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Periplasmic protein thiol:disulfide oxidoreductases of *Escherichia coli*

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Abstract

Disulfide bond formation is part of the folding pathway for many periplasmic and outer membrane proteins that contain structural disulfide bonds. In *Escherichia coli*, a broad variety of periplasmic protein thiol:disulfide oxidoreductases have been identified in recent years, which substantially contribute to this pathway. Like the well-known cytoplasmic thioredoxins and glutaredoxins, these periplasmic protein thiol:disulfide oxidoreductases contain the conserved C-X-X-C motif in their active site. Most of them have a domain that displays the thioredoxin-like fold. In contrast to the cytoplasmic system, which consists exclusively of reducing proteins, the periplasmic oxidoreductases have either an oxidising, a reducing or an isomerisation activity. Apart from understanding their physiological role, it is of interest to learn how these proteins interact with their target molecules and how they are recycled as electron donors or acceptors. This review reflects the recently made efforts to elucidate the sources of oxidising and reducing power in the periplasm as well as the different properties of certain periplasmic protein thiol:disulfide oxidoreductases of *E. coli*. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Dsb protein; Cytochrome *c* maturation; C-X-X-C motif; Periplasmic protein folding; Redox control; Thioredoxin

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1. Introduction

Genetic and biochemical studies have led to the discovery of an array of protein thiol:disulfide oxidoreductases in *Escherichia coli* (see also [1–4] for reviews). The function of these proteins is either the oxidative formation of disulfide bonds, which is often necessary for folding and stability of secretory proteins, the reduction of non-native disulfides, or the isomerisation of disulfide bonds in proteins, especially when wrong disulfide bonds were formed.

Generally, thiol:disulfide oxidoreductases are characterised by two features. (i) They share an active site containing two cysteines, arranged in a C-X-X-C motif (Fig. 1), which are either in the reduced state forming two thiols or in the oxidised state forming an intramolecular disulfide bond. This motif represents the active site of proteins that interact directly with cysteines or cystines in the target molecules. This interaction leads to the formation of a kinetically unstable intermediate, the so-called mixed disulfide. (ii) Despite very low primary sequence similarity, most of these proteins show the same overall tertiary structure known as the thioredoxin-like fold [5].

In the bacterial cytoplasm, the protein thiol:disulfide oxidoreductases comprise the thioredoxin and glutaredoxin systems [6]. They catalyse reductive processes such as the transfer of electrons to ribonucleotide reductase and reduction of disulfide bonds. This reflects the fact that the cytoplasm is a reducing environment.

In the periplasm, disulfide bond (Dsb) formation is a necessary part of the protein folding pathway of many cell envelope proteins [2]. Here, oxidation of dithiols is catalysed rapidly and in a rather unspecific way by DsbA and DsbB. Further periplasmic thiol:disulfide oxidoreductases are known, which are responsible for the transfer of reducing power towards the cell envelope and for reduction and isomerisation of randomly formed disulfide bonds.

Studies with the apparently strongest dithiol oxidising protein of the periplasm, DsbA, and with the strongest disulfide reducing protein of the cytoplasm, thioredoxin (Trx1), revealed that the redox properties of protein thiol:disulfide oxidoreductases are strongly influenced by the intervening X-X dipeptide between the active-site cysteines [7–11]. DsbA and thioredoxin variants, in which the X-X dipeptide was mutated so as to mimic the active site of another thiol:disulfide oxidoreductase, have a redox potential that is shifted in the direction of the redox potential of that latter protein. In the case of the thioredoxin mutant variants, their in vivo redox properties correlated well with their intrinsic redox potentials. Their ability to complement a *trxA* mutant (deficient in Trx1) increased with decreasing redox potential. Thus, the low redox potential of Trx1 is clearly an important factor for its in vivo function [12]. Interestingly, active-site variants of DsbA, which were more than 1000-fold weaker oxidants than the wild-type protein, could still functionally replace DsbA under normal growth conditions [7]. In the case of DsbA the redox potential may, therefore, be less critical than initially assumed [9]. A further important factor influencing the redox function of protein thiol:disulfide oxidoreductases is the redox environment in the compartment where these proteins are located [9,13,14]. The cytoplasm is a more reducing environment, whereas the periplasm is more oxidising. For example, it was possible to complement a deficiency of periplasmic DsbA with artificially secreted thioredoxin variants even though these variants had a much lower redox potential than DsbA [9]. The low efficiency of wild-type Trx1 complementing a *dsbA* mutant for disulfide bond formation was suggested to be due to a slow rate of polypeptide oxidation [9,13]. Additionally, it was proposed that Trx1, despite having a normal in vivo function as a reductant, is able to act as an oxidant in the cytoplasm depending on its own redox state. In the absence of thioredoxin reductase that catalyses the reduction of thioredoxin by NADPH, Trx1 seemed to assist the formation of disulfide bonds in cytoplasmically expressed alkaline phosphatase (PhoA) [14].

Comparison of the amino acid sequence surrounding the conserved C-X-X-C motif does not lead to the identification of an extended common sequence motif for the periplasmic protein thiol:disulfide oxidoreductases and thioredoxin (Fig. 1). Only limited similarities between some members can be observed. Several protein thiol:disulfide oxidoreductases contain an aromatic residue N-terminal to the C-X-X-C motif (DsbA, DsbD, DsbG, CcmG, thioredoxin). Like in thioredoxin, this aromatic residue is a conserved tryptophan in the reducing proteins DsbD and CcmG. Moreover, in protein thiol:disulfide oxidoreductases, which either reduce a disulfide bond to a dithiol or reduce a disulfide bond in a protein for isomerisation and reoxidation, a positively charged residue follows the C-X-X-C motif (DsbC, DsbD, DsbG, CcmG, thioredoxin). By contrast, the C-X-X-C motif of DsbB and CcmH is

```
DsbA: GAPQVLEFFSFFCPHCYQFEEVLHISDN
DsbB: ALWFQHVMLLKPCVLCIYERCALFGVLG
DsbC: EKHVITVFTDITCGYCHKLHEQMADYNA
DsbD: GKPVMLDLYADWCVACKEFEKYTFSDPQ
DsbG: APVIVYVFADPFCPYCKQFWQQARPWVD
CcmG: GKPVLLNVWATWCPTCRAEHQYLNQLSA
CcmH: EQQFRQLTEELRCPKCQNNSIADSNSMI
Trx1: DGAILVDFWAEWCGPCKMIAPILDEIAD
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Fig. 1. Alignment of the sequences surrounding the active site of the periplasmic protein thiol:disulfide oxidoreductases and of cytoplasmic thioredoxin (Trx1) of *E. coli*. The strictly conserved cysteine residues are written in black boxes, while residues which show only partial similarity are shaded grey. Twelve amino acids are shown on each side of the conserved C-X-X-C motif: DsbA: aa 18–45; DsbB: aa 29–56; DsbC: aa 86–113; DsbD: aa 391–418; DsbG: aa 114–141; CcmG: aa 68–95; CcmH: aa 31–58; Trx1: aa 20–47.

neither preceded by an aromatic, nor followed by a positively charged residue. Comparison of a specific oxidoreductase with its respective homologues in other organisms reveals that the sequence in the vicinity of the C-X-X-C motif is conserved within a group of oxidoreductases. So far, the functional relevance of this sequence conservation is not known. Exchanges of charged residues in the vicinity of the active site of DsbA did not significantly influence the redox potential of the protein [15,16]. Further particular segments of the amino acid sequence that are conserved within particular subfamilies may represent surfaces for the interaction with target proteins. For example, CcmG homologues contain a short sequence of high similarity in their C-terminal, hydrophilic domain [17,18]. In the DsbD protein, several cysteines, prolines and glycines are conserved in putative transmembrane helices. The cysteine residues are located in an extended segment of sequence conservation and have been suggested to function in the transfer of reducing equivalents from the cytoplasm into the periplasm [19,20]. They are also conserved in the cytochrome *c* maturation protein CcdA of *Bacillus subtilis*, which shows overall homology to the central core of the DsbD protein [21].

Recent studies have also revealed that the function of some of the protein thiol:disulfide oxidoreductases is not limited to the oxidation and reduction of dithiols and disulfide bonds, but might be extended to a chaperone-like activity preventing aggregation of some substrate proteins independently of the presence of cysteine residues [22–25]. In this review we address the function of every so far known periplasmic protein thiol:disulfide oxidoreductase of *E. coli* and summarise the recent findings of their complex interactions to maintain the redox balance in the periplasm.

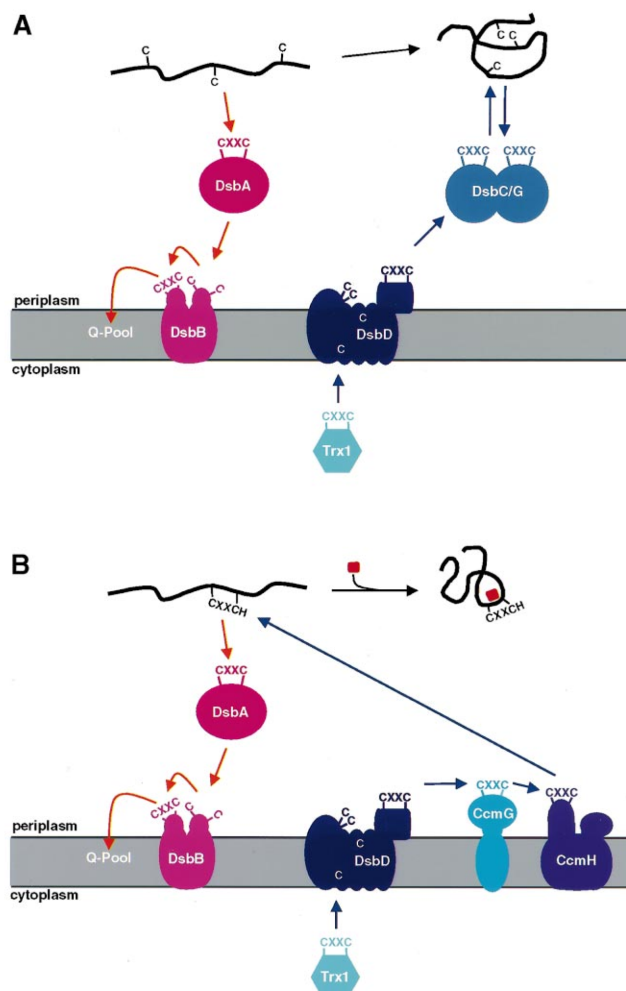

Fig. 2. Model of disulfide bond formation catalysed by various protein thiol:disulfide oxidoreductases in the periplasm and subsequent protein folding (see also [129,130]. A: Disulfide bond formation in a periplasmic protein with an uneven number of cysteine residues, which in this example are non-consecutively connected in the mature product. B: Redox pathway of cytochrome *c* maturation. Proteins with oxidative activity are shown on the left (depicted in red), while proteins with reductive/isomerisation activity are shown on the right (in blue). Red and blue arrows indicate the flow of electrons. Black arrows indicate the folding pathway of the polypeptide.

2. The oxidising pathway in the *E. coli* periplasm

2.1. DsbA

The periplasmic thiol:disulfide oxidoreductase DsbA was independently discovered by two groups [26,27], although an earlier report [28] had already pointed out the existence of a periplasmic protein disulfide oxidoreductase activity. Since then, the protein, which is responsible for the formation of disulfide bonds in newly translocated proteins, has been studied in much detail.

E. coli DsbA is a soluble, monomeric 21.1-kDa protein that contains a single catalytic dithiol/disulfide pair within the active-site sequence C-X-X-C (Figs. 1 and 2) [26,27]. The active site of the protein is trapped experimentally in the oxidation state [29,30]. DsbA catalyses the random oxidation of reduced, unfolded proteins in an extremely rapid disulfide exchange reaction [29–33]. The standard redox potential of purified DsbA (E_0') = −0.125 V is in agreement with the oxidising properties of this protein [34,35]. For comparison, thioredoxin, which functions as a disulfide reductant in the cytoplasm, has a standard redox potential of −0.270 V [10]. The oxidising power of DsbA derives from the low pK_a value of the first cysteine residue (C_{30}), which is about 3.5, suggesting that DsbA transfers its disulfide bond very easily onto substrates in thiol disulfide exchange reactions [36]. The low pK_a of C_{30} is thought to be mainly determined by the two residues (histidine and proline) between the active-site cysteines [7,8].

Insertion mutations in the *dsbA* gene (*dsbA*::*kan1*, *dsbA*::Tn*5*) lead to a pleiotropic phenotype, underlining the central role of DsbA in the periplasm. In particular,

dsbA mutants are sensitive to reduced dithiothreitol (DTT) and benzylpenicillin, and lack active alkaline phosphatase, β-lactamase and the outer membrane protein OmpA [26,27,37]. Other processes that involve proteins requiring disulfide bonds for activity are also severely affected: for example, motility due to disrupted flagellar assembly, and infection with phage M13 because of a defect in F pilus assembly [26,38]. Moreover, functional P-type, type I and type IV fimbriae are missing in *dsbA* mutants [23,39,40]. For the assembly of P fimbriae, it has been shown that not only the oxidising activity is required, but also a chaperone-like activity that is exerted apparently by DsbA [23]. This finding was supported recently by Sauvonnet and Pugsley [22], who found that secretion of pullulanase requires DsbA, but is independent of disulfide bond formation, suggesting a chaperone-like activity for DsbA. This activity of DsbA was further ascertained by in vitro refolding assays with D-glyceraldehyde-3-phosphate dehydrogenase [24]. Further phenotypes include the lack of holocytochrome *c*, defects in enterotoxin I secretion and in folding of periplasmic Cu,Zn superoxide dismutase as well as sensitivity to Cd^{2+}, Zn^{2+} and Hg^{2+} [41–45].

DsbA homologues of *E. coli* have been found in several proteobacteria of the γ-subdivision, with sequence identities between 28% and 97%: *Shigella flexneri* [46], *Salmonella typhimurium* [47], *Erwinia chrysanthemi* and *Erwinia carotovora* [48,49], *Haemophilus influenzae* [50], *Vibrio cholerae* [51,52], *Azotobacter vinelandii* [53], *Yersinia pestis* [54], *Klebsiella* sp. [55], *Salmonella typhi* and *Salmonella enteritidis* [56]. Further homologous proteins have been identified by blast search in *Pseudomonas syringae* (accession number: AAB92367), *Pseudomonas aeruginosa* (accession number: P95460), *Legionella pneumophila* (accession number: P50024) and *Enterobacter amnigenes* (accession number: AF012826.1). As far as the phenotypes of *dsbA* mutants in pathogenic bacteria have been analysed, deficiencies in the biogenesis of fimbriae, enterotoxin secretion and invasiveness have been described, making DsbA a protein of putative medical interest [22,39,40,46,52,54,57]. The *A. vinelandii dsbA* mutant strains were impossible to characterise because of their extremely poor growth [53]. It is interesting that most *dsbA*-null mutants are viable. This raises the question of whether there is another source for a remaining disulfide bond-forming activity.

2.2. DsbB

The protein that is responsible for re-oxidation of DsbA is called DsbB (Fig. 2). Its gene was identified by searching for *E. coli* mutants defective in disulfide bond formation [37,38,58]. Like *dsbA* insertion mutants, the *dsbB* insertion mutants (*dsbB*::Tn*10*, *dsbB*::*kan5*) exhibit a pleiotropic phenotype being deficient in disulfide bond formation in periplasmic proteins and sensitive to DTT and benzylpenicillin. The sensitivity to benzylpenicillin is likely due to the lack of the two disulfide bonds in the penicillin-binding protein 4, which are required for its enzymatic activity [37,59]. Cytochrome *c* maturation was also found to be inhibited in *dsbB* mutants [60,61]. Topological analysis of the 20-kDa protein revealed four transmembrane helices with both termini localised in the cytoplasm [62]. Four of the six cysteine residues, which are located in the two periplasmic domains of the protein, are required for DsbB activity, whereas the remaining two are not [62]. The two cysteine residues in the first periplasmic loop are arranged in the conserved motif C_{41}-X-X-C_{44} (Fig. 1), while the two cysteine residues located in the second periplasmic loop are separated by 25 amino acids (C_{104} and C_{130}). The hydrophilic loops of DsbB probably do not form a thioredoxin-like fold, as these domains neither are large enough nor contain conserved residues apart from the cysteines (Fig. 1). Nevertheless, evidence has arisen that DsbB functions as a thiol:disulfide oxidoreductase re-oxidising DsbA. In addition to the fact that the *dsbA* and the *dsbB* single mutants as well as the *dsbA*-*dsbB* double mutants exhibit the same phenotype, the DsbA protein accumulated in the reduced state in *dsbB* mutants [37,58]. What is more, a disulfide-bonded complex between DsbA and DsbB could be identified [63–65]. Analysis of the conditions allowing formation of such a DsbA-DsbB complex led to the proposal of the following reaction cycle for the regeneration of oxidised DsbA: the disulfide bond of C_{104}-C_{130} of DsbB is transferred onto reduced DsbA by the nucleophilic attack of C_{30} of DsbA; subsequently, C_{104} and C_{130} of DsbB are re-oxidised by intramolecular transfer of the disulfide bond of C_{41}-C_{44} [63–65]. The recycling of fully oxidised DsbB is discussed in Section 5.1.

Homologous proteins have been identified in several proteobacteria of the γ-subdivision: *S. flexneri* (accession number: Q54155), *Vibrio alginolyticus* (accession number: Q56578), *H. influenzae* [66], *P. aeruginosa* [67] as well as in proteobacteria of other subdivisions: *Burkholderia cepacia* [68], *Rickettsia prowazekii* [69] and *Campylobacter jejuni* [70]. Furthermore, a putative DsbB homologue has been identified in the Gram-positive bacterium *Bacillus subtilis* (accession number: AF021803.1) [71]. DsbB of *B. cepacia* was found to be able to restore motility and protease secretion of an *E. coli dsbB* mutant, and it was suggested that it is needed for disulfide bond formation in extracellular protease of *B. cepacia* [68].

3. Reducing power in the periplasm

3.1. DsbD

The *dsbD* gene, also called *dipZ*, was initially discovered because of its effect on cytochrome *c* biogenesis [72,73]. At the same time, another group localised the gene in the *cutA* locus, whose three gene products (CutA1, CutA2 and CutA3) seemed to confer copper tolerance to the cell [74]. The predicted polypeptide encoded by *cutA2* cor-

responds to DsbD and contains the C-X-X-C motif. This motif has also been identified as a metal-binding motif in several bacterial and eukaryotic proteins such as CopZ, CCS, Atx1, CCH, and HAH1 [75–80]. Since other sequence features of copper-binding proteins are not present, it was proposed that CutA2/DsbD might have an important chaperone-like function for an unknown protein, which in turn is directly involved in either copper uptake or efflux [74]. Independently, the *dsbD* gene has also been identified in a genetic screen as an extragenic suppressor of *dsbA* mutants: mutations in the *dsbD* gene resulted in partial phenotypic compensation of *dsbA* and *dsbB* mutations [81]. The suppression depended on the presence of DsbC [82]. DsbD is a 565-amino acid polypeptide that is integrated into the membrane with nine putative transmembrane helices [83]. At the C-terminus, DsbD contains a 16-kDa periplasmically oriented domain (Fig. 2), which shares 40–45% sequence identity with eukaryotic protein disulfide isomerase [60,81]. This domain contains a C-X-X-C motif (amino acids 480–483) that is surrounded by some conserved residues of thioredoxins (Fig. 1). Moreover, sequence alignments for several DsbD homologues revealed that four further cysteines are strictly conserved, of which two (C_{122} and C_{128}) are located in the first periplasmic loop and two on the cytoplasmic and periplasmic side of the second and fifth transmembrane helix, respectively [19,83]. These cysteines were shown to be essential for DsbD function [83]. Lack of the DsbD protein led to some of the phenotypic defects observed with other *dsb* mutants such as hypersensitivity to DTT and benzylpenicillin [81]. Whereas *dsbA-dsbD* and *dsbB-dsbD* double mutants resulted in at least a partial restoration of the wild-type phenotype, a *dsbC-dsbD* double mutant (for DsbC see Section 4.1) showed a more complex phenotype with restoration of some characteristics, but also additive effects [81]. For example, motility was not affected in either a *dsbC* or *dsbD* single mutant, but the double mutant was not as motile as the single mutants. In *dsbD* mutants, DsbA and DsbC accumulated in their oxidised forms [81]. Taken together, DsbD appears to play a reducing role in the otherwise oxidising environment of the periplasm [72,81].

DsbD homologues have also been identified in other bacteria. The most closely related ones are DsbD of *H. influenzae* [66] and DipZ of *P. aeruginosa* [19]. The latter was found to be involved in cytochrome *c* biogenesis. However, in contrast to a *dsbD* mutant of *E. coli*, holoprotein formation in a *P. aeruginosa dipZ* mutant was only attenuated [19]. Another DsbD homologue has been analysed in *S. typhimurium*, which, although it is less conserved, functions as a suppressor for copper sensitivity (ScsB), a feature that has also been observed for DsbD of *E. coli* [74,84]. In *Pantoea citrea* a gene was identified, which might be involved in the induction of pink disease in pineapple; this gene was called *dsbC*, although its gene product shares 28% amino acid sequence identity with DsbD of *E. coli* (accession number: AAD38449). Two further proteins that show weak homology to the mentioned DsbD homologues are DsbD of *Chlamydia pneumoniae* (accession number: AAD18924) and of *Chlamydia trachomatis* [85]. They possess an extension at the N-terminus, but share the thioredoxin-like motif at the C-terminus. Moreover, members of a whole family of much smaller proteins possess low similarity to the core region of DsbD, but lack the C-terminal segment with the thioredoxin-like motif. The best-studied member is CcdA of *B. subtilis*, which has been shown to be also required for cytochrome *c* maturation [21].

3.2. CcmG and CcmH: specialised oxidoreductases

Two further proteins with a conserved C-X-X-C motif have been identified in the periplasm of *E. coli*. These are CcmG, also called DsbE ([2], accession number: P33926), and CcmH. Studies devoted to cytochrome *c* biogenesis in *E. coli* led to the identification of the genes required for the formation of *c*-type cytochromes [86,87]. Homologous proteins in other bacteria are encoded by similar gene clusters like CcmG and CcmH and are also required for cytochrome *c* maturation. Both proteins have been shown to function in the redox pathway of cytochrome *c* maturation [87–90].

CcmG is a 20-kDa protein with an N-terminal membrane anchor and faces the periplasm with its hydrophilic C-terminal domain containing the active site (Fig. 2B) [89]. Around the C-X-X-C motif several additional residues are conserved between CcmG and the reductases Trx1 and DsbD, suggesting that CcmG also assumes the thioredoxin fold (Fig. 1).

Homologues of CcmG have been identified in many bacteria; the best-studied ones are HelX of *Rhodobacter capsulatus* [91,92], CycY of *Bradyrhizobium japonicum* [17,93], CcmG of *Paracoccus denitrificans* [94], and CycY in *Rhizobium leguminosarum* [95]. They are all essential for cytochrome *c* maturation. CcmG mutants of *P. denitrificans* are additionally affected in maturation of cytochrome aa_3 [94]. Further homologues have been identified in *P. citrea* (accession number: AAD19543), *H. influenzae* [66], *Pseudomonas fluorescens* [96,97], *Allochromatium vinosum* (accession number: AAB04631), and *Rhodobacter sphaeroides* (accession number: AAB61910). In particular, members of the CcmG family share a conserved sequence G-V-X-G-X-P-E in the C-terminal part of the protein that may specify protein–protein interactions [17,18]. Detailed biochemical characterisation of CycY of *B. japonicum* revealed a rather reducing redox potential for the soluble C-terminal domain ($E_0' = -0.217$ V), supporting the view that this species of thiol:disulfide oxidoreductases plays a reducing role during cytochrome *c* maturation [17].

Active-site mutants of CcmG and HelX have been tested for function in *E. coli* and *R. capsulatus*, respectively. Each cysteine residue of the active site was changed

to a serine, either individually or both together. In *E. coli*, it has been shown that *ccmG* mutants having one or both cysteine residues of the active site replaced by serine produce strongly decreased levels of holocytochrome *c*. Furthermore, cytochrome *c* maturation could be restored when CcmG active-site mutants were grown in the presence of reducing compounds such as cysteine or 2-mercaptoethanesulfonic acid. Remarkably, a *ccmG* in-frame deletion mutant was irreversibly defective [89,94,98]. In *R. capsulatus*, the active-site mutants were tested under conditions of photosynthetic growth, for which *c*-type cytochromes are absolutely essential, and shown to be lethal under these conditions [92]. It was concluded that CcmG is especially required for the reducing pathway of cytochrome *c* maturation [89].

In contrast to various other bacterial oxidoreductases, CcmG did not show thiol:disulfide reductase activity in the classical insulin reduction assay, suggesting a high specificity of CcmG for its reaction partner [17,92]. Likewise, a *ccmG* deletion mutant did not influence the activity of alkaline phosphatase (PhoA), a protein that needs to form two intramolecular disulfide bonds to assume an active conformation. By contrast, formation of a correctly disulfide-bonded PhoA structure has been shown to depend on the general thiol:disulfide oxidoreductases DsbA, DsbB, DsbC and DsbD [26,37,72,81,82,99]. The fact that CcmG does not affect the activity of alkaline phosphatase is a further hint at the high specificity of CcmG for its substrate [17,94].

CcmH is a membrane-bound protein exposing the conserved motif L-R-C-X-X-C into the periplasm (Figs. 1 and 2B) [90]. The *E. coli* CcmH protein is special in that it appears to be a hybrid of the two cytochrome *c* maturation proteins CcmH (also called Ccl2 and CycL) and CcmI (also called CycH), which are usually found in Gram-negative bacteria instead. Its N-terminal domain contains the C-X-X-C motif, whereas its C-terminal domain corresponds to CcmI. Interestingly, the C-terminal hydrophilic domain of *E. coli* CcmH was shown not to be essential for cytochrome *c* maturation [87,90]. Homologues with respect to the N-terminal domain of *E. coli* CcmH have been found in *B. japonicum* [100], *Rhizobium etli* [101], *R. leguminosarum* [102], *Sinorhizobium meliloti* [103], *R. capsulatus* [104], *P. denitrificans* [105], *P. fluorescens* [96, 97], *H. influenzae* [66], and *P. citrea* (accession number: AAD19544). The N-terminal hydrophobic sequence of the *R. capsulatus* CcmH homologue Ccl2 appears to be a cleavable signal sequence [92]. It has been suggested that the N-terminal signal sequence of CcmH of *E. coli* is also cleaved after translocation to the periplasm, thus resulting in a protein with two periplasmic domains that are linked by two transmembrane helices [90].

Under anaerobic growth conditions, when *E. coli* normally synthesises *c*-type cytochromes, only the second cysteine residue of the C-X-X-C active site is essential for cytochrome *c* maturation. However, under aerobic growth conditions, both cysteine residues are required [90]. During anaerobic nitrite respiration, *E. coli* synthesises the NrfF protein that is homologous to the N-terminal domain of CcmH and contains the C-X-X-C motif. NrfF is believed to be involved specifically in the biogenesis of NrfA, the cytochrome c_{552} subunit of the formate-dependent nitrite reductase [106,107]. The role of the CcmH homologue Ccl2 in *R. capsulatus* during cytochrome *c* maturation has also been studied [92]. Site-directed mutagenesis of the active-site cysteines did not allow growth under anaerobic photosynthetic conditions, when *c*-type cytochromes are essential.

Several lines of evidence indicate that CcmH has a reducing function during cytochrome *c* maturation. First of all, the *R. capsulatus* Ccl2 protein is able to reduce the haem-binding domain of an apocytochrome *c* peptide in vitro [92]. Secondly, active-site mutants of *E. coli* CcmH could be restored for cytochrome *c* formation with the reducing compound 2-mercaptoethanesulfonic acid [90]. Finally, Gabbert and colleagues found that expression of *ccl2* is six-fold enhanced under aerobic (i.e., more oxidising) versus anaerobic conditions [108]. It is concluded that, although the region encompassing the C-X-X-C motif does not resemble other thioredoxin-like proteins, CcmH is involved in the redox pathway of cytochrome *c* biogenesis [5,90,92]. Therefore, both proteins, CcmG and CcmH, are assigned to the family of periplasmic redox proteins in *E. coli*.

4. Isomerisation of disulfide bonds

4.1. DsbC

The *dsbC* gene was identified in a genetic selection for its ability to rescue sensitivity to reduced DTT of Tn*10*-mutagenised *E. coli* [99]. In *E. chrysanthemi*, a gene encoding the homologue of *E. coli* DsbC was independently identified by using *E. chrysanthemi* DNA libraries to complement an *E. coli* mutant with the intention of cloning the *dsbA* homologue [109]. *E. coli dsbC* mutants have a defect in disulfide bond formation in periplasmic proteins, but this defect is not as strong as in *dsbA* and *dsbB* mutants [29,99]. On the other hand, overexpression of *dsbC* almost restores the wild-type phenotype of cells lacking DsbA and/or DsbB and, reciprocally, overexpression of *dsbA* can complement the lack of DsbC. The redox state of DsbA is not affected by DsbC, implying that DsbA and DsbC constitute an independent and parallel system of thiol oxidation [99]. DsbC is a soluble, periplasmic protein that forms a homodimer consisting of two 23.5-kDa subunits (Fig. 2A). Each subunit assumes a thioredoxin-like fold containing the C-X-X-C motif, which forms an unstable, reactive disulfide [110,111]. In wild-type cells, the cysteines of the active site are found predominantly in the thiol form [20,29]. In addition, DsbC has a second set of

cysteines, which forms a putative structural disulfide bond (C_{141} and C_{163}) [111]. Biochemical characterisation of DsbC revealed that, like DsbA, DsbC has dual activities in vitro depending on its redox state. In the oxidised state, it transfers the disulfide bond of the active site onto a reduced target protein, whereas in the reduced state it can act as disulfide isomerase and shuffle mispaired disulfides [29]. However, in vivo characterisation showed that, while DsbA merely oxidises cysteines on the substrate, DsbC efficiently isomerises preformed disulfide bonds [29,81,82,111]. Sone and colleagues developed an elegant experimental system supporting the view of disulfide isomerase activity for DsbC: using alkaline phosphatase with an uneven number of cysteine residues due to mutagenesis, they identified the formation of correct as well as incorrect combinations of disulfide bonds, depending on the presence or absence of DsbC [112]. The isomerase activity of DsbC has been attributed to a stronger substrate binding than that of DsbA [113]. The reason why *dsbC* mutants show a much milder phenotype than *dsbA* mutants may be that very little disulfide isomerisation is required for growth, because only very few periplasmic and outer membrane proteins have more than two disulfide bonds per monomer [29]. Moreover, it was suggested that disulfide bond formation by DsbA might occur simultaneously with polypeptide translocation, and, if the cysteine pairings are consecutive in the primary sequence, probably only the correct ones are formed [29]. It was also reported that *dsbC* mutants lose the ability to correctly fold periplasmic proteins with multiple disulfide bonds, but are not affected in the folding and oxidation of proteins with only one disulfide bond such as the OmpA protein [82]. Recently, it was shown that DsbC can promote the in vitro reactivation of denatured D-glyceraldehyde-3-phosphate dehydrogenase during refolding. Since this enzyme does not possess any disulfide bond, the activity of DsbC in reactivation was ascribed to a chaperone activity independent from the isomerase activity [25].

Proteins displaying significant sequence identity to DsbC of *E. coli* and *E. chrysanthemi* are known from *S. typhimurium* (accession number: P55890), *H. influenzae* [66], and *P. aeruginosa* (accession number: AAC16483).

4.2. What is the function of DsbG?

Only recently, a further periplasmic thiol:disulfide oxidoreductase called DsbG has been identified in *E. coli*. This protein is synthesised as a precursor of 27.5 kDa and processed in the periplasm to a 25.7-kDa mature species (Fig. 2A) [114]. Besides sharing sequence similarity with the thiol:disulfide oxidoreductase DsbC (29%), DsbG is also homodimeric and forms a reactive disulfide bond in the C-X-X-C motif [115]. The characterisation of DsbG in vivo and in vitro led to the assumption that the protein exhibits a thiol:disulfide oxidoreductase activity in the periplasm; however, its precise function has not yet been determined [114–116]. The results obtained from its characterisation are partly inconsistent. The *dsbG* gene was identified on the basis of its ability to confer resistance to high concentrations of reduced DTT in a *dsbB* mutant, when present in multiple copies [114]. In a *dsbG* mutant background, reduced periplasmic proteins accumulated. Furthermore, the mutant cells were not viable unless *dsbA* and *dsbB* were overexpressed or oxidising compounds were added to the growth medium [114]. Andersen and colleagues proposed an oxidising function for DsbG because of the residual oxidation still observed in a *dsbA* mutant [114]. In a recent publication, Bessette and coworkers suggested that DsbG might act as a thiol:disulfide isomerase [115]. They were able to partly rescue the defect in formation of active multi-disulfide proteins in a *dsbC* mutant background through overexpression of *dsbG* [115]. In contrast to the earlier work, they were unable to confirm either the lethal phenotype of a *dsbG* mutant or its defect in folding of periplasmic proteins [114,115]. Thus, they concluded that DsbG is redundant under the tested conditions or has a limited set of substrates for which it is needed [115]. Stewart et al. reported that DsbG can be reduced by DsbD [83]. All in all, the precise function of DsbG is still not known.

5. Electron flow pathways of protein thiol:disulfide oxidoreductases

5.1. How are DsbA and DsbB re-oxidised?

Recent work of Kobayashi and co-workers suggested that the oxidative power of the DsbA/DsbB system is recycled via the electron transfer system of the respiratory chain (Fig. 2) [117,118]. *E. coli hemA* and *ubiA-menA* mutants, defective in haem and quinone biosynthesis, respectively, markedly accumulated the reduced form of DsbA in haem- or quinone-depleted cells; moreover, DsbB accumulated first in a reduced form, and then in a disulfide-linked complex with DsbA [117]. Restoration of the respiratory chain in these mutants quickly converted DsbA to the free oxidised form [117]. This study was extended by the characterisation of the redox states of the essential cysteine residues of DsbB. The two pairs of cysteines have contrasting properties. The C-terminally located disulfide bond between C_{104} and C_{130} is unstable and requires the presence of the N-terminally located disulfide bond formed between C_{41} and C_{44} [118]. By contrast, the cysteine residues of the C_{41}-X-X-C_{44} motif form a disulfide bond that seems to be refractory even to reduction by reducing agents. Remarkably, this resistance requires that DsbB is integrated into a membrane that contains the normal set of respiratory components and that oxygen is present [118]. It was assumed that the C_{41}-X-X-C_{44} motif of DsbB is maintained in the oxidised form due to the coupling to O_2 via the respiratory chain. Hence, whenever

C_{41} and C_{44} are reduced, they are re-oxidised immediately [118]. A similar model has been suggested by Bader and co-workers [119]. They showed that membranes containing catalytic amounts of DsbB can rapidly re-oxidise DsbA. As the reaction strongly depended on the presence of oxygen, they also concluded that oxygen serves as the final electron acceptor for the disulfide bond formation system of DsbA/DsbB [119]. Corroborative for the requirement of the respiratory chain for DsbA/DsbB recycling is also the recent finding that a low ubiquinone content leads to thiol sensitivity [120]. This phenotype corresponds to the thiol sensitivity observed for various *dsb* mutants. Intrigued by these facts, Bader and colleagues studied the recycling of DsbB in more detail. They proved in vitro that disulfide bond formation and respiratory electron transport are coupled directly [121]. They succeeded in demonstrating that DsbB uses quinones as electron acceptors, thus allowing various choices for electron transport to support disulfide bond formation under aerobic and anaerobic growth conditions (Fig. 2) [121]. They not only identified quinones as the direct electron acceptor of DsbB, but also solved the mystery of how DsbB is recycled under anaerobic growth conditions. In this case, other terminal electron acceptors, such as nitrate, nitrite and fumarate, accept electrons from the corresponding terminal reductases [121].

5.2. *How do electrons reach the periplasm?*

Some reactions in the periplasm involve the reduction of disulfide bonds, for example isomerisation reactions catalysed by DsbC and DsbG or the preparation of cysteine residues for the covalent ligation of haem during cytochrome *c* biogenesis (Fig. 2). Therefore, there must be a source of reducing equivalents for the otherwise oxidising environment of the periplasm. More and more evidence for an electron flow from the cytoplasm to the periplasm has accumulated recently. Mutants lacking either the cytoplasmic thioredoxin Trx1 or one of the periplasmic thiol:disulfide oxidoreductases DsbC or DsbD display similar phenotypes, implying that they function in the same pathway [82]. Alkaline phosphatase accumulated in a misfolded state in *trxA*, *dsbC* and *dsbD* mutants, but this defect was restored by the addition of low amounts of reduced DTT. Further support for the reducing pathway consisting of thioredoxin, DsbC and DsbD was obtained in an analysis of the redox state of DsbC in *trxA* and *dsbD* mutants: the DsbC protein was oxidised completely in cells lacking either Trx1 or DsbD, whereas in the wild-type, the active site of DsbC was in the reduced state [20].

The postulated flow of electrons from the cytoplasm to the periplasm is presented in Fig. 2A [20,83]. The thioredoxin reductase/thioredoxin system transfers electrons onto DsbD, which is supposed to act as a bridge between cytoplasm and periplasm. Next, DsbD reduces DsbC and other periplasmic target proteins, whose functions depend on their reduced state in the periplasm. It is still not known whether direct interactions occur between the Trx1 and DsbD and the DsbD and DsbC pairs, or whether further components are required. However, DsbD apparently plays a central role in the transport of reduction power over the inner membrane [83]: site-directed mutagenesis of the six conserved cysteines revealed that each of them contributes to the function of this protein. A mechanism of electron transduction through the membrane was proposed, which involves the passage of electrons along cysteines within DsbD. From the topological analysis it was proposed that C_{182} is the only conserved cysteine close to the cytoplasmic side of the membrane and thus likely the first electron acceptor in DsbD. A cascade of electron transfer reactions involving formation and resolving disulfide bonds between pairs of cysteines within DsbD would result in a reduced C-X-X-C motif on the periplasmic side of the membrane that can reduce oxidised C-X-X-C motifs of periplasmic disulfide oxidoreductases such as DsbC or CcmG. Although the idea of a membrane-internal, catalytically active disulfide bond that is formed transiently between cysteines close to the inner and outer face of individual transmembrane helices is attractive, it carries with it the problem of how the spatial distances are overcome. Most likely, large conformational changes of the protein would be required.

6. Cytochrome *c* biogenesis: an example of the complexity of a redox pathway in protein maturation

Cytochrome *c* maturation has been shown to require various thiol:disulfide oxidoreductases [41,61,72,88,89, 122]. *E. coli* is a facultative anaerobe that does not naturally synthesise *c*-type cytochromes during aerobic growth. During anaerobic growth, however, it can produce five *c*-type cytochromes depending on the available electron acceptor [123]. In the presence of trimethylamine-*N*-oxide (TMAO), the membrane-bound *c*-type cytochrome TorC is induced as part of the major TMAO reductase pathway [124]. Four *c*-type cytochromes are synthesised during anaerobic growth with nitrite: the *c*-type cytochromes of the formate-dependent nitrite reductase, NrfA and NrfB, and the *c*-type cytochromes of the periplasmic nitrate reductase, NapB and NapC [86,125]. They are either localised as soluble proteins in the periplasm (NrfA, NapB) or attached to the cytoplasmic membrane on the periplasmic side (NrfB, NapC). A special feature of *c*-type cytochromes is the covalent attachment of the vinyl side chains of haem to the cysteines of the conserved C-X-X-C-H motif in apocytochrome *c*, which must be reduced for haem binding. When the apoprotein is translocated to the periplasm, it is most likely to be in the reduced state, and haem addition should in principle be possible. The fact that extra reductants such as CcmG and CcmH are required for cytochrome *c* maturation [89,90] raises the

question of whether this requirement is due to a rapid, non-specific oxidation of the cysteines by DsbA. Does the CcmG/CcmH system re-reduce oxidised apocytochrome *c*, or does it prevent oxidation of newly translocated apocytochrome by DsbA? To distinguish between these possibilities one can ask whether oxidised apocytochrome *c* is an obligatory intermediate of the cytochrome *c* maturation pathway. It has been found that neither *dsbA* nor *dsbB* mutants contain detectable amounts of holocytochrome *c* [41,60,61]. Since cytochrome *c* maturation in these mutants could be rescued by the addition of disulfide compounds to the growth medium, it was inferred that the DsbA/DsbB system exerts an oxidising function during cytochrome *c* biogenesis [61]. However, under our experimental conditions we have not been able so far to reproduce the DsbA effect (R. Fabianek, unpublished results). If DsbA is indeed required for cytochrome *c* maturation, its role may be not only be to pairwise oxidise the cysteines of C-X-X-C-H motifs, but also to prefold the apocytochrome polypeptide in order to make it more accessible to haem ligation.

Along the same line, cytochrome *c* maturation has also been found to be affected in *dsbD* and *trxA* mutants [60,72,98,122]. In this case, restoration of cytochrome *c* biogenesis was achieved by the addition of thiol reductants to the medium [98,122]. It is conceivable that – as suggested for the reduction of DsbC – reduction equivalents necessary for the re-reduction of the haem-binding motif in apocytochrome *c* are passed across the cytoplasmic membrane by the thioredoxin system via DsbD. By contrast, a *dsbC* mutant was not affected in cytochrome *c* maturation. Although overexpression of DsbC complements various other *dsbA* mutant phenotypes, it does not restore formation of *c*-type cytochromes [60].

The characterisation of the effects of the general oxidoreductases on cytochrome *c* maturation in connection with the features of the two cytochrome *c* maturation-specific oxidoreductases CcmG and CcmH leads to a preliminary model for the redox pathway of cytochrome *c* maturation in *E. coli* (Fig. 2B): the newly translocated apocytochrome *c* is the target for oxidation by DsbA/DsbB, which initiate the folding pathway of the protein [41]. Subsequently, the haem-binding site must be re-reduced for haem attachment. This requires the participation of thioredoxin, DsbD, CcmG and CcmH [41,89,90,98,122]. It has been suggested that CcmH directly interacts with apocytochrome *c* by reducing the disulfide bond of the haem-binding site. Then, this interaction is resolved by CcmG, resulting in reduced apocytochrome *c* that is released for the covalent haem attachment, and in recycled CcmH. The oxidised CcmG protein is recycled via DsbD/Trx1 [90]. Although the *R. capsulatus* CcmG and CcmH homologues have been shown to interact in vitro [92], direct protein–protein interaction between components of the cytochrome *c* maturation pathway in vivo has not yet been shown. Clearly, the identification of true mixed disulfide intermediates will be required to describe a precise mechanism of the redox reactions participating in this process.

Cytochrome *c* maturation is one of several pathways for posttranslational protein modification, which requires a proper arrangement of cysteines, and therefore involves protein disulfide oxidoreductases. In contrast to processes like pilus assembly or activation of extracellular enzymes, which require the formation of disulfides, cytochrome *c* maturation demands that the polypeptide is protected from the formation of undesired disulfides, while protein folding must still occur. A balance of oxidative as well as reductive steps, exerted by general and specialised oxidoreductases, may be the solution to this complex problem. Certainly, other periplasmic proteins that use cysteines for cofactor binding and/or enzymatic activity face a similar problem, and thus may require other specific protein disulfide oxidoreductases yet to be discovered.

7. Conclusion

Recent work has resulted in substantial progress in elucidating the electron flow pathways for maintenance of catalysis of disulfide bond formation and isomerisation in the *E. coli* periplasm. It is now clear that the DsbA/DsbB system is re-oxidised via the respiratory chain. The direct acceptors of electrons from DsbB are ubiquinone or menaquinone. The advantage of the quinone molecules is their ability to move freely in the cytoplasmic membrane and to interact with a broad variety of target molecules such as dehydrogenases and quinol oxidases. Furthermore, they may also act as acceptors for the protons which emerge during oxidation of thiol groups. It remains a question of how electrons find their way from the apparently periplasmically localised C-X-X-C motif of DsbB to the quinone pool in the cytoplasmic membrane. Moreover, it is not clear how *E. coli* reoxidises DsbB under fermentative growth conditions without an added electron acceptor. Perhaps the endogenous formation of fumarate and succinate during its mixed acid fermentation might be sufficient for reoxidation of DsbB.

Reducing power for the reduction and isomerisation reactions in the periplasm is derived by an inverse electron flow from the cytoplasm via the cytoplasmic membrane into the periplasm. Participants are Trx1 in the cytoplasm, DsbD in the cytoplasmic membrane and the respective target molecules in the periplasm like DsbC, DsbG, and proteins involved in the cytochrome *c* maturation pathway. The ultimate source of the electrons seems to be NADPH, whose electrons are transferred via thioredoxin reductase to Trx1. Does direct interaction occur between Trx1 and DsbD and between DsbD and its targets? It is conceivable that DsbD functions as a mediator of electrons through the cytoplasmic membrane. Several residues are conserved in the transmembrane helices, among them

two cysteine residues, which may be able to form a reversible disulfide bond.

While the oxidative branch of the periplasmic redox network involving DsbA and DsbB is known relatively well at both the genetic and the biochemical levels, the reducing pathway(s) appear(s) to be more complex, and mostly genetic information has been gained. Direct protein–protein interactions have been described neither for the common protein thiol:disulfide oxidoreductases such as DsbD and DsbC, nor for the cytochrome *c* maturation-specific redox proteins such as CcmG and CcmH.

How special is *E. coli* in using such an elaborated network of periplasmic protein thiol:disulfide oxidoreductases to control redox-dependent protein folding? Whole genome sequences tell us that only close relatives of *E. coli* such as *H. influenzae* contain a similar set of these proteins. For example, from BLAST search analysis, DsbA appears to be restricted to the γ-proteobacteria, which raises the question of whether extracytoplasmic, enzyme-catalysed protein disulfide formation does not occur in other bacteria or is facilitated by a different system. The closest relative of DsbA in the Gram-positive *B. subtilis* is YvgV (accession number: CAB15353): with only about 20% amino acid identity over the entire length of the polypeptide it contains a putative signal sequence and the conserved C-X-X-C motif. However, it is not known whether this protein is a functional homologue of DsbA. Likewise, a putative DsbB homologue (about 26% amino acid sequence identity) containing all four conserved cysteines is present in *B. subtilis* (YolK; accession number: CAB14062), which could be the oxidant of YvgV. For the special case of cytochrome *c* biogenesis, neither of these two components has been identified in a genetic screen; rather, the CcdA protein with some similarity to DsbD, but lacking the redox-active C-X-X-C motif, was discovered [21]. Because *B. subtilis* is also missing the reducing branch of the cytochrome *c* maturation-specific oxidoreductases CcmG and CcmH homologues, it is entirely unclear how redox control for cytochrome *c* maturation is exerted in this organism.

It is not too surprising that oxidative folding of secreted proteins in the endoplasmic reticulum of eukaryotes is not controlled exclusively by glutathione and protein disulfide isomerase [126]. Recently, a protein called Ero1p has been identified, which is essential for disulfide bond formation [127,128]. Ero1p is associated with the membrane surrounding the endoplasmic reticulum and has been suggested to be functionally equivalent to DsbB [127,128]. The identification of different protein thiol:disulfide oxidoreductases in the endoplasmic reticulum indicates that this class of proteins provides a wide-spread mechanism for redox-dependent control of protein folding and maturation.

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