A Periplasmic Reducing System Protects Single Cysteine Residues from Oxidation

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The thiol group of the amino acid cysteine can be modified to regulate protein activity. The Escherichia coli periplasm is an oxidizing environment in which most cysteine residues are involved in disulfide bonds. However, many periplasmic proteins contain single cysteine residues, which are vulnerable to oxidation to sulfenic acids and then irreversibly modified to sulfinic and sulfonic acids. We discovered that DsbG, which contains a CXXC motif, located within a thioredoxin fold, is kept reduced by DsbD. The structure of DsbG resembles that of DsbC, but the dimensions of the DsbG cleft are larger and its surface is less hydrophobic. It has thus been predicted that DsbG preferentially interacts with proteins that are folded or partially folded. However, the substrates of DsbG are not known and its function has remained obscure.

We sought to clearly define the function of DsbG by identifying its substrates. We first used a global proteomics approach to compare the proteome of a dsbG mutant strain to that of a wild type but did not find a single protein that was affected by the absence of DsbG (table S1). To trap DsbG to its substrates, we produced the DsbG\textsubscript{CXXA} mutant, in which an alanine replaces the second cysteine of the CXXC motif. This approach has been used to trap thioredoxin substrates (4). DsbG\textsubscript{CXXA} was purified under denaturing conditions. DsbG and slower migrating bands were present in the purified sample (fig. S1). Addition of the reducing agent dithiothreitol (DTT) led to the disappearance of most of these bands and the corresponding increase of DsbG, which suggests that the upper bands corresponded to DsbG bound to unknown proteins.

The complexes were separated by two-dimensional gel electrophoresis (Fig. 1B). Three periplasmic proteins, YbiS, ErfK, and YnhG, were potential substrates of DsbG. The cysteine proteins Ef-Tu, DnaK, and Fur were also identified but probably represent false positives that react with DsbG during cell lysis. Indeed, Ef-Tu has highly reactive cysteines and has also been found in a complex with DsbA (5).

The three periplasmic proteins are homologous proteins belonging to the same family of L,D-transpeptidases, which catalyze the cross-linking of peptidoglycan for cell wall synthesis (fig. S1). Because they possess a sole cysteine, essential for activity (6), these proteins are not likely in need of a disulfide isomerase but rather of a reductase to rescue their cysteine from oxidation within the oxidizing periplasm. To investigate this further, we studied the interaction between DsbC and is also a V-shaped dimeric protein, with a thioredoxin fold and a CXXC motif that is kept reduced by DsbD. The structure of DsbG resembles that of DsbC, but the dimensions of the DsbG cleft are larger and its surface is less hydrophobic. It has thus been predicted that DsbG preferentially interacts with proteins that are folded or partially folded. However, the substrates of DsbG are not known and its function has remained obscure.

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DsG and YbiS, the most active of the three L,D-transpeptidases (7).

We modified the cysteine of purified YbiS with 2-nitro-5-thiobenzoate (TNB) and monitored the reduction of this residue by following the release of the TNB anion (Fig. 2A) (8). The release of TNB was faster when YbiS-TNB was incubated with reduced DsbG than with DsbC (Fig. 2B), which suggests that DsbG catalyzes the reaction more efficiently. Thus, YbiS and, presumably by extension, the other homologous L,D-transpeptidases are substrates for DsbG.

We sought to confirm that DsbG interacts with YbiS in vivo. Expression of DsbGCXXA in a dsbA strain led to the appearance of a band of ~70 kD, detected by antibodies to both YbiS and DsbG (Fig. 2C). This band migrated with the size expected for a DsbG-YbiS complex and was sensitive to DTT. In contrast, no YbiS-DsbC complex was detected when a DsbCXXS mutant was expressed in a dsbC strain. Thus, DsbG specifically interacts with YbiS in vivo.

The fact that we trapped YbiS in complex with DsbG implied that the cysteine of YbiS oxidizes in the periplasm and suggested that YbiS requires functional DsbG to maintain its reduced, catalytically active state. To test the oxidation state of YbiS, samples were taken from dsbG, dsbC, dsbCdsbG, and wild-type strains grown in stationary phase, a condition under which reactive oxygen species (ROS) accumulate. Reduced thiols were modified with methoxy-polyethylene glycol (mPEG), a 5-kD molecule that covalently reacts with free thiols, leading to a shift on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). More oxidized YbiS was observed in dsbG strains (~60%) than in wild-type (~40%) and dsbC (~40%) strains (Fig. 2D), indicating that YbiS can be oxidized in vivo and that it preferentially depends on DsbG for reduction. The accumulation of oxidized YbiS was somewhat greater in the dsbCdsbG mutant (~70%), which suggests that DsbC is able to partially replace DsbG.

Both DsbG and DsbC depend on electrons provided by DsbD to stay reduced in the periplasm (9). In the absence of DsbD, both proteins are found oxidized and are thus inactivated (1). To confirm that inactivation of DsbG and DsbC leads to increased oxidation of YbiS by ROS, we studied the effect of dsbD deletion on the oxidation state of YbiS in mutant strains in which ROS accumulate (10). Deletion of dsbD caused increased oxidation of YbiS in a strain lacking the catalase KatE and the alkyl hydroperoxidase system AhpCF (Fig. 2E). Thus, electrons flowing from the cytoplasm to DsbG and DsbC via DsbD keep the single cysteine of YbiS reduced.

We next asked how the single cysteine residues of DsbG substrates are oxidized. Oxidized glutathione (GSSG), which is present in the E. coli periplasm (11), could potentially react with the cysteine of the transpeptidases (RSH) to form a glutathionylated adduct (RSSG). We expressed DsbGCXXA in a strain lacking gamma-glutamylcysteine synthase (gshA), the first enzyme of the glutathione biosynthesis pathway. The formation of the YbiS-DsbG complex was still observed, even when bacteria were grown in minimal media (Fig. S2). Thus, although we cannot rule out that a small fraction of YbiS may indeed be glutathionylated, S-glutathionylation is not the primary oxidation product in YbiS.

We next considered whether the cysteine of YbiS might be oxidized to a sulfenic acid (Cys-SOH) by oxidants present in the periplasm. Sulfenic acids are highly reactive groups that tend either to react rapidly with other cysteine residues present in the vicinity to form a disulfide bond or to be further oxidized to sulfonic or sulfurous acids (12). Sulfenic acids can also be stabilized by electrostatic interactions within the micro-environment of certain proteins when no other cysteine is present. YbiS reacts with a genetically encoded probe based on the redox-regulated domain of Yap1 (13), a yeast transcription factor that reacts with electrophilic cysteines such as sulfenic acids (14). Thus, the active site cysteine residue of YbiS may be prone to sulfenylation.

Because of their high reactivity, sulfenic acids are often difficult to identify. To determine whether the cysteine residue of YbiS could form a stable sulfenic acid, we used DAz-1, a probe that is chemically selective for sulfenic acids (15). In addition, DAz-1 contains an azide chemical handle that can be modified to append a biotin moiety, allowing detection of the labeled proteins by streptavidin-conjugated horseradish peroxidase (Strep-HRP). Purified YbiS was labeled by DAz-1, indicating that YbiS undergoes sulfenic acid formation in vitro (Fig. 3A). Moreover, incubation of YbiS with H2O2 led to an increase in protein labeling. The presence of the sulfenic acid modification was further verified by mass spectrometry (Fig. S3). By contrast, no labeling was observed for YbiS in a strain lacking the catalytic cysteine. Although in some proteins, such as the organic peroxide sensor OhrR (16), cysteine sulfenylates condense with a backbone amide to generate a cyclic sulfenamide, this modification was not observed with YbiS.

We confirmed that YbiS forms a sulfenic acid in vivo by labeling the oxidized protein directly in living cells using DAz-2, an analog of DAz-1 with improved potency (17). A probe that is chemically selective for sulfenic acids (15). In addition, DAz-1 contains an azide chemical handle that can be modified to append a biotin moiety, allowing detection of the labeled proteins by streptavidin conjugated horseradish peroxidase (Strep-HRP). Purified YbiS was labeled by DAz-1, indicating that YbiS undergoes sulfenic acid formation in vitro (Fig. 3A). Moreover, incubation of YbiS with H2O2 led to an increase in protein labeling. The presence of the sulfenic acid modification was further verified by mass spectrometry (Fig. S3). By contrast, no labeling was observed for YbiS in a strain lacking the catalytic cysteine. Although in some proteins, such as the organic peroxide sensor OhrR (16), cysteine sulfenylates condense with a backbone amide to generate a cyclic sulfenamide, this modification was not observed with YbiS.

We confirmed that YbiS forms a sulfenic acid in vivo by labeling the oxidized protein directly in living cells using DAz-2, an analog of DAz-1 with improved potency (17). After biotinylation of the probe, sulfenic acid-modified proteins were captured on streptavidin beads. Immunoblot analysis with antibody to YbiS shows that YbiS is present in the DAz-2–labeled protein fraction (Fig. 3B). Likewise, recombinant His-tagged YbiS could be modified in vivo by DAz-2. After enrichment on a Ni2+ column,
YbiS was biotinylated and detected by Strept-HRP (Fig. 3C). Control reactions carried out in the ybiS strain (Fig. 3B) or in the absence of DAz-2 (Fig. 3C) gave no detectable protein labeling, as expected.

To determine whether DsbG and DsbC control the level of sulfonylation in the periplasm, wild-type, dsg, dsbc, and dsbcDsgb strains were grown in stationary phase, sulfenic acids were labeled in living cells, and periplasmic extracts were prepared. After biotinylation of DAz-2, samples were analyzed by immunoblot using both Strept-HRP and an antibody to YbiS. Several periplasmic proteins were labeled by DAz-2, including a band migrating at the same position as YbiS (Fig. 4). This in vivo snapshot of the global sulfenic acid content of a subcellular compartment reveals that sulfenic acid formation is a major posttranslational modification in the periplasm. Although the biotinylated bands were observed in periplasmic extracts prepared from all strains, the labeling was more intense in the samples prepared from dsbcDsgb mutants. This indicates that DsbG and DsbC are part of a periplasmic reducing system that controls the level of cysteine sulfonylation in the periplasm and provides reducing equivalents to rescue oxidatively damaged secreted proteins.

We propose the following model for the control of cysteine sulfonylation in the periplasm and the protection of single cysteines in this oxidizing compartment (fig. S4). In the periplasm, many proteins contain an even number of cysteines (18) that form disulfide bonds and are thus protected from further oxidation. However, some proteins, including YbiS, contain either a single cysteine or an odd number of cysteines (18). Because they are not involved in disulfides, these cysteines would, without protection, tend to be vulnerable to oxidation and form sulfenic acids. Sulfenic acids, unless they are stabilized within the micro-environment of the protein, are susceptible to reaction with small molecule thiols to form mixed disulfides, as in the organic peroxide sensor OhrR (16), or to further oxidation to sulfinic and sulfonic acids. Oxidizing a catalytically active thiol inactivates the protein, necessitating a system in the periplasm that could rescue single cysteine residues from oxidation. DsbG, whose negatively charged surface is better suited to interact with folded proteins, appears to be a key player in this system. DsbC, whose inner surface is lined with hydrophobic residues, seems to be designed to interact with unfolded proteins to correct non-native disulfides. In parallel to this function, DsbC could also serve as a backup for DsbG. Both DsbC and DsbG are kept reduced by DsbD. Thus, the electron flux originating from the cytoplasmic pool of reduced nicotinamide adenine dinucleotide phosphate provides the reducing equivalents required for both the correction of incorrect disulfides and the rescue of sulfenylated orphan cysteines. Sulfenic acid formation is pervasive in certain eukaryotic cells, both as unwanted products of cysteine oxidation by ROS and in enzyme catalysis and signal transduction (14, 19). Proteins from the thioredoxin superfamily are widespread and have been identified in most genomes. Thus, some of these thioredoxin superfamly members may play similar roles in controlling the global sulfenic acid content of eukaryotic cellular compartments.

Fig. 3. YbiS forms a stable sulfenic acid in vitro and in vivo. (A) Sulfenic acid modification of YbiS or YbiS<sub>C186A</sub>, untreated or exposed to one or three equivalents of H<sub>2</sub>O<sub>2</sub>. Sulfenylation was detected by immunoblot with Strept-HRP. Equal protein loading was verified by reprobing with antibody to YbiS. Detection of (B) endogenous and (C) recombinant YbiS labeled in vivo with DAz-2.

Fig. 4. Protein sulfenic acids accumulate in the periplasm of dsbcDsgb strains. The asterisks denote a band corresponding to YbiS.

References and Notes
20. We thank G. Connerotte and H. Degand for technical help, and E. VanShaftingen, M. VeigadaCunha, F. Swisser, P. Leverrier, A. Hiniker, J. Bardwell, and U. Jakob for discussions. J.F.C. and P.M. are Chercheur Logistique of the Fonds de la Recherche Scientifique. M.D. is a research fellow of the Fonds pour la formation à la Recherche dans l’Industrie et dans l’Agriculture, and J.M. is a project leader of the VIB. This research was supported by grants from the FNRS to J.F.C. and from the Leukemia and lymphoma Society (Special Fellows Award 3100-07) and the American Heart Association (Scientist Development Grant 0835419N) to K.S.C.
21. Conflict of Interest Statement: Patent protection has been applied for for the DAz-1 and DAz-2 chemical probes. These compounds will soon be commercially available from Cayman Chemical (Ann Arbor, MI, USA). For inquiries regarding the DAz probes: katesc@umich.edu.

Supporting Online Material
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Materials and Methods
Figs. S1 to S4
Tables S1 to S4
References