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Overexpression of the rhodanese PspE, a single cysteine-containing protein, restores disulfide bond formation to an *Escherichia coli* strain lacking DsbA

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Summary

Escherichia coli uses the DsbA/DsbB system for introducing disulfide bonds into proteins in the cell envelope. Deleting either *dsbA* or *dsbB* or both reduces disulfide bond formation but does not entirely eliminate it. Whether such background disulfide bond forming activity is enzyme-catalyzed is not known. To identify possible cellular factors that might contribute to the background activity, we studied the effects of overexpressing endogenous proteins on disulfide bond formation in the periplasm. We find that overexpressing PspE, a periplasmic rhodanese, partially restores substantial disulfide bond formation to a *dsbA* strain. This activity depends on DsbC, the bacterial disulfide bond isomerase, but not on DsbB. We show that overexpressed PspE is oxidized to the sulfenic acid form and reacts with substrate proteins to form mixed disulfide adducts. DsbC either prevents the formation of these mixed disulfides or resolves these adducts subsequently. In the process, DsbC itself gets oxidized and proceeds to catalyze disulfide bond formation. Although this PspE/DsbC system is not responsible for the background disulfide bond forming activity, we suggest that it might be utilized in other organisms lacking the DsbA/DsbB system.

Keywords

rhodanese; disulfide bond formation; protein disulfide isomerase; sulfenic acid; DsbA; DsbC

Introduction

The formation of disulfide bonds in proteins is an enzymatic process. In bacteria, the enzymes that catalyze disulfide bond formation exhibit a thioredoxin-like fold and contain the typical Cys-X-X-Cys active site motif of thioredoxin family proteins (Kadokura *et al.*, 2010). In *E. coli*, the enzyme that introduces disulfides is the periplasmic protein DsbA,

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Supporting information
The following supplementary material is available for this article online:
Figs. S1–S3; Tables S1–S5; supplementary experimental procedures.

which in its active form contains a disulfide bond between the two catalytic cysteines (Bardwell *et al.*, 1991). In the reaction between DsbA and substrate proteins, electrons are transferred from the substrate's cysteines to the disulfide bond of DsbA, resulting in a disulfide-bonded substrate and a reduced DsbA (Kadokura *et al.*, 2010). DsbA must be reoxidized to maintain its activity, a step carried out by DsbB (Bardwell *et al.*, 1993). DsbB, a cytoplasmic membrane protein, passes electrons from DsbA to membrane-embedded quinones (Kobayashi *et al.*, 1997). DsbA is localized to the bacterial periplasm in Gram-negative bacteria and homologues of it in Gram-positive bacteria are membrane-bound with their DsbA-like domain facing into the extra-cytoplasmic space. Since DsbA and DsbB are localized to the cell envelope, disulfide-bonded proteins are generally found only amongst proteins exported from the cytoplasm.

Although DsbA is essential for efficient disulfide bond formation, strains deleted for the *dsbA* (or *dsbB*) genes still exhibit some disulfide bond formation, albeit at rates orders of magnitude lower than strains with the DsbA/DsbB pathway intact (Bardwell *et al.*, 1991). This background activity could be due to a pathway in the cell envelope with low activity or to air oxidation due to the presence of oxygen. To test whether there may be another disulfide bond-forming pathway, we have, in the past, selected for mutants that conferred higher disulfide bond-forming activity to a Δ*dsbA* strain. This selection yielded mainly mutations in the gene *dsbD*, a protein required to maintain the protein disulfide isomerase DsbC in the reduced state (Rietsch *et al.*, 1996). Our results suggested that the oxidized DsbC that accumulated in the *dsbD* mutant was sufficient to increase disulfide bond formation, although nowhere close to the extent seen in a wild-type strain. Nevertheless, the finding that a double *dsbA dsbC* mutant still exhibited the same background oxidative activity seen in the single *dsbA* mutant indicated that the DsbC protein was not responsible for that activity.

Here, we again sought to identify an enzyme that might provide the background activity by looking for a gene which, when overexpressed using multi-copy expression plasmids constituting a clone bank of *E. coli*, could increase that activity. To our surprise, we found that overexpression of a periplasmic protein, PspE, which contains only a single cysteine, significantly increased disulfide bond formation in many proteins in a Δ*dsbA* strain. PspE belongs to the rhodanese family of proteins whose members have been implicated in certain biosynthetic pathways in the bacterial cytoplasm (Adams *et al.*, 2002; Cipollone *et al.*, 2007). The ability of overexpressed PspE to promote disulfide bond formation in the Δ*dsbA* strain requires DsbC, but not DsbB. Remarkably, the cysteine of overexpressed PspE is oxidized to the sulfenic acid form and reacts with substrate proteins to form mixed disulfide-bonded adducts. Our results suggest a novel pathway whereby this oxidized PspE cooperates with DsbC to promote disulfide bond formation in folding proteins in the periplasm.

Results

Overexpression of PspE confers motility to *E. coli ΔdsbAD* cells in a DsbC-dependent fashion

E. coli strains lacking either *dsbA* or *dsbB* are non-motile because efficient disulfide bond formation is required for the stability of FlgI, a flagellar structural protein (Dailey *et al.*, 1993). Consequently, proteins that restore disulfide bond formation significantly in these strains also confer motility. We overexpressed the *E. coli* proteome from a high-copy plasmid library (6–12 kb genomic inserts) in a Δ*dsbBCDGompA* strain to identify enzymes that possess low disulfide bond forming activity but are able to rescue motility defects when produced at high levels. In this search, we used a strain lacking DsbD and DsbC to avoid oxidized DsbC from contributing to background activity and also lacking OmpA, a highly expressed disulfide-bonded protein, as it may monopolize much of the increased activity

(Rietsch *et el.*, 1996). We also deleted *dsbG* in this strain, with the assumption that oxidized DsbG may potentially be a source of disulfide bonds as well. We isolated 17 clones with reproducibly improved motility when compared to the parent strain that were not due to restoration of the DsbA/B pathway (Table S1). Sequencing of the plasmids isolated from these clones revealed that 4 of them contain an N-terminal fragment of Braun's lipoprotein (Lpp) encoded as a chimeric fusion to a small part of the plasmid backbone. This Lpp chimera is most effective (among other clones) in restoring motility when overexpressed. However, because this construct does not produce an endogenous protein, we did not further investigate it. 8 of the 17 clones contain multiple genes on the overexpressing plasmid and we were unable to readily infer much information from them. The last 5 clones contain either the entire, or a portion of the, *psp* (*p*hage *s*hock *p*rotein) operon. The overlapping regions of these inserts suggest that *pspE* (the last gene in the operon) was responsible for the increased motility in these clones. To confirm our analysis, we constructed a plasmid containing only *pspE* and showed that overexpression of PspE alone does indeed confer motility to the Δ*dsbBCDGompA* strain (Fig. 1A, *left panel*). However, PspE overexpression did not rescue motility defects in the Δ*dsbACDGompA* strain, suggesting that DsbA may be necessary for the observed effect.

To ascertain the requirement for DsbA, we assessed the effects of overexpression of PspE on motility in a variety of *dsb* background strains. We found that while overexpression of PspE does confer motility to a Δ*dsbA* strain (Fig. 1A, *middle panel*), it does not improve motility in a Δ*dsbAC* strain (Fig. 1A, *right panel*). Motility is conferred on a strain only when one of the two proteins DsbA or DsbC is present (Fig. 1A and S1); thus, DsbA is not required for this PspE-dependent activity. Since PspE is a periplasmic protein, it appears that PspE works in conjunction with either DsbA or DsbC to improve disulfide bond formation in the periplasm. Interestingly, PspE overexpression has the most pronounced effect on the motility of a Δ*dsbAD* strain, implying that DsbC may be better able to cooperate with PspE for promoting disulfide bond formation.

Overexpression of PspE restores disulfide bond formation in *E. coli ΔdsbAD* cells

The motility assay is an indirect way for studying disulfide bond formation in the periplasm. Therefore, we asked whether PspE overexpression has a direct effect on disulfide bond formation. We examined the oxidation state of an outer membrane lipoprotein (RcsF) via chemical alkylation of free Cys in the protein. RcsF is a known substrate of DsbA (Kadokura *et al.*, 2004); the native form of RcsF contains two disulfides formed between its four Cys residues (Leverrier *et al.*, 2011; Rogov *et al.*, 2011). In the absence of *dsbA*, the myc-tagged RcsF protein is almost completely alkylated, indicating that it is in the reduced state (Fig. 1B). PspE overexpression increases the amount of oxidized (non-alkylated) RcsF in all *dsb* strains examined except the Δ*dsbAC* strain and promotes disulfide bond formation quite effectively in the Δ*dsbAD* strain, both findings consistent with results from motility assays. Using the Δ*dsbAD* strain, we further show that PspE overexpression promotes disulfide bond formation in four other DsbA substrates, YodA, ZnuA, OmpA and DppA, although not in Bla (Fig. 2) (Kadokura *et al.*, 2004). As was found for FlgI (Hizukuri *et al.*, 2006), we detected less (or no) protein when disulfide bond formation was not restored (no PspE overexpression; Fig. 2), reflecting the instability of proteins in the absence of their structural disulfide bonds which protect them against periplasmic proteases. These findings show significant generality in the PspE-dependent oxidation pathway.

Oxidized DsbC accumulates in *E. coli* Δ*dsbAD* cells when PspE is overexpressed

DsbC has a major role to play in this novel PspE-dependent oxidation pathway. The finding that disulfide bond formation promoted by PspE is most effective in a Δ*dsbA* strain where *dsbD* is also deleted is consistent with a model in which the redox state of DsbC is important

for DsbC-dependent oxidative protein folding that can occur in the absence of DsbA (Rietsch *et al.*, 1996; 1997). In the presence of DsbD, the active site cysteines of DsbC are kept reduced, allowing DsbC to function normally as a reductase or isomerase of misoxidized proteins in the periplasm. However, in the absence of DsbD, the oxidized form of DsbC would no longer be reduced and can act as an oxidant instead. DsbG and CcmG (DsbE), the two other substrates of DsbD, are not involved in this oxidation pathway, since PspE-dependent oxidation does not work without DsbA and DsbC (Fig. 1A and 1B). Furthermore, removing DsbG has no effect on disulfide bond formation upon PspE overexpression (Fig. 1A and 1B, compare Δ*dsbAG* to Δ*dsbA* strains).

To determine whether overexpression of PspE alters the redox state of DsbC's active site cysteines, we examined the oxidation state of DsbC in Δ*dsbA* and Δ*dsbAD* strains via alkylation. DsbC contains two disulfide bonds, a structural disulfide bond that is formed largely in the absence of the DsbA/B system presumable due to the background oxidative activity, and a disulfide bond in its Cys-X-X-Cys active site that is kept in the reduced state by DsbD (Rietsch *et al.*, 1997). In the absence of DsbD, the DsbC Cys-X-X-Cys active site is completely oxidized, although this effect is also dependent on the presence of DsbA and DsbB. Without the DsbA/B system, the paucity of disulfide-bonded proteins lowers the levels of misoxidized proteins that would be reduced by DsbC (itself getting oxidized), thus eliminating the need for DsbD to reduce DsbC. Therefore, in the Δ*dsbA or* Δ*dsbAD* strain, the DsbC active site disulfide bond appears to be completely reduced at steady state (Fig. 1C) (Rietsch *et al.*, 1997). Given that there is an increase in disulfide bond formation in the Δ*dsbAD* strain which is dependent on DsbC, however, there must be a small, undetectable amount of the oxidized form present. Remarkably, DsbC becomes completely oxidized when PspE is overexpressed in the Δ*dsbAD* strain (Fig. 1C). Since oxidized DsbC is capable of partially replacing DsbA in introducing disulfide bonds in substrate proteins (Rietsch *et al.* 1996; Bader *et al.*, 2001; Segatori *et al.*, 2006; Pan *et al.*, 2008), the observed effects of PspE overexpression may, in part, be due to the oxidation of DsbC. It follows that this pathway becomes really efficient in a strain deleted for *dsbD* in which DsbD is no longer available to keep DsbC in a reduced state; this is evident from the lack of oxidized DsbC at steady state when PspE is overexpressed in a Δ*dsbA* strain where DsbD is also expressed (Fig. 1C).

The active site cysteine residue in overexpressed PspE is oxidized to the sulfenic acid form

PspE is a single-domain rhodanese with a reactive cysteine in its active site that can be oxidized to form persulfide (PspE-S-SH) or sulfenic acid (PspE-S-OH) derivatives (Cheng *et al.*, 2008; Li *et al.*, 2008). These derivatives of rhodaneses have been shown to be capable of oxidizing the Cys-X-X-Cys motif of thioredoxins (Nandi *et al.*, 2000). Therefore, since DsbC is a member of the thioredoxin family, an explanation for how PspE overexpression can result in the oxidation of DsbC is that PspE directly oxidizes DsbC. To test this hypothesis, we set out to determine if overexpressed PspE is indeed oxidized in the cell. We first showed that overexpression of the Cys-to-Ala mutant of PspE eliminates the ability of PspE to confer motility to the Δ*dsbAD* strain (Fig. 3A), indicating that the active site Cys is absolutely required for activity. We then isolated periplasmic extracts from PspE-overexpressing strains pre-treated with iodoacetamide (IAM, free sulfhydryl reactive) and dimedone (sulfenic acid reactive), and analyzed the samples by mass spectrometry (MS) after EndoLysine C digestion. We identified and quantified any modification on the active site Cys of PspE by comparing the signal intensities of the Cys-containing peptide on MS using single reaction monitoring (SRM) analysis. Essentially no IAM-modified peptide (0.07%, n=5) was detected (Fig. 3B and Table S2), suggesting that the active Cys of overexpressed PspE is not a free thiol. We found that PspE is almost exclusively found

modified by dimedone (S-dimedone) (99.6%, n=5, Figs. 3B, 3C, S2 and Table S2). As dimedone is specific for sulfenic acids (Reddie *et al.*, 2008), this indicates that a significant portion of the active site Cys is converted to the sulfenic acid form, and is thus activated to react with and oxidize the normally reduced active site cysteines of DsbC. By contrast, we show that another periplasmic rhodanese YnjE, which does not confer motility to the Δ*dsbAD* strain when overexpressed (Fig. 3A), exists as a mixture of unmodified (S-IAM), sulfenylated (S-dimedone), sulfinylated (SO_2H) and sulfonylated (SO_3H) forms (Fig. 3B and Table S2). The strong correlation between the pronounced effect PspE overexpression has on the motility of the Δ*dsbAD* strain and the high levels of the sulfenylated species suggests that this oxidized form of PspE is the active species necessary for promoting disulfide bond formation through DsbC.

Overexpressed PspE reacts with substrate proteins to form mixed-disulfide adducts that are subsequently resolved by DsbC

PspE may directly oxidize DsbC, which can promote disulfide bond formation in the periplasm. If this is true, it may be possible to detect mixed disulfide-bonded intermediates between PspE and DsbC. Therefore, we overexpressed PspE (fused with a C-terminal myc-tag to facilitate detection) in various *dsb* strains and looked for cross-links between PspE and DsbC. We did not observe any PspE-DsbC adducts in this experiment. Surprisingly, however, we found that the bacteria accumulate mixed disulfide-bonded complexes between PspE and other Cys-containing proteins, particularly in the absence of DsbC (Fig. 4A, Δ*dsbAC*). Therefore, it appears that a role of DsbC is either to prevent formation of mixed-disulfide complexes formed between PspE and other (substrate) proteins or to resolve them once they have been formed (Fig. 5). If the former explanation is correct, then DsbC could do this by reducing PspE and becoming oxidized in the process. If the latter explanation is correct, the reaction of DsbC with the mixed disulfide could proceed to completion in two ways (Fig. 5). In the first model, DsbC reduces the mixed disulfide to yield reduced PspE, reduced substrate and oxidized DsbC (Shouldice *et al.*, 2010). Such a reaction could explain why we observe oxidized DsbC in the Δ*dsbAD* strain upon PspE overexpression (Fig. 1C), which can go on to catalyze disulfide bond formation in the periplasm. In the second model, DsbC forms a mixed disulfide with the substrate, resolution of which results in the formation of an oxidized substrate with its native disulfide bond and a reduced DsbC. This latter pathway is comparable to a true isomerization reaction.

We established that DsbC can act as a disulfide isomerase in this system by asking if the active site mutant of DsbC containing only the nucleophilic Cys of the Cys-X-X-Cys motif ($DsbC_{CXXS}$) is able to resolve the observed mixed-disulfide adducts (Fig. 4B). A DsbC with only its nucleophilic Cys available could attack the mixed disulfide PspE-substrate complex, yielding a DsbC-substrate mixed disulfide. However, that mixed disulfide can only be resolved by attack of a second substrate cysteine on the mixed disulfide, yielding DsbC with its single Cys and an oxidized substrate. Our results show that the DsbC mutant with only its nucleophilic Cys present can still resolve the PspE mixed disulfide adducts (Fig. 4B). Importantly, at the same time, we also demonstrate that $DsbC_{CXXS}$ confers motility to a Δ*dsbACD* strain overexpressing PspE (Fig. 4C), indicating that disulfide bond formation is partially restored with this DsbC mutant, albeit at lower efficiency than DsbC. Taken together, our data suggest that in strains with wild-type DsbC, PspE-promoted disulfide bond formation proceeds via two parallel pathways: one mediated by oxidized DsbC (following reduction of oxidized PspE or mixed-disulfides) and the other dependent on reduced DsbC functioning as an isomerase (to resolve mixed-disulfides).

PspE does not account for background disulfide bond formation in a Δ*dsbA* strain

This study was initiated to determine whether we could detect an enzyme that was responsible for the background disulfide bond formation seen in an *E. coli* Δ*dsbA* strain. To determine whether PspE might provide this activity, we deleted the *pspE* gene and introduced the deletion into a Δ*dsbA* strain. Removing *pspE* does not appear to affect motility in a wild-type or Δ*dsbA* strain (Fig. S3A). As assessed by disulfide bond formation in OmpA, in the absence of PspE protein, there is no reduction in the background activity in a Δ*dsbA* strain (Fig. S3B).

Discussion

We and others have sought alternative pathways for disulfide bond formation in the periplasm of *E. coli*, yielding information on the array of possible mechanisms for this process that may be available to organisms. Several ways of restoring higher levels of disulfide bond formation to a *dsbA* null mutation have been described. These mechanisms include attachment of a signal sequence to cytoplasmic thioredoxins (Debarbieux *et al.*, 1998; Jonda *et al.*, 1999; Masip *et al.*, 2004) or glutaredoxins to allow their export to the periplasm (Eser *et al.*, 2009). Exported wild-type thioredoxin becomes oxidized by DsbB (Jonda *et al.*, 1999), while a mutant thioredoxin with a Cys-Cys-X-Cys motif forms a dimeric complex containing an iron-sulfur center (Masip *et al.*, 2004), both of which can serve as an oxidant in the periplasm. Disulfide bonds introduced by exported glutathione likely derived from the formation of a glutaredoxin-glutathione complex in the periplasm (Eser *et al.*, 2009), which can serve as a source of oxidation of cysteines in cell envelope proteins. Other approaches to increase disulfide bond formation in the periplasm rely on increasing the levels of oxidized DsbC, which can act as an oxidant itself (Rietsch *et al.* 1996; Bader *et al.*, 2001; Pan *et al.*, 2008). These include eliminating DsbC reduction by removing DsbD (Rietsch *et al.* 1996), or allowing DsbB to oxidize DsbC by altering DsbB specificity (Pan *et al.*, 2008) or disrupting the dimeric structure of DsbC (Bader *et al.*, 2001). In this paper, we have discovered yet another way for improving disulfide bond formation through increasing the levels of oxidized DsbC. We found that overexpression of PspE, a periplasmic rhodanese, partially restores disulfide bond formation in a Δ*dsbA* strain via a process dependent on DsbC, but not DsbB. We demonstrate that the active site cysteine residue of PspE is oxidized to the sulfenic acid form, which reacts with Cys-containing proteins to form mixed-disulfide adducts (Figs. 4 and 5). DsbC resolves these adducts either by 1) reducing the mixed-disulfides, 2) catalyzing disulfide bond rearrangement or 3) reducing oxidized PspE, thus preventing formation of the mixed disulfides. In the first and third pathways, DsbC itself becomes oxidized and can proceed to introduce disulfide bonds in substrate proteins. In the second pathway, disulfide isomerization yields disulfide bonds directly in the PspE-crosslinked proteins. The end result of any of these pathways is an overall increase in disulfide bond formation in the periplasm upon PspE overexpression. Contrary to our initial hypothesis, however, we found that this PspE/DsbC disulfide bond forming system is not responsible for the background oxidative activity in Δ*dsbA* or Δ*dsbB* strains, as deleting *dsbC* (Rietsch *et al.*, 1996) or *pspE* (Fig. S3) does not eliminate residual oxidation in the periplasm.

DsbA can also work with PspE to catalyze disulfide bond formation. Qualitatively, PspE/DsbA restores motility to a Δ*dsbBC* strain (Fig. S1) to a level comparable to that conferred by PspE/DsbC in a Δ*dsbAB* strain (Fig. 1A). While DsbC can participate in this system as a reductase and isomerase, it is harder to imagine how DsbA come into play. Reduced DsbA has been shown to possess some reductase (Bardwell *et al.*, 1991) and isomerase activity (Akiyama *et al.*, 1992; Wunderlich *et al.*, 1993; Joly *et al.*, 1994) *in vitro*. Furthermore, it was reported recently that overproducing DsbA and the periplasmic chaperone/protease DegP in a Δ*dsbC* strain also lacking DsbB function enhances disulfide isomerization

activity *in vivo* (Ren *et al.*, 2011). Therefore, it is possible that DsbA behaves as an isomerase in the context of PspE-mediated oxidation *in vivo*. We think it is unlikely that DsbA acts as a reductase since oxidized DsbA would be generated and should restore motility close to wild-type levels, a phenotype that is not observed in our experiments.

It is intriguing that the effect on improving disulfide bond formation is specific to PspE but not to another periplasmic rhodanese YnjE. This discrimination may be due to the fact that the oxidation pathway proceeds via mixed-disulfides between the rhodanese domain and substrate proteins, therefore requiring that the active site Cys on the rhodanese domain be rather accessible. PspE is a single-domain rhodanese with its Cys-containing active site near the surface of the protein (Li *et al.*, 2008). In contrast, YnjE is a triple-tandem-domain rhodanese and only the last rhodanese domain contains the conserved active site Cys residue (Hanzelmann *et al.*, 2009). In the crystal structure of this protein, the second rhodanese domain sits over the active site in the third domain; therefore, despite having more than 50% (n=3) of YnjE oxidized to the sulfenic acid form, the active site may not be accessible to react with substrate proteins to form mixed disulfides and thus promote disulfide bond formation like PspE.

One interesting question is whether the PspE/DsbC system described here may be operative in other bacteria. The DsbA/B system is the most widespread disulfide bond forming pathway in Gram-negative bacteria (Dutton *et al.*, 2008). Some organisms like *Mycobacterium tuberculosis* use the bacterial VKOR homolog (vitamin K epoxide reductase) in place of DsbB as the oxidant for DsbA. Other bacteria, such as *Geobacter sulfurreducens* and *Aquifex aeolicus*, do not contain DsbA and DsbB/VKOR homologs but are predicted to have an oxidizing periplasm. Both organisms live in unique environments, so it is not surprising that they utilize a different mechanism for disulfide bond formation. To determine whether the PspE/DsbC system might be used by other bacteria, we searched in these genomes for the presence of a single-domain periplasmic rhodanese like PspE and a disulfide isomerase like DsbC, which in combination would allow the system to function. Both organisms express a single-domain rhodanese (*G. sulfurreducens*: GSU_0398 and *A. aeolicus*: aq_1599) in the periplasm, although the latter is not a clear homolog of PspE (Giuliani *et al.*, 2010). Since these bacteria are both capable of utilizing sulfur compounds for respiration, it is possible that these PspE-like rhodaneses can be easily activated to react with protein substrates. We also found DsbC homologs in *G. sulfurreducens* (GSU_0850) and *A. aeolicus* (aq_2093), but more importantly, we cannot find DsbD homologs in either organism. Both organisms only contain CcdA homologs, a class of proteins belonging to the DsbD superfamily but which are not capable of reducing DsbC-like proteins (Katzen *et al.*, 2002; Cho *et al.*, 2012). Furthermore, homologs of another recently-described DsbD superfamily member, ScsB, which can reduce DsbC-like proteins, are also not found in *G. sulfurreducens* and *A. aeolicus* (Cho *et al.*, 2012). Taken together, these observations suggest that it is possible that a system analogous to PspE/DsbC may function efficiently for disulfide bond formation in *G. sulfurreducens* and *A. aeolicus*.

The physiological role of PspE in *E. coli* is not known. Although PspE has been shown to account for 85% of the total rhodanese activity in the cell (Cheng *et al.*, 2008), deleting or overexpressing the protein (Adams *et al.*, 2002) does not confer cyanide sensitivity or resistance (a proposed role for rhodaneses), respectively, relative to wild-type cells. As a part of the *psp* operon, PspE production may be induced under stress conditions, such as filamentous phage infection (Darwin, 2005). However, it is not induced under other conditions that activate the phage shock response, such as heat stress and osmotic stress (Cheng *et al.*, 2008). In fact, PspE is also produced from its own promoter (distinct from the promoter for the *psp* operon), suggesting that it may have additional roles independent of the phage shock response (Darwin, 2005).

When dimedone was added to the culture, we found the Cys residue of PspE to be covalently modified by this molecule. As dimedone specifically modifies cysteine sulfenic acids (Reddie *et al.*, 2008), this indicates that PspE can be oxidized to a sulfenic acid *in vivo*. Moreover, quantification of the modification by SRM analysis suggests that a large part of the protein is sulfenylated in the periplasm. In a previous report, overexpressed and purified His-tagged PspE was found to exist as a mixture of free thiol and persulfide (one or two additional sulfur atoms) species, which can detoxify cyanide *in vitro* (Cheng *et al.*, 2008), but the sulfenylated form of the protein was not detected. However, detection of underivatized cysteine sulfenic acid is notoriously difficult as this modification can rearrange into other species or be further oxidized, which could explain the discrepancy. The discrepancy could also stem from differences arising from growth conditions, media, expression levels, epitope-tagging and/or modification-trapping methods employed during analysis. The fact that we did not detect the persulfide form of PspE in the SRM analysis does not rule out that this modification may also exist *in vivo*, together with the sulfenic acid derivative. However, as dimedone does not react with persulfides (K. S. Carroll, unpublished), our data indicate that sulfenylation of PspE does occur in the periplasm. This conclusion is in agreement with other reports that showed that the catalytic cysteine of proteins from the rhodanese family can be oxidized to a sulfenic acid (Nandi *et al.*, 2000), including the *E. coli* periplasmic protein YnjE which presents a sulfenic acid in its crystal structure (Hanzelmann *et al.*, 2009).

The almost complete oxidation of PspE's cysteine to the sulfenic acid form is somewhat puzzling as these bacteria are not being grown under oxidative stress conditions. The levels of sulfenylation of other periplasmic proteins containing a single cysteine residue, though not overexpressed in the cell, are significantly lower (YbiS, ~7.5%; AraF, ~5% (unpublished data)). Therefore, our observation implies that the active site cysteine of PspE is particularly prone to oxidation. How PspE becomes oxidized to the sulfenic acid form remains to be determined; one possible source of oxidation is molecular oxygen. Our finding raises questions for further studies that could focus on the structure and redox activity of the purified PspE protein itself as well as on the impact of PspE overexpression on the physiology of *E. coli*.

Experimental procedures

Strains and plasmids

Strains and plasmids used in this study are listed in Tables S3 and S4, respectively.

Motility assay

Motility of bacterial strains containing empty vector or pDSW204-*pspE* was assayed on soft agar (~0.3%) plates. A freshly-streaked colony was picked for each strain and stabbed into M63/glucose soft agar plates containing ampillicin and 1 mM IPTG (for induction of *pspE* overexpression). If strains also contain pBAD33-*dsbC* plasmids, chloramphenicol and 0.2% L-arabinose were included in the soft agar plates. Plates were then incubated (facing up) at 30 °C for > 2 days.

Chemical alkylation

Briefly, a 5-ml culture was grown to OD_{600} ~0.5 in M63/glucose minimal medium with the appropriate antibiotics at 30 °C. A 500-μl culture aliquot was then transferred to a 1.5-ml tube, and 80 μl of trichloroacetic acid (TCA, 70% in water, Sigma) was added. The mixture was incubated on ice for 20 min and precipitated proteins were pelleted at 18,000 × *g* for 10 min at 4 °C. The protein pellet was washed with 600 μl ice-cold acetone, and left to air dry at room temperature for 5 min. Following that, the TCA-precipitated proteins were either

directly subjected to alkylation or first reduced before alkylation. For reduction of disulfide bonds, TCA-precipitated proteins were incubated in 100 μl 100 mM Tris.HCl, pH 8.0 containing 0.1% SDS and 100 mM dithiothreitol (DTT, Invitrogen) for 30 min at room temperature. M63 (700 μl) medium was added and the reduced proteins were re-precipitated with TCA and further washed with acetone. Precipitated proteins (reduced or not) were then solubilized in 80 μl 100 mM Tris.HCl, pH 6.8 containing 1% SDS and 12.5 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS, Invitrogen), 12.5 mM maleimide-PEG2000 (Malpeg2k, NOF) or 20 mM *N*-ethylmaleimide (NEM, Sigma). The mixture was gently vortexed for 20 min at room temperature, and subsequently incubated for 30–40 min at 37 °C. Non-reducing 3X-SDS sample buffer (40 μl) was then added to each sample, heated for 10 min at 100 °C and 15 μl of sample was applied to SDS-PAGE directly. Tris.HCl polyacrylamide (4–20% or 12%) gels were used (running conditions: 150 V for 1 h). The proteins were transferred onto PVDF membranes and immunoblotted with the appropriate antibodies.

Mass spectrometry – single reaction monitoring (SRM)

A single colony of HK295 containing pDSW204-*pspE* was inoculated into 5 ml of M63/glucose medium containing ampicillin and grown overnight at 37°C aerobically. Cells from the overnight culture were diluted 100-fold into 60 ml of fresh M63/glucose medium containing ampicillin supplemented with IPTG (1 mM). At OD_{600} ~0.6, 600 μl of 1 M dimedone (final concentration of dimedone is 10 mM) and 6 ml of 500 mM iodoacetamide (final concentration of iodoacetamide is 50 mM) were added. The culture was then incubated for 5 min at 37°C with aeration, harvested by centrifugation at 5,000 rpm for 15 min at 4°C and the pellet was resuspended in 6 ml of TSE buffer (100 mM Tris.HCl, pH 8, 20% sucrose, 1 mM EDTA) containing 10 mM dimedone and 50 mM iodoacetamide. After 20 min of incubation at 4°C with shaking, the sample was centrifuged for 15 min at 10,000 rpm at 4°C. The 6 ml supernatant (periplasmic extract) was kept and concentrated until 600 μl.

For quantification of the relative abundance of each form, 20 μl of the periplasmic extract were TCA-precipitated and digested O/N at 30 °C in 50 μl of 0.1 M NH_4HCO_3, 2 M urea, pH 8, containing 0.5 μg of Endo Lys-C and 5 μl was analyzed by LC-MS as described (Pyr Dit Ruys *et al.*, 2012). Briefly, the mass spectrometer was operated in data-dependent mode to follow up 5 single reaction monitoring (SRM) transitions corresponding to the different thiol modifications; an internal EndoLys-C peptide from PspE was used as a reference. For setting up the SRM transitions to monitor the Cys-containing PspE peptide (VYCNAGRQSGQAK), 5 μg of recombinant PspE was alkylated with iodoacetamide (IAM) or oxidized with hydrogen peroxide in the presence of dimedone (DMD) and then digested as described above. The peptides were analysed by LC-MS/MS to allow characterization of gas-phase fragmentation for all peptide species and determination of the best daughter ion to be selected for SRM assay (see Table S2). The SRM transitions for YnjE were chosen according to the same procedure (see Table S2) except that trypsin was used to produce the Cys-containing YnjE peptide (AWNIKPEQQVSFYCGTGWR). Abundances of each molecular species were obtained by peak intensities integration and their relative abundances were calculated as a percentage from the total area for all forms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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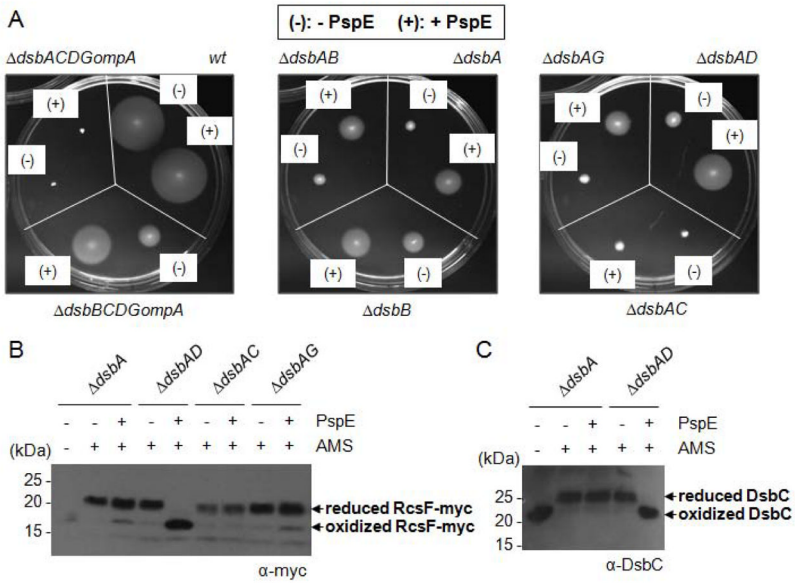

Figure 1.

Overexpression of PspE restores disulfide bond formation in *E. coli* Δ*dsbAD* cells in a DsbC-dependent fashion. (A) Plate assays to monitor motility of various *E. coli dsb* mutants overexpressing PspE. Strains overexpressing (+) or not overexpressing (−) PspE contain pDSW204-*pspE* or empty vector, respectively. Plates contain 1 mM IPTG for induction of expression. (B) and (C) Immunoblots showing the oxidation states of (B) myc-tagged RcsF and (C) DsbC in *E. coli dsb* mutants overexpressing PspE, as judged by chemical alkylation with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). All strains constitutively express myc-tagged RcsF from low copy plasmid pAM238-*rcsF-myc*. Strains overexpressing (+) or not overexpressing (−) PspE contain pDSW204(Δ*lacI*^{q})-*pspE* or empty vector, respectively. No IPTG was added during growth.

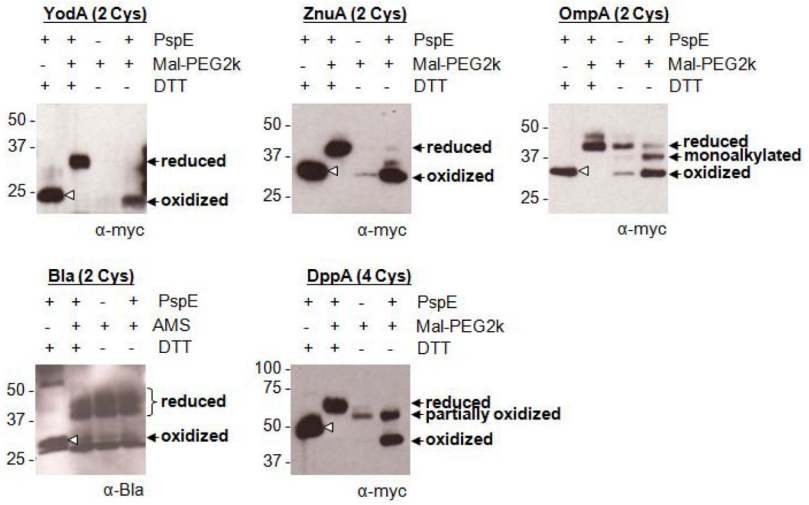

Figure 2.

Overexpression of PspE restores disulfide bond formation of known DsbA substrates in *E. coli* Δ*dsbAD* cells. Immunoblots showing the oxidation states of various DsbA substrates, as judged by chemical alkylation using AMS or maleimide-PEG2000 (Malpeg2k). Strains express myc-tagged substrates (YodA, ZnuA, OmpA, DppA) from the low copy plasmid pAM238. Strains overexpressing (+) or not overexpressing (−) PspE contain pDSW204(Δ*lacI*^{q})-*pspE* or empty vector, respectively. β-Lactamase (Bla) was expressed from the pDSW204 vectors. The number of cysteine residues in each protein is indicated in parenthesis. The first two lanes of each panel are controls to show the gel positions of reduced substrate protein before (open arrowheads) and after alkylation. The former provides a reference for the position of the oxidized substrate protein (following alkylation) for most substrates, except for YodA and DppA where the disulfide bonds cause a gel shift. The latter provides a reference for the position of reduced substrate protein (following alkylation). Dithiothreitol (DTT) was used for reducing disulfide bonds.

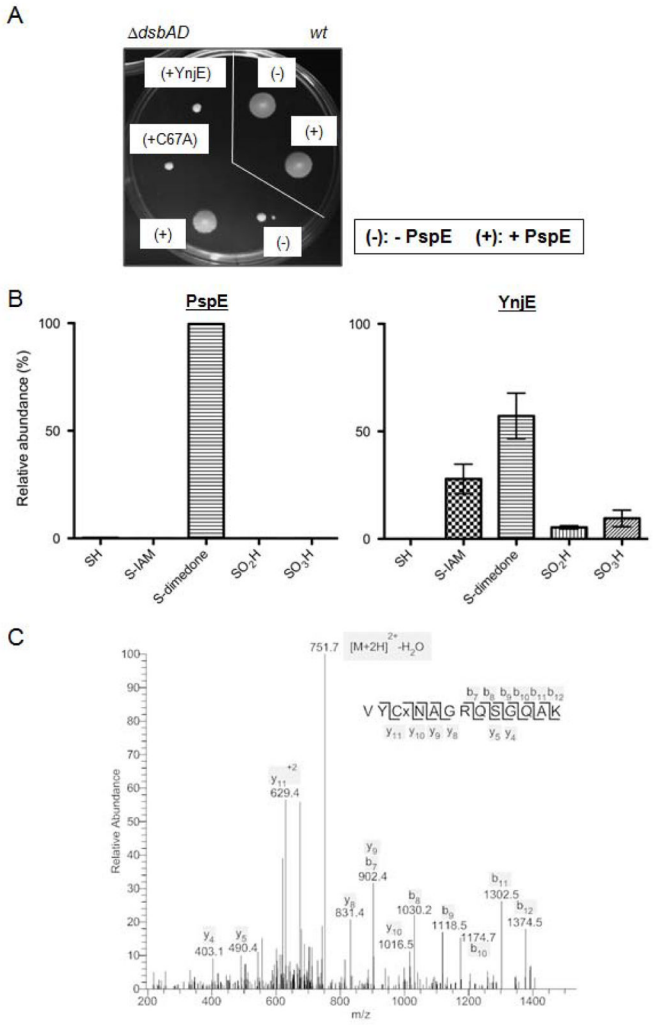

Figure 3.

The active site Cys residue in PspE is required for disulfide bond formation and is oxidized to the sulfenic acid form. (A) Plate assays to monitor motility of *E. coli* Δ*dsbAD* cells overexpressing wild-type PspE, mutant $PspE_{C67A}$ and another periplasmic rhodanese YnjE. Strains overexpressing PspE (+), $PspE_{C67A}$ (+C67A), YnjE (+YnjE) or not overexpressing (−) PspE contain pDSW204-*pspE*, -$pspE_{C67A}$, -*ynjE* or empty vector, respectively. Plates contain 1 mM IPTG for induction of expression. (B) Quantification by MS and SRM of relative peak intensities of Cys-containing peptide of overexpressed PspE (n=5) and YnjE (n=3) with different modifications. SH = free thiol; S-IAM = covalent adduct formed between SH and iodoacetamide; S-dimedone = covalent adduct formed between sulfenic acid and dimedone; SO_2H = sulfinylated; SO_3H = sulfonylated (for details, see Table S2). (C) Tandem mass (MS/MS) spectrum of the dimedone-modified Cys-containing peptide of PspE (*m/z* = 760.7 for a doubly charged ion). The mass difference between the y_{11} and y_{10} (+241 Da) fragments reveal that Cys_{67} (+103 Da) of PspE exists as a sulfenic acid modified by dimedone (+138 Da) (for details, see Fig. S2).

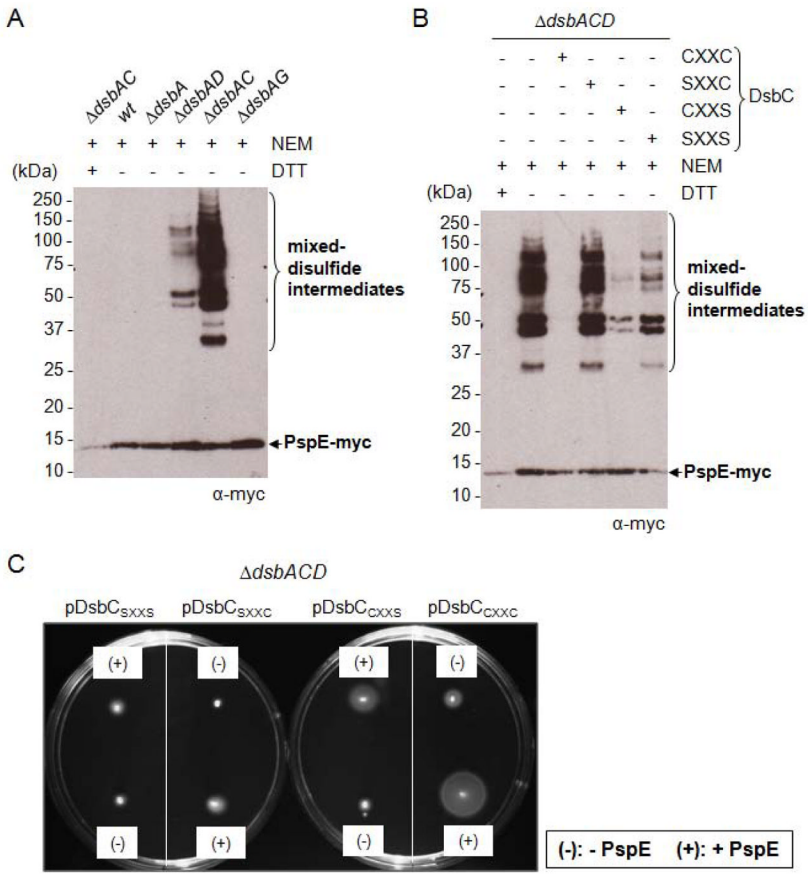

Figure 4.

Overexpressed PspE reacts with substrate proteins to form mixed-disulfide adducts that are subsequently resolved by DsbC. (A) Immunoblot revealing the formation of high-molecular-weight mixed-disulfide-bonded intermediates between myc-tagged PspE and protein substrates. (B) Immunoblot showing the effects of expressing wild-type or mutant DsbCs in *E. coli* Δ*dsbACD* cells overexpressing PspE-myc. Proteins were alkylated with *N*-ethylmaleimide (NEM). (C) Plate assays to monitor motility of *E. coli* Δ*dsbACD* cells expressing various DsbC mutants in *E. coli* Δ*dsbACD* cells overexpressing PspE. PspE-myc or PspE was overexpressed from pDSW204-*pspE-myc* or pDSW204-*pspE* (induced by 1 mM IPTG). The DsbC proteins were expressed from pBAD33-*dsbC* (pDsbC$_{CXXC}$), pBAD33-*dsbC*$_{C98S}$ (pDsbC$_{SXXC}$), pBAD33-*dsbC*$_{C101S}$ (pDsbC$_{CXXS}$) and pBAD33-*dsbC*$_{C98S/C101S}$ (pDsbC$_{SXXS}$), respectively (induced by 0.2% arabinose).

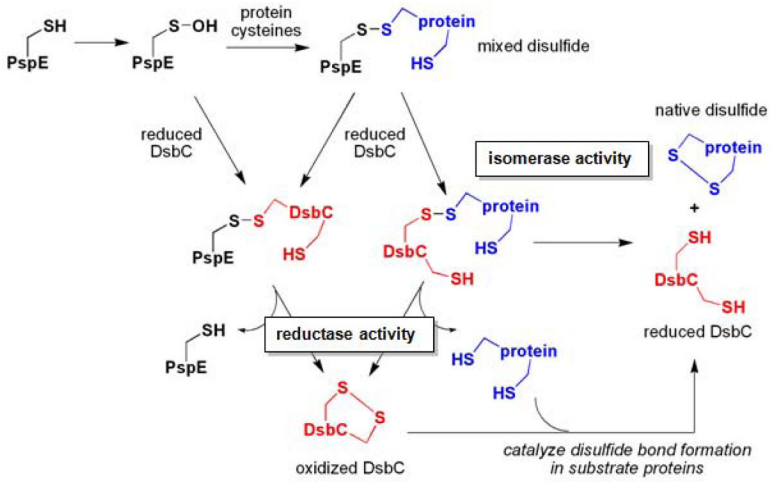

Figure 5.
The proposed PspE/DsbC pathway for disulfide bond formation in the periplasm.