disulfide bond formation during the translocation of proteins into the periplasm, favors joining pairs of cysteines as they appear in order in the amino acid sequence [30]. As a result, formation of non-native disulfide bonds can occur particularly in cases where the mature form of the protein has two or more disulfides that are formed between pairs of cysteines that do not appear in order in the amino acid sequence of the protein (also termed nonconsecutive disulfides) [59, 60]. Any aberrant disulfide must be isomerized for the protein to attain native conformation. DsbC, the bacterial counterpart of PDI, is a homodimer, each subunit containing an N-terminal dimerization domain and a C-terminal catalytic domain possessing a CXXC redox center [61, 62]. It was first identified in a genetic search for DTT sensitive *E. coli* mutants, and was shown to be an effective catalyst of disulfide isomerization *in vitro* [62, 63]. However, evidence for the *in vivo* function of DsbC in proofreading aberrant disulfides came indirectly from a genetic screen aimed at finding alternative pathways for disulfide bond formation [64]. Suppressor mutations were detected that restored disulfide bond formation to a strain deleted for the *dsbA* gene. The mutations obtained reduced or eliminated the activity of the gene for a membrane protein DipZ, later named DsbD, or of the genes for components of the thioredoxin reduction pathway, *trxA* and *trxB*. Further, the restoration of disulfide bond formation in these mutant strains was dependent on the presence of DsbC, suggesting that DsbC was directly replacing DsbA as the oxidant of cysteine-containing substrates. These apparently complex findings were explained by the discovery that a pathway of electron transfer was occurring in the cell in which DsbC is reduced by the cytoplasmic membrane protein DsbD, which, in turn, is reduced by cytoplasmic thioredoxins. In *dsbD* or *trxA* null mutants, this electron flow is stopped resulting in the accumulation of oxidized DsbC. The oxidized DsbC can then replace DsbA. These results indicated that DsbC is naturally maintained in the reduced state. The maintenance of DsbC in the reduced state is consistent with its functioning as an isomerase since the first step in repairing incorrectly formed disulfide bonds is an attack on such bonds by a reduced cysteine in the isomerase, as had been observed in the *in vitro* experiments [62]. Further studies showed that DsbC has a chaperone activity and is required for the proper folding of a variety of proteins with multiple nonconsecutive disulfides, including periplasmic acid phosphatase and penicillin-insensitive murein endopeptidase MepA, and heterologously expressed human urokinase and plasminogen activator vtPA [60, 64–68].

DsbC initiates the isomerization process by engaging with mis-paired thiols in substrates, forming a mixed disulfide with one of the substrate thiols using its more N-terminal active-site reduced thiol (cysteine 98). Mechanistically, there are two modes by which disulfide isomerization can proceed and lead to DsbC-promoted rearrangement of disulfides (Figure 3). In the first mode, a nucleophilic attack by the appropriate substrate thiol on the DsbC-substrate mixed disulfide results in the release of a reduced DsbC and the formation of a

new substrate disulfide. In this sense, DsbC is acting as a true disulfide isomerase. In the second mode, DsbC functions as a disulfide reductase. A nucleophilic attack is initiated by the more C-terminal active site cysteine of DsbC (cysteine 101), resulting in the release of a reduced substrate and an oxidized DsbC. The reduced DsbC is then re-generated by electrons supplied by DsbD. The formation of the correct disulfide bonds in the substrate protein, thus reduced by DsbC, is presumed to form by another round of oxidation performed by DsbA, perhaps in conjunction with proper folding of the substrate protein [69].

Several lines of evidence are consistent with the model in which incorrectly formed disulfide bonds in proteins are first reduced by an enzyme such as DsbC and then reoxidized by oxidants such as DsbA. The evidence does not rule out the possibility that direct isomerization may make a small contribution to this corrective process. First, a mutant DsbC protein in which the second cysteine of the CXXC motif is replaced by alanine is strongly defective in its ability to assist the folding of urokinase, a eukaryotic protease containing multiple nonconsecutive disulfides [68]. However, it is possible that such a mutation could inactivate the escape pathway by yielding a trapped DsbC in mixed-disulfides with substrates, thus reducing the effective concentration of free DsbC [70]. The second line of evidence is that the accumulation of a reduced DsbC in *dsbA*, *dsbD* double mutant (as opposed to oxidized DsbC in *dsbD*) suggests that oxidized DsbC is ordinarily generated as a result of its action in reducing disulfides of substrate proteins [64]. These and other observations strengthen the argument that isomerization takes place by rounds of reduction of incorrectly formed disulfides by DsbC and substrate re-oxidation by DsbA [69, 71].

Studies on the reduction of DsbC by DsbD have revealed an intriguing inter-domain cascade of thiol-disulfide exchange reactions within DsbD that shuttles electrons across the cytoplasmic membrane to the periplasm [68, 72, 73]. DsbD is a three-module membrane protein composed of a cytoplasmic transmembrane domain (DsbD β), flanked by two soluble periplasmic domains (DsbD α and DsbD γ). Each of these three domains contains a pair of redox active cysteines. Through a poorly understood mechanism, the transmembrane embedded cysteines of DsbD β receive electrons directly from a thioredoxin molecule. These electrons are then transferred to the C-terminal domain, which in turn passes the electrons to the N-terminal domain before they eventually reach DsbC. This positions DsbD and its family of homologues with diverse functions as the only known class of enzymes through which electrons are directly shuttled across the cytoplasmic membrane to the periplasm [74].

In addition to DsbC, DsbD and its homologues in various bacteria contribute to the maintenance of a number of different periplasmic enzymes in the reduced state. Among these is DsbG, a homologue of DsbC as shown by both sequence (26% identity) and by structure comparisons [75]. DsbG was originally detected in a search for *E. coli* genes whose expression from a multicopy plasmid suppressed the dithiothreitol (DTT) sensitivity of a *dsbB* mutant [76]. On the basis of this phenotype, DsbG was mistakenly proposed to be another thiol oxidant in the periplasm that could promote disulfide bond formation. However, this behavior of DsbG was simply due to its activity as an oxidoreductase catalyzing thiol-disulfide exchange reactions. In fact, while DsbG can support disulfide isomerization in the periplasm, although to a lesser extent than DsbC, its main function

appears to be the protection of cysteine thiols from oxidative damage [77, 78]. In the presence of reactive oxygen species, cysteine thiols can undergo oxidation to the sulfenic acid species ($-SOH$). These species, if unreduced to the thiol form, will be irreversibly oxidized to the sulfinic and sulfonic acid forms, which would be particularly damaging for enzymes that use cysteines in their active-sites [79]. DsbG's role as an enzyme that repairs oxidative damage to cysteines has been demonstrated with the *E. coli* periplasmic L, D transpeptidases [78].

Another class of proteins that can be reduced by electrons transferred across the cytoplasmic membrane by DsbD-like proteins is the thiol-dependent peroxiredoxins. These enzymes are efficient scavengers of peroxides and play an important role in cellular antioxidant defense mechanisms [80]. In the catalysis process of converting harmful peroxides to water, a peroxiredoxin active-site thiol is oxidized to sulfenic acid which must be reduced to the thiol form before the enzyme can engage with peroxides in a new reaction cycle [81]. The reduction of the sulfenic acid species in the active-site of cytoplasmic peroxiredoxins usually is catalyzed by thioredoxins or thioredoxin domains of proteins [74, 82]. In *E. coli*, the only known peroxiredoxins localize to the cytoplasm. However, some proteobacteria, such as *Caulobacter crescentus*, do have a cell envelope peroxiredoxin (PprX) and its recycling is achieved by a thioredoxin-like protein, TlpA [74]. The electron source for TlpA is the cytoplasmic membrane protein ScsB which shares some domain homology to *E. coli* DsbD and functions very similarly to DsbD. Thus, with electrons derived from the cytoplasm, DsbG and TlpA have very similar functions, helping protect cysteine-containing proteins from oxidative damage [74]. How common this particular protective mechanism is in other oxidizing cellular environments, such as the ER and IMS of mitochondria has not yet been determined. Nevertheless, in the ER peroxides generated by various cellular processes can be rerouted to tap into the oxidative protein folding pathway [83]. For instance, the ER localized peroxiredoxin 4 (PRDX4), in addition to Ero1 mediated oxidation, utilizes peroxides to catalytically supply oxidizing equivalent to Protein Disulfide Isomerase (PDI) which in turn can oxidize nascent polypeptides that transverse the ER [84].

One question posed by the existence of both thiol reducing and oxidizing pathways in the same compartment is how they can co-exist without interfering with each other? If reducing equivalents supplied by DsbC/DsbD would tap into the DsbA/DsbB system by the oxidation of DsbC by DsbB or reduction of DsbA by DsbD, this would elicit futile cycling and may eventually deplete the cytoplasmic NADPH reservoir [85]. As it will become apparent throughout this review, all of the pathways of electron transfer between thioredoxin enzymes and other thiol-redox proteins, in both the cytoplasm and periplasm, appear to require intermediary proteins that do not contain a thioredoxin-like domain [72, 86]. In other words, electron transfer between DsbA and DsbC is not observed. Additionally it appears that segregation of reducing and oxidizing pathways is maintained by structural and kinetic barriers. For instance, the homodimeric arrangement of DsbC exerts steric constraints that preclude its interaction with and subsequent oxidation by DsbB [87]. In addition, the α -helical domain of DsbA is thought to prevent its interaction with and subsequent reduction by DsbD α [88].

Disulfide Bond Formation in the Cytoplasm

The cytoplasm is considered an electron-rich compartment. Powered by at least two dedicated reduction machineries, electrons are extracted from NADPH and the tripeptide glutathione to supply various electron-requiring biochemical processes, including deoxyribonucleotide synthesis and oxidative stress responses [89–91]. It is estimated that in a T-cell within S-phase more than 100,000 disulfides are generated per second as a result of deoxyribonucleotide generation by ribonucleotide reductase (RNR) [92]. These disulfides obviously require efficient reduction mechanisms in order to complete the catalytic cycle of RNR. In *E. coli*, there are two major reducing pathways in the cytoplasm: i) the flavoenzyme thioredoxin reductase (*trxB*) and its redox partners *trxA* and *trxC*, and ii) the glutaredoxins and the couple glutathione/glutathione reductase (*gor*) [93]. These two pathways form a cysteine-based network of electron hubs that appear to show some redundancies and overlapping substrates (Figure 4). For instance, viability of *E. coli* under aerobic conditions is observed even when either *trxB* or *gor* is compromised, but not when both are missing [94]. However, addition of a reducing agent (e.g. DTT) rescues *trxB*, *gor* lethality.

In addition to the example of RNR, disulfide bonds form and break continuously in the cytoplasm as part of other cellular regulatory and catalytic mechanisms. However, it has long been thought cytoplasmic proteins are generally devoid of structural disulfide bonds, in part because only few had been found. Early studies on the secretion of β -lactamase, containing two consecutive and non-essential disulfides, showed that the formation of disulfide bond does not happen until the protein is secreted to the periplasm [9]. Further, when growth factors and hormones, which sometimes are heavily decorated with disulfide bonds, are made recombinantly in the cytoplasm of *E. coli*, they tend to aggregate as insoluble inclusion bodies [95–97]. This phenomenon is attributed, at least in part, to the adverse effects on the folding and stability of these proteins in the absence of the formation of disulfide bonds. There have been only sporadic reports showing the presence of structural disulfide bonds for some proteins when made recombinantly in the cytoplasmic of *E. coli*; interestingly, the vast majority of these cases involved proteins of thermophilic origin [23, 98, 99]. Nevertheless, Nucleocytoplasmic Large DNA Viruses (NCLDV) do encode enzymes that promote the formation of stable disulfide bonds in viral proteins expressed in the cytoplasm of their host. In poxviruses this process is essential for virus maturation, replication and virulence. The generation of these cytoplasmic bonds is carried out by dedicated catalysts encoded in the viral DNA. For example, Vaccinia virus carries three proteins, G4L, E10R and A2.5L that constitute a complete pathway for the formation of disulfide bonds in a variety of poxviral proteins [100–102]. The mechanism involved is reminiscent of other pathways in the ER or intermembrane space of mitochondria, in which a sulfhydryl oxidase belonging to the ERV1/ALR family, E10R, and its redox partner A2.5L directly oxidize a thioredoxin-like protein, G4L, which in turn oxidizes viral substrates F9L and L1R [14, 100]. Although a similar pathway has not been discovered in bacteriophages, sulfhydryl oxidases are widespread in the viral domain of both eukaryotic and bacterial life [103].

It had been thought for many years that disulfide-bonded proteins were not present in the cytoplasm of cells. The existence of significant numbers of proteins with disulfide bond in the cytoplasm of certain organisms was not recognized until 2002. An approach in which the counting of cysteines in proteins was used suggested that some extremophiles, in contrast to mesophiles, have an abundance of even number of cysteine residues within cytoplasmic proteins [23]. The high fraction of proteins with even numbers of cysteines was proportional to the optimum growth temperature of the extremophile; hyperthermophiles that grow optimally above 80°C exhibit this property. For example, *Pyrobaculum Aerophilum* and *Aeropyrum pernix* with optimal growth temperatures of 101°C and 95°C, respectively, have up to 78% and 68% of their cytoplasmic proteins of known crystal structures containing disulfide bridges [24]. This apparent evolutionary adaptation can be easily explained by the protein stabilizing effect of disulfide bonds for organisms growing at high temperatures [104, 105]. These observations appeared to contradict the prevailing dogma that the cytoplasm is a reducing environment in which structural disulfide bonds in proteins cannot form or are immediately reduced. However, it is coherent with the growing understanding that neither the cytoplasm nor the periplasm can be considered strictly a reducing or oxidizing environment. The cytoplasm of *E. coli* which was considered a strictly reducing environment can be altered in a *trxB* null mutant or by the introduction of a sulfhydryl oxidase to allow cytoplasmic disulfide bond formation (discussed below) [106–108]. In other words, the absence of disulfide-bonded proteins in the cytoplasm of most organisms is not due to a reducing environment of the cytoplasm, but rather to the absence of a cytoplasmic enzyme catalyst (such as DsbB) that could promote disulfide bond formation.

The native pathway and the source of oxidizing equivalents in the cytoplasm of Crenarchaeal organisms have recently been studied. Comparative phylogenetic analysis has shown that the most disulfide-rich thermophilic prokaryotes do have in common a candidate for a presumed cytoplasmic enzyme catalyst of disulfide bond formation [24]. The crystal structure of the enzyme shows that it has a two-domain arrangement belonging to the thioredoxin/glutaredoxin families, and each domain has a characteristic CXXC motif [109, 110]. *In vitro* data also shows that PDO from various hyperthermophiles is capable of only catalyzing thiol:disulfide exchange reactions; in other words PDO is a disulfide shuttle and not an oxidase [111]. As such, it has no net input on the formation or breakage of protein disulfides and it must itself acquire disulfide bonds before it can participate as an oxidant. Despite the importance of these findings in the implication of PDO in disulfide bonds maintenance, they do not, however, directly demonstrate its exact function *in vivo*, nor do they explain the molecular mechanisms by which *de novo* sulfhydryl oxidation takes place in the cytoplasm of hyperthermophiles.

In a study aimed at engineering a cytoplasmic disulfide bond formation pathway in *E. coli* (See section on evolution in the cytoplasm), it was shown that hyperthermophilic Crenarchaea have two copies of a VKOR homologue (Figure 5) [108]. Membrane topology prediction algorithms of these homologues from *Aeropyrum Pernix* (ApVKORin and ApVKORout) and experimental data (Boyd D et.al unpublished observations) showed that each protein has four transmembrane segments but assumes opposite membrane topology; i.e. the redox active sites of one copy faces the cytoplasm while the other faces the

extracellular space. When expressed in *E. coli*, the two hyperthermophilic VKOR homologous promote disulfide bond formation one in the cytoplasm and the other in the periplasm, depending on the putative localization of their redox active sites. Their function in promoting disulfide bond formation depends on the presence in *E. coli* of a DsbA in the same compartment in which the active sites of ApVKOR are located, consistent with what is known regarding the mechanism of VKOR from other organisms. These findings strongly suggest that the cytoplasmically oriented copy, i.e. ApVKORin, contributes to disulfide bond formation in the cytoplasm of *Aeropyrum Pernix* by regenerating the oxidized form of a cytoplasmic enzyme, perhaps analogous to DsbA. The evolutionary process by which some Crenarchaeal species appear to generate VKORs that function in disulfide bond formation in two compartments is a common one [112]. A gene duplication event, followed by an alteration of the bias of charged amino acids that normally reside in the cytoplasmic leaflet of the inner membrane according to the positive-inside-rule. It can be seen in Figure 5 that there may have been at least two such events within Crenarchaea. The *Aeropyrum pernix* VKORs are closely related to one another. Among Thermoproteales the *Pyrobaculum* inward and outward facing VKORs are closer to one another than those of other taxa, Sulfolobales for example, while the *Vulcanisaeta* and *Caldivirga* inward facing VKORs are unrelated to any others while the outward facing VKORs in the same strains are of the Thermoproteales type. It is not clear what the topology of the progenitor VKOR was, but since protein synthesis has probably evolved in an anaerobic and reducing environment, topology inversion was likely towards the cytoplasm. If the latter assumption is correct, then such an evolutionary step might have played a pivotal role in the survival of the last universal common ancestor, which is hypothesized to be a hyperthermophile [113–115]. Those Crenarchaea that have two oppositely oriented VKORs appear have diverse oxygen growth requirements. For example *Aeropyrum pernix* and *Pyrobaculum Aerophilum* are strictly aerobic and microaerophilic, respectively, whereas *Pyrobaculum Arsenaticum* is an obligate anaerobe [116–118]. This suggests that a requirement of a catalyst for cytoplasmic disulfide bond formation is possible regardless of the oxygen availability. It must be borne in mind that not all hyperthermophilic organisms that are predicted to be enriched in disulfide-bonds have two copies of VKOR. Thus, it is still unclear by what route/mechanisms these organisms promote the formation of disulfide bonds in their cytoplasmic proteins.

Laboratory-Based Evolution of Pathways of Protein Disulfide Bond Formation in the Prokaryotic Cell Envelope

One way to gain further insights into the possible mechanisms of disulfide bond formation is by evolving new pathways in strains that are missing one or more components of the disulfide bond formation machinery. In addition to yielding surprising findings regarding the plasticity of redox enzymes, this approach has also provided a valuable tool in deciphering the complexity of the components of the system involved. There are various ways by which the bacterium *E. coli* can be evolved or engineered to overcome deficiencies in native disulfide bond formation pathways (Figure 6). We will discuss below the efforts that have been made using *E. coli* as a model, starting from the laboratory-evolved oxidation and

isomerization pathways in the periplasm, before we conclude with a description of these novel pathways in the cytoplasm (Figure 7).

Laboratory-evolved oxidation pathways in the cell envelope

Complementation of a *dsbA* deletion strain can be achieved, remarkably, with a variety of thiol-disulfide exchange catalysts (oxidoreductases). In one case, the normally cytoplasmic TrxA of *E. coli* was forced to be exported to the periplasm by attachment of a signal sequence to its amino-terminus. In a *dsbA* mutant background, the exported thioredoxin promotes disulfide bond formation to a measurable degree in a DsbB-dependent manner [119–121]. This activity is greatly enhanced when the thioredoxin CXXC redox motif is replaced with that of DsbA. Similar results have been obtained with *E. coli* thioredoxin 2 (TrxC) and with eukaryotic PDI or even a fragment of it that includes only the N-terminal thioredoxin domain [122, 123]. Additionally, the thiol redox protein DsbC, also a thioredoxin homologue, can be altered by rational design, or by random mutagenesis and selection to behave as a substrate of DsbB. Disruption of the dimerization propensity of DsbC by mutations in the alpha-helix that joins the N-terminal dimerization domain with the C-terminal thioredoxin domain renders the enzyme oxidizable by DsbB [87]. A similar outcome can be achieved by selecting for DsbB mutants that can receive electrons from DsbC, thereby bypassing the need for DsbA [124]. These DsbB mutants mapped to the horizontal amphiphilic $\frac{1}{2}$ helix which was implicated in the regulation of electron flow from DsbA [33, 36]. No DsbB mutants were found that directly oxidize cysteines within folding substrates (i.e. without the need for DsbA or DsbC). This finding is in contrast to what has been found with yeast Ero1, where it is possible to obtain mutants that circumvent PDI by directly oxidizing ER nascent proteins.

Iron-sulfur cluster thioredoxin

All of these aforementioned laboratory-evolved examples are essentially replacing a thiol-disulfide exchange catalyst with another. In other words, the oxidizing capacity of the periplasm, which is set by the action of DsbB and quinone, has not been manipulated. There have been multiple attempts to reconstitute pathways for oxidative protein folding in the absence of the entire sulfhydryl oxidation machinery, e.g. both DsbA and DsbB. One common denominator of these various attempts is that the route by which electrons reach their ultimate acceptor does not involve quinones. These laboratory-evolved machineries include I) the engineering of a novel sulfhydryl oxidase, II) the overexpression of a protein containing highly reactive cysteine residue (e.g. a periplasmic rhodenase), and III) glutaredoxins exported to the periplasm.

By asking whether one can evolve an alternative mechanism for disulfide bond formation based on an existing scaffold, Masip et al. engineered a pathway by imposing evolutionary pressure on TrxA. The authors used a strain that not only lacks DsbA and DsbB, but also lacks two genes for cytoplasmic redox proteins, TrxB and Gor, thus rendering the cytoplasm more oxidizing. They then asked whether thioredoxin, when exported to the periplasm, would promote disulfide bond formation in such a strain. The rationale behind their proposal was, given that thioredoxin accumulates in the oxidized form in a *trxB*, *gor* strain, if it is

targeted to the periplasm via the Tat pathway, which can export folded protein post translationally, it might be able to function as a disulfide carrier and substitute for both DsbA and DsbB. When the researchers found that wild-type thioredoxin exported in this strain was unable to complement DsbB/DsbA, they randomized the thioredoxin redox motif CGPC and screened mutants that would complement. They found that the thioredoxins with the altered motifs CACA and CACC were able to promote disulfide bond formation. By forming a [2Fe-2S]-bridged dimer, two thioredoxin molecules carrying CACA motifs were able to acquire a novel oxygen-dependent sulfhydryl oxidase activity. A similar oxidizing capacity and use of iron-sulfur cluster has been observed for poplar glutaredoxin C1 (GrxC1) [125]. Using cysteines from a pair of glutathione molecules, two GrxC1 subunits, each carrying YCPWC active site, incorporate an intra-subunit bridging [2Fe-2S] cluster. This enzymic arrangement is capable when targeted to the periplasm of complementing DsbAB. It remains uncertain if GrxC1 is capable of catalyzing sulfhydryl oxidation using molecular oxygen or whether it uses GSSG as electron acceptor [125].

Glutaredoxins and glutathione

Whereas defects of a *dsbB* deletion strain can be alleviated, at least partially, by adding a substantial amount of GSSG (>5 mM) or cystine to the growth media, the same is not true in the absence of DsbA, where GSSG cannot replace it [15]. This suggests that GSSG, while it can oxidize DsbA to some extent, is unable to act as a direct oxidant for protein thiols in the periplasm. Nevertheless, a periplasmically-localized glutaredoxin 3 can substitute for both DsbB and DsbA, even without the exogenous supplement of glutathione [123]. This implies that glutaredoxin 3 (Grx3) is capable of mediating transfer of electrons from folding periplasmic proteins to oxidized glutathione. The action of this glutaredoxin was, however, dependent on the cytoplasmic biosynthesis of glutathione. These findings were taken as an evidence for the presence of glutathione in the periplasm. It remains to be determined how GSSG is generated in the periplasm, since only reduced glutathione can be exported by a putative ABC transporter [126].

Overexpression of periplasmic rhodanase PspE

A third approach to generating alternative disulfide bond-forming pathways arose out of a study aimed at finding the reason(s) behind the observed residual oxidation in strains deleted for the *dsbA* and *dsbB* genes [127]. Transforming an *E. coli* library of gene-sized fragments on a multicopy plasmid library into the *dsbA*, *dsbB* mutant yielded a gene, *pspE*, whose overexpression suppressed defects in motility. The *pspE* gene codes for a thiosulfate:cyanide sulfurtransferase, also known as rhodanese [128]. The complementation of the strain deleted for *dsbA* and *dsbB* by *pspE* was dependent on the presence of DsbC and complemented more strongly when *dsbD* was deleted, thus preventing reduction of any oxidized DsbC. Rhodanases transfer sulfur atoms from thiosulfate to thiophilic acceptors such as cyanides and thiols providing the sulfur for such compounds as thiamine and thiouracil [129, 130]. The mechanism of action of rhodanases involves a conserved catalytic cysteine residue with a highly reactive thiol group that forms a persulfide [131]. This thiol group can also undergo oxidation to the sulfenic acid derivative [132]. Results in this study indicate that the sulfenic acid derivative of overexpressed PspE represents a major fraction of the protein [127]. Both

species can potentially oxidize DsbC which then could oxidize other thiol-containing proteins in the periplasm and replace DsbA (and DsbB). Such a pathway reverses the normal function of DsbC in disulfide breakage, as did some of the novel pathways described above.

Although the physiological function of certain rhodanases in biosynthetic pathways is known, the function of the rhodanase PspE in the periplasm is not understood. Also, the source of the ultimate oxidizing agent of PspE in this novel pathway described here has not been identified. Nevertheless, the finding raises the possibility that there may be some bacterial species that lack DsbA, DsbB/VKOR and, DsbD and that utilize a periplasmic rhodanase/DsbC pathway to promote protein disulfide bond formation.

Evolved isomerization pathways

Ordinarily, in many bacteria, the enzymes that catalyze disulfide bond formation and disulfide bond isomerization are distinct from each other. However, it is clear that the two processes can be carried out by a single protein, given the examples of the dual activities of eukaryotic PDI and from rational genetic design experiments in prokaryotes. While the measured reduction potentials of DsbA and DsbC are similar, a stark structural difference between the two is the domain organization and the dimeric form of DsbC. DsbC acquires a V-shaped conformation as two monomers join at the C-terminal dimerization domain. The cleft formed by the two arms of the V-shaped conformation is thought to accommodate misfolded substrate polypeptides with incorrect disulfide linkages that then undergo reshuffling. PDI, a four-domain oxidoreductase capable of performing thiol oxidation as well as disulfide isomerization, shares similar structural features with DsbC [133]. Both PDI and DsbC are organized structurally in such a way that their redox-active sites are facing each other inside the cavity of the V-shaped arrangement [61]. Deletions in the alpha-helix that connects the two domains of DsbC abolish its resistance to oxidation by DsbB, while it retains its capacity to be reduced by DsbD [87]. Therefore, a DsbC with truncations in the linker region between its two domains maintains the isomerization activity while conferring on DsbC the ability to catalyze oxidation, i.e. replacing DsbA. Remarkably, when the C-terminal dimerization domain of DsbC is fused to DsbA, the resultant chimera is also capable of performing oxidation as well as complex isomerization reactions [134]. A similar outcome is achieved when the dimerization domain of the proline cis/trans isomerase FkpA, which is also V-shaped, is fused to DsbA [85]. These chimeras are functional in isomerization reactions as long as the point of fusion between DsbA and the dimerization domain favours a configuration in which the active sites (CXXC) are juxtaposed, resembling the original arrangement in an intact dimer of DsbC.

Another example, in which an alternate means of performing isomerization of mis-oxidized protein was selected for, is linked to a selection for antibiotic resistance in a strain expressing an altered β -lactamase. Selecting for ampicillin resistance of an *E. coli* strain that was deleted for *dsbC* and carried a β -lactamase with a few additional cysteines introduced into it, yielded strains that bypassed the need for DsbC for isomerization by massively overproducing DsbA (a 50 fold increase) [135]. The overproduction caused DsbA to shift towards the reduced state (50% oxidized probably as a result of outrunning the capacity of DsbB to reoxidize DsbA). Thus by having an abundance of reduced DsbA, correct disulfide

bond formation can be achieved, at least partially, by rounds of oxidation-reduction carried out by the same catalyst, DsbA. It is unclear from these findings how efficient these strains are in disulfide isomerization as compared to a strain expressing DsbC, and whether DsbD is required for the process (since the rate of DsbA reduction by DsbD is insignificant). The isomerization capacity of such a strain is obviously undermined by the supply of electrons from the reduced fraction of DsbA. In other words it is limited by DsbA:substrates stoichiometry. Nevertheless, it raises the possibility that some bacteria may catalyze oxidation and isomerization by a single protein [136].

Laboratory-evolved oxidation pathways in the cytoplasm

Studies in bacterial genetics have paved the way over the years for the construction of a plethora of *E. coli* strains with novel cytoplasmic redox properties (Figure 7). As mentioned above, cytoplasmically localized alkaline phosphatase (PhoA) is inactive, due to the failure to have its essential disulfides formed. This property allows a genetic selection for disulfide bond formation in the cytoplasm by finding conditions under which the activity of the enzyme is required for growth of the bacteria. Since alkaline phosphatase is a promiscuous phosphomonoesterase, if it were active in the cytoplasm, it should be able to replace important metabolic enzymes such as serine-1-phosphate phosphatase, required for serine biosynthesis, or fructose-1,6-bisphosphatase, essential for gluconeogenic growth. Selection for mutants that restore viability, in mutants lacking one or the other of these two enzymes made it possible to isolate *E. coli* mutants capable of functionally expressing cytoplasmic PhoA [106]. The mutations were mapped to *trxB* and they allowed PhoA to acquire its essential disulfides. Analysis of these suppressor mutations showed that in a *trxB* mutant, TrxA and TrxC accumulate in the oxidized form and can oxidize PhoA to the active form [106]. The naturally disulfide-reducing enzymes TrxA and TrxC, are now acting as oxidants (as they do when exported to the periplasm; see above) [137]. But what is the source(s) of TrxA oxidation in the cytoplasm? During ribonucleotide reduction, class I RNR requires two electrons from the dithiols of thioredoxins to generate deoxyribonucleotides. Hence electrons of PhoA are passing successively to ribonucleotides via RNR and thioredoxins, resulting in the latter's oxidation [90]. Additionally, one can imagine that disulfide bond formation mediated by thioredoxins in a *trxB* mutant can also be due to their oxidation resulting from their response to oxidative stress [89, 138, 139].

Disruptions in other reducing pathways, e.g. inactivation of the *gor* gene or of the glutathione biosynthesis pathway, also give rise to an increased phosphatase activity of cytoplasmic PhoA, but to a much lower extent than in *TrxB* (4–5 fold lower) [94]. This is probably due to the more efficient reduction of substrates by the TrxA, C/TrxB system compared to that by glutathione and glutaredoxins. However, a double *trxB*, *gor* mutant, which only can grow when rescued by the presence of the reductant dithiothreitol (DTT), shows high levels of cytoplasmic disulfide bond formation when the DTT is removed [140]. Further, the non-viable doubly mutant strain accumulates suppressors at a high frequency when DTT is removed from the growth media [140]. The suppressors are gain-of-function mutations in the *ahpC* gene encoding alkyl hydroperoxidase that convert this enzyme into a glutathione reductase [141]. These suppressor mutations, which restore growth to the double mutant by increasing the cytoplasm's capacity to provide reducing equivalents to RNR, still

maintains high levels of active alkaline phosphatase. This latter strain, also known commercially as Origami™ (Novagen), has been widely used for the production of recombinant disulfide-bonded proteins in the cytoplasm of *E. coli* [142]. For cytoplasmic expression of proteins with multiple nonconsecutive disulfides, a disulfide isomerase, such as DsbC, is also required for efficient reshuffling and correct pairing of cysteines [140][143][144].

The evolved mechanisms for the generation of disulfide bonds in the cytoplasm that we have described so far do not utilize proteins that are normally dedicated to introducing disulfide bonds into proteins. Rather, these novel pathways are based on redirecting the activities of proteins that are ordinarily involved in other cytoplasmic regulatory and catalytic processes. However, recent approaches have succeeded in altering the capacity of the *E. coli* cytoplasm to make disulfide bonds by expressing in the cytoplasm enzymes that are dedicated catalysts of disulfide bond formation in other non-bacterial organisms. Interestingly, the expression of a mitochondrial intermembrane space sulfhydryl oxidase, Erv1, significantly boosts the capacity of the *E. coli* cytoplasm to promote structural disulfide bonds, even though the cytoplasmic reducing pathways are genetically intact [107][143]. Presumably, Erv1 is using molecular oxygen to oxidize substrates, such as cytoplasmic alkaline phosphatase, but it is also possible that an intermediary molecule, such as TrxA is mediating substrate oxidation. The use of sulfhydryl oxidases also proved to be useful for *in vitro* synthesis of disulfide-bonded proteins using wheat germ extracts [145].

In a similar approach, the native periplasmic DsbB/DsbA system was engineered to redirect oxidizing equivalent from the quinone pool to the cytoplasm [108]. By combining the inversion of the topology of the membrane protein DsbB in such a way that its active-site cysteines are facing the cytoplasm, and the expression of a cytoplasmic version of DsbA, it was possible to reconstruct oxidative protein folding in the cytoplasm by linking sulfhydryl oxidation to the respiratory chain via quinones. It was also possible to engineer DsbB variants that would catalyze disulfide bond formation in two compartments (cytoplasm and the periplasm) simultaneously; presumably by having a dual orientation in the membrane. This laboratory-engineered pathway prompted the search for naturally evolved, DsbB-like catalyst that would also promote cytoplasmic disulfide bond formation by adopting an inverted membrane topology. While a bioinformatics search did not find a DsbB homologue with such properties, it did however result in the identification of inverted VKORs from *Crenarchaea*.

Perspective and Conclusions

Since the discovery of DsbA 20 year ago, a tantalizing diversity of prokaryotic disulfide bond formation pathways has emerged. Nevertheless, there is some commonality in catalysis mechanisms and in pathways of electron flow from substrates to ultimate electron acceptors.

For many bacterial pathogens, whether Gram-negative or Gram-positive, virulence is mediated by cellular protein structures that are stabilized by one or more disulfides. Indeed, when disulfide bond formation pathways are compromised, for example in *Pseudomonas aeruginosa*, this pathogen exhibits a reduced virulence [146, 147]. Thus, targeting of

disulfide bond formation pathways appears to be an attractive approach for the development of antimicrobials and anti-virulence agents [148]. The future may yield inhibitors of these pathways of both basic and applied interest as some reports in literature have begun to address this concept [149, 150].

Despite advances in our understanding of oxidative protein folding, the efficient production of disulfide-bonded proteins is still one the bottlenecks in the development of protein-based pharmaceuticals. The discovery of ApVKORin and DsbBin should add to the available arsenal of redox enzymes and *E. coli* mutant strains which are currently being used for the fine-tuning of recombinant protein expression in the cytoplasm. The universe of disulfide bond metabolism is ever-expanding as the discovery of new mechanisms continues, and there is much yet to unravel regarding the complexity of redox pathways across life.

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Highlights

- Disulfide bond formation in proteins is a catalyzed process
- Prokaryotic organisms show diversity in their capacity to form protein disulfide bonds
- Novel disulfide bond formation pathways can be revealed by genetic selection and rational design

Figure 1.

Pathways for native disulfide bond formation in the periplasm of *E. coli*. DsbB oxidizes DsbA, which in turn oxidizes proteins secreted into the periplasm. DsbD shuttles electrons via thioredoxins from the cytoplasm to reduce DsbC and DsbG which participate in native disulfide bond formation by reducing incorrect bonds and preventing cysteines from hyperoxidation to sulfinic and sulfonic acid intermediates.

Figure 2.

DsbB and VKOR are central to oxidative protein folding in prokaryotes. DsbB and VKOR share similar overall architecture but with active-site domain chirality. The enzymes contain two pairs of conserved cysteines that are located in two extracytoplasmic loops. Both enzymes use a quinone (represented by a hexagon with Q) as a cofactor which oxidizes a nearby CXXC redox-active site. The second pair of active site cysteines is located in an adjacent loop and receives a disulfide bond intermolecularly from the CXXC motif. Both enzymes are responsible for oxidizing a DsbA which introduces disulfides into a variety of protein substrates. *Synechococcus sp.* VKOR has a DsbA domain which is C-terminally fused to the 4-helix bundle via a 5th transmembrane helix.

Figure 3.

Rearrangement of a four-cysteine protein containing two disulfide bonds. Starting from the reduced state, DsbC can form transiently an intermolecular mixed disulfide with oxidized substrates. The rearrangement process of disulfide connectivities can occur by either a direct isomerization reaction, or by cycles of reduction/oxidation. The nucleophilic attack by the C-terminal active site of the isomerase gives rise to the escape pathway, which resolves kinetically trapped isomerase-substrate complexes.

Figure 4.

The reducing pathways of the cytoplasm in *E. coli*. Electrons derived from NADPH are funneled through glutathione (GSH) and small proteins that belong to the thioredoxin superfamily to several biochemical pathways. The two branches of the reducing pathways can overlap in their function giving a functional plasticity to ensure efficient flux of electrons to a variety of enzymes such as: MsrA (Methionine sulfoxide reductase), Phosphoadenylyl-sulfate (PAPS) reductase, Ribonucleotide reductase (RNR), Oxygen response regulon (OxyR) and Arsenate reductase (ArsC).

Figure 5.

Phylogenetic relationships among Crenarchaeal VKORs. Crenarchaeal VKORs were found using Blast [151]. Hmmer3 [152] was used to construct Hidden Markov models for aligned VKORs and to search in the genomes of 47 Crenarchaeota found among 2416 genomes downloaded from Genbank May 23, 2013. 42 VKORs were found among 31 of the Crenarchaeal strains. These were aligned with MUSCLE [153]. The alignments were trimmed to 70% of its original length with Gblocks and trees were constructed with Phym13 [154]. Orientation was determined with Scampi [155].

The tree generally conforms to the phylogeny of the organisms (as indicated by the taxonomy with the exception of the Acidilobales VKORs which lie within the Thermoproteales). The inward and outward facing VKORs (“i” for inward and “o” for outward) of *Aeropyrum pernix*, which is a member of the Desulfurococcales, are each other’s nearest neighbor in the tree. Pyrobaculum inward facing VKORs are more closely related to outward facing Pyrobaculum VKORs than to others (Sulfolobus VKORs for example). *Vulcanisaeta moutnovskia* and *Calddivirga maquilingensis*, both belonging to the Thermoproteales, inward facing VKORs are not closely related to any other VKORs while the outward facing ones are close the other outward facing thermolobales VKORs. In the tree shown “*Sulfolobus islandicus* o” represents ten *S. islandicus* and one *S. solfataricus* P2 outward facing VKORs which have highly similar sequences. “*S. acidocaldarius* o” represents three nearly identical outward facing VKORs from three strains. “*S. solfataricus* o” represents two similar outward facing VKORs from strains P2 and 98. The *Metallosphaera cuprina* VKOR inward facing is predicted as outward facing by other topology predictors.

Figure 6.

Laboratory-evolved disulfide bond formation pathways in the periplasm of *E. coli*. The oxidation of protein thiols to disulfides is an electron abstraction process. DsbA ordinarily mediates this process. In the absence of either or both of DsbA and DsbB, one can rationally evolve several routes by which electrons can pass from substrates undergoing oxidative protein folding and shuttled to various electron acceptors. These evolved strains have various capacities for the catalysis of disulfide bond formation, which can also be dependent on the nature of the disulfide-containing substrate used. This includes I) Periplasmic targeting of a mutant version of *E. coli* thioredoxin, TrxA (CACC) which forms a [2Fe-2S]-coordinated dimer, or II) of *E. coli* poplar glutaredoxin overcome the need for DsbAB. III) DsbC can replace DsbA when the *E. coli* rhodanase (*pspE*) is overexpressed from a plasmid. VI) In a DsbD strain, DsbC shifts to the oxidized state presumably by reducing sulfenic acid species. V) DsbB and DsbC ordinarily do not interact. However, a mutation in either the short membrane-localized α -helix of DsbB (DsbB*), or in the dimerization domain of DsbC (DsbC*) can break the interaction barrier and bypass the need for DsbA. VI) TrxA and TrxC as well as eukaryotic PDI when targeted to the periplasm can also complement DsbA.

Figure 7.

Laboratory-evolved disulfide bond formation pathways in the cytoplasm of *E. coli*.

Structural disulfide bonds do not naturally occur in the cytoplasm of *E. coli*. However, the redox state of the cytoplasm can be altered to permit the oxidation of thiols and the pairing of cysteines to form structural disulfides. This is achieved by disruption of either or both of the main reducing pathways in *E. coli*, Gor and TrxB. In Gor strain electron pass from protein substrates to glutaredoxins and then to oxidized glutathione (GSSG), whereas in a TrxB strain electrons are passed from substrates to thioredoxins and then to RNR. Alternatively, the introduction of the sulfhydryl oxidase Erv1 to the cytoplasm links oxidative protein folding directly to molecular oxygen. The inversion of membrane orientation of *E. coli* DsbB (DsbBin), or the use of a naturally inverted VKOR (VKORin), along with the co-expression of a cytoplasmic DsbA also allows cytoplasmic disulfide bond formation.