Oxidative stress, protein damage and repair in bacteria

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Abstract | Oxidative damage can have a devastating effect on the structure and activity of proteins, and may even lead to cell death. The sulfur-containing amino acids cysteine and methionine are particularly susceptible to reactive oxygen species (ROS) and reactive chlorine species (RCS), which can damage proteins. In this Review, we discuss our current understanding of the reducing systems that enable bacteria to repair oxidatively damaged cysteine and methionine residues in the cytoplasm and in the bacterial cell envelope. We highlight the importance of these repair systems in bacterial physiology and virulence, and we discuss several examples of proteins that become activated by oxidation and help bacteria to respond to oxidative stress.

Aerobic environments
Environments that are characterized by the presence of free molecular oxygen (O_2).

Univalent electron donors
Compounds that transfer electrons (one at a time) onto an electron acceptor.

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The gain of single electrons by oxygen (O_2) generates partially reduced reactive oxygen species (ROS), including superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), which can damage cells and cause cell death. In bacteria that grow in aerobic environments, ROS can form endogenously through the reaction between O_2 and univalent electron donors, such as metal centres, dihydroflavin cofactors ($FADH_2$ cofactors) and quinones[1]. In particular, ROS, reactive nitrogen species (RNS) and reactive chlorine species (RCS) arise in environments that are hostile to bacteria; for example, in phagocytic cells such as macrophages and neutrophils[2–4].

The exposure of cells to ROS, RNS and RCS damages cellular components, including DNA, membrane lipids and proteins. Therefore, most organisms produce catalases, peroxiredoxins and superoxide dismutases, which are enzymes that react with harmful oxidants and convert them to harmless products by neutralizing them before they cause damage to cellular components[1]. Oxidative damage can result in covalent modifications that destabilize and inactivate proteins. For example, arginine, lysine, proline and threonine can be carbonylated[5], histidine can be modified to oxo-histidine[6], and tyrosine can react with RNS to form nitrotyrosine[7]. The amino acids cysteine (Cys) and methionine (Met) are particularly sensitive to oxidation owing to the electron-rich sulfur atom in their side chain. In this Review, we will first describe the mechanisms of Cys and Met oxidation in proteins and then focus on the systems that repair oxidatively damaged proteins in the cytoplasm and the bacterial cell envelope. We will also discuss how some bacterial proteins become activated through oxidation. Last, the role of Cys and Met repair pathways in bacterial physiology and virulence will be discussed.

Mechanisms of Cys and Met oxidation

Oxidation of Cys residues. The Cys side chain is a powerful nucleophile owing to the presence of a thiol (–SH) functional group. Thiols are mildly acidic, because of the weakness of the S–H bond. The pK_a of free Cys is ~8.6 (REF. 8), but in proteins this value can be substantially modified. For example, the presence of positively charged residues in proximity to a thiol may decrease its pK_a value by 3–4 units, which favours its deprotonation and the formation of a thiolate ($–S^-$) at physiological pH. Thiolates are more potent nucleophiles and readily react with electrophilic species and oxidants. Reactions with two-electron oxidants, such as peroxides and peroxynitrite, form sulfenic acids (–SOH) (FIG. 1a), whereas reactions with one-electron oxidants, such as $O_2^{\bullet-}$ and $OH^{\bullet}$, produce thiyl radicals ($RS^{\bullet}$). Thiyl radicals can also react with $OH^{\bullet}$ to form sulfenic acids[9]. Sulfenic acids are highly reactive species that either form a disulfide bond (S–S) with a proximal Cys or are irreversibly oxidized to sulfinic acid ($–SO_2H$) and sulfonic acid ($–SO_3H$)[10] (FIG. 1a). In addition, sulfenic acids can react with low-molecular-weight thiols or they can be stabilized in specific protein environments by polar amino acids or specific hydrogen bonds[11]. The reactivity of thiol groups with oxidizing molecules varies depending on the oxidant and the accessibility of the thiol reactive group. We refer

Metal centres
Metal atoms that are required for the structure or catalytic action of certain proteins.

Dihydroflavin cofactors
($FADH_2$ cofactors). Reduced forms of flavin adenine dinucleotide (FAD), which is a covalently bound redox moiety that is required by certain oxidoreductases to exert their function.

Quinones
Organic compounds that are composed of a polar redox-active head group coupled to a lipid side chain that varies in both length and degree of saturation. Quinones primarily function as electron transporters.

Oxidants
Compounds that cause other molecules to lose electrons.

Oxidation
A process in which electrons are lost by a molecule.

Nucleophile
A chemical species that is attracted by a positive charge and is able to donate a pair of electrons.

pK_a
The negative logarithm of the acid dissociation constant (pK_a = –log K_a). pK_a is a quantitative measure of the strength of an acid in solution; the lower the pK_a value, the stronger the acid.

Deprotonation
The removal of a proton (a hydrogen cation (H^+)) from an acid in an acid–base reaction. The species formed is the conjugate base of that acid.

Thiolate
A deprotonated form of a thiol functional group.

Electrophilic species
A chemical species that has affinity for electrons.

Low-molecular-weight thiols
Highly reactive non-protein compounds that contain thiol functional groups (–SH), such as glutathione (GSH), mycothiol or bacillithiol.

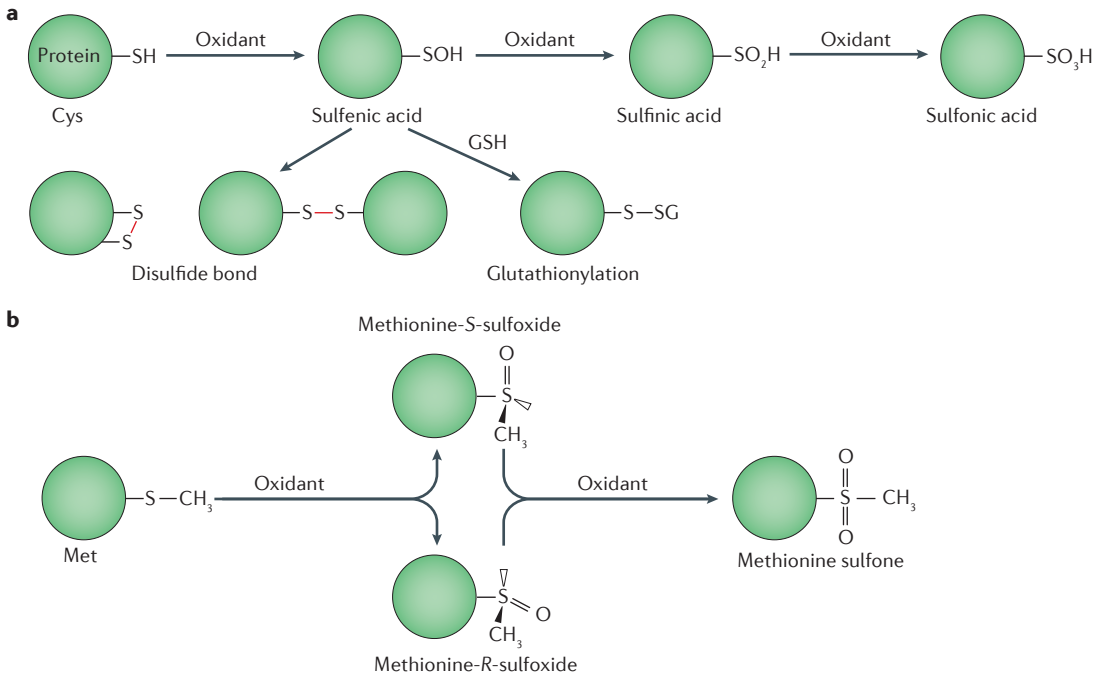


Figure 1 | **Oxidation of Cys and Met residues by oxidants. a** | Cys oxidation. Cys residues (–SH) can be oxidized (for example, by reactive oxygen species (ROS), reactive chlorine species (RCS) and reactive nitrogen species (RNS)) to sulfenic acids (–SOH), which can be further oxidized to sulfinic acid (–SO_2H) and sulfonic acid (–SO_3H). Alternatively, sulfenic acid can react with a second Cys residue to form an intra-disulfide or inter-disulfide bond (S–S; highlighted in red), or with glutathione (GSH) to form a glutathionylated protein (S–SG). **b** | Met oxidation. The oxidation of Met (–S–CH_3) produces methionine sulfoxide (–SO–) in either the (*R*-) or (*S*-) diastereoisomeric form, owing to the presence of an asymmetric sulfur centre. Further oxidation produces methionine sulfone (–SO_2–).

the reader to the following reviews for more information on the chemical mechanisms that are involved and examples of cellular processes that involve the formation of sulfenic acid (see REFS 9,12,13).

Oxidation of Met residues. The sulfur atom in the thioether side chain of Met can be oxidized to sulfoxide, thereby converting Met into methionine sulfoxide (Met-O)[14]. Met-O has two diastereoisomeric forms (*R*- and *S*-)[15,16] (FIG. 1b). Met-O residues can be further oxidized to methionine sulfone (Met-O_2)[14,17], which is an irreversible and rare modification[17]. Most biologically relevant oxidants convert Met to Met-O in either one-electron or two-electron oxidation reactions[14,18]. One-electron oxidation leads to the formation of a sulfide radical cation intermediate, to which a second electron can be transferred to produce Met-O, whereas two-electron oxidation directly leads to the formation of Met-O. The rates at which oxidants react with Met vary; for example, the rate of reaction with $OH^•$ and hypochlorous acid (HOCl) is rapid[19,20], whereas the rate is slower with peroxynitrite ($ONOO^-$) and H_2O_2 (REFS 14,21).

Repair of Cys and Met in the cytoplasm

Repair of oxidized Cys residues. In the cytoplasm, oxidized Cys residues are reduced to the thiol state by various oxidoreductases, including thioredoxins (Trxs) and glutaredoxins (Grxs). Trxs from all domains of life share a highly conserved protein fold that is composed of five β-strands surrounded by four α-helices, and a CXXC catalytic motif[22] (FIG. 2a). The amino-terminal Cys of this motif, which is mostly present as a thiolate under physiological conditions, initiates the reduction reaction by attacking an oxidized substrate and then forms a mixed-disulfide intermediate[22] (FIG. 2a). This reaction triggers the deprotonation of the second Cys residue, which is stabilized by hydrogen bonds that are formed with backbone amides of active site residues[22]. The deprotonated Cys then engages in nucleophilic attack of the mixed-disulfide intermediate, which releases an oxidized Trx and a reduced substrate with a thiol group. Regeneration of the reduced CXXC motif in Trx is carried out by thioredoxin reductase, which is a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent flavoenzyme[22] (FIG. 2a).

Escherichia coli encodes two Trxs, thioredoxin 1 (Trx1) and Trx2. Trx1, which is expressed at higher levels than Trx2 under physiological conditions, reduces disulfide bonds that form in the catalytic intermediates of enzymes such as ribonucleotide reductase, phosphoadenosine-phosphosulfate (PAPS) reductase, thiol peroxidase and bacterioferritin comigratory protein (Bcp). The reduction of these catalytic intermediates is essential for the maintenance of the catalytic activity of these enzymes. Recently, 257 proteins that are involved in many cellular processes, including energy metabolism, biosynthesis, transcription and regulation of cell shape, were found to require Trx1 for activity.

Diastereoisomeric forms
The forms adopted when a molecule has multiple chiral centres. Methionine sulfoxide (Met-O) contains two chiral centres: the α-carbon and the sulfur. Met-*S*-O and Met-*R*-O refer to the (*S*-) and (*R*-) configurations of the sulfur, respectively.

Oxidoreductases
Enzymes that catalyse electron transfer from a donor (reductant) to an acceptor (oxidant).

Reduction
A process in which electrons are gained by a molecule.

Mixed-disulfide
An intermolecular covalent bridge (–S–S–) formed between two thiol groups from two different proteins or peptides.

Iron–sulfur clusters
Inorganic prosthetic groups that are composed of iron and sulfur atoms, which can act as catalysts, redox sensors or structural elements.

Glutathione
A major redox buffer. Glutathione reduces disulfide bonds by acting as an electron donor. Once oxidized, glutathione can be reduced by glutathione reductase, using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. Glutathione is composed of a tripeptide of L-cysteine, L-glutamate and glycine. In its oxidized form, two glutathione tripeptides are connected by a disulfide bond.

It was hypothesized that these proteins contain Cys residues that are sensitive to oxidation and therefore require Trx1 to repair oxidative damage and thus maintain their activity and function[23]. By contrast, the function of Trx2, which is a unique zinc-containing Trx[24], is poorly defined. However, the expression of Trx2 is induced by OxyR (BOX 1), a H_2O_2 sensor, which suggests that Trx2 carries out a specific function during oxidizing conditions[25,26].

E. coli mutants that lack Trx1 and Trx2 can survive oxidative damage owing to the activity of Grxs[27]. Grxs from all domains of life share some structural similarity to Trxs[28,29]. Most Grxs contain a CXXC catalytic motif and are therefore referred to as dithiol enzymes, whereas the rest have a CXXS catalytic motif and are referred to as monothiol enzymes[29]. In *E. coli*, the dithiol Grx1, Grx2 and Grx3 enzymes are involved in the reduction of oxidized Cys in substrate proteins[29], whereas the monothiol Grx4 enzyme contributes to the assembly of iron–sulfur clusters[30]. Similarly to Trxs, dithiol Grxs react with oxidized proteins and become oxidized when the target disulfide is reduced (FIG. 2b). Dithiol Grxs are then reduced by glutathione (GSH; a low-molecular-weight thiol), which reacts with the first Cys in the CXXC motif to form a mixed-disulfide intermediate. This mixed-disulfide intermediate then reacts with a second GSH molecule, which results in the release of a reduced Grx and an oxidized glutathione molecule (GSSG). GSSG is reduced to GSH by glutathione reductase, which is an NADPH-dependent enzyme (FIG. 2b).

GSH, which is present at high intracellular concentrations[27], reacts with ROS, RCS and RNS and acts as a redox buffer to protect cells from oxidative damage. In addition, GSH can prevent the irreversible oxidation of Cys by reacting with sulfenic acids in substrate proteins, forming glutathione–protein mixed disulfides[12], and therefore prevents the formation of sulfinic acids. Some bacteria use alternative low-molecular-weight thiols to protect proteins from oxidative damage, such as mycothiol in *Mycobacterium* spp.[31] and bacillithiol in *Bacillus* spp.[32].

Repair of oxidized Met residues. The repair of oxidized Met is carried out by methionine sulfoxide reductases (Msrs), which are found in most living organisms[33].

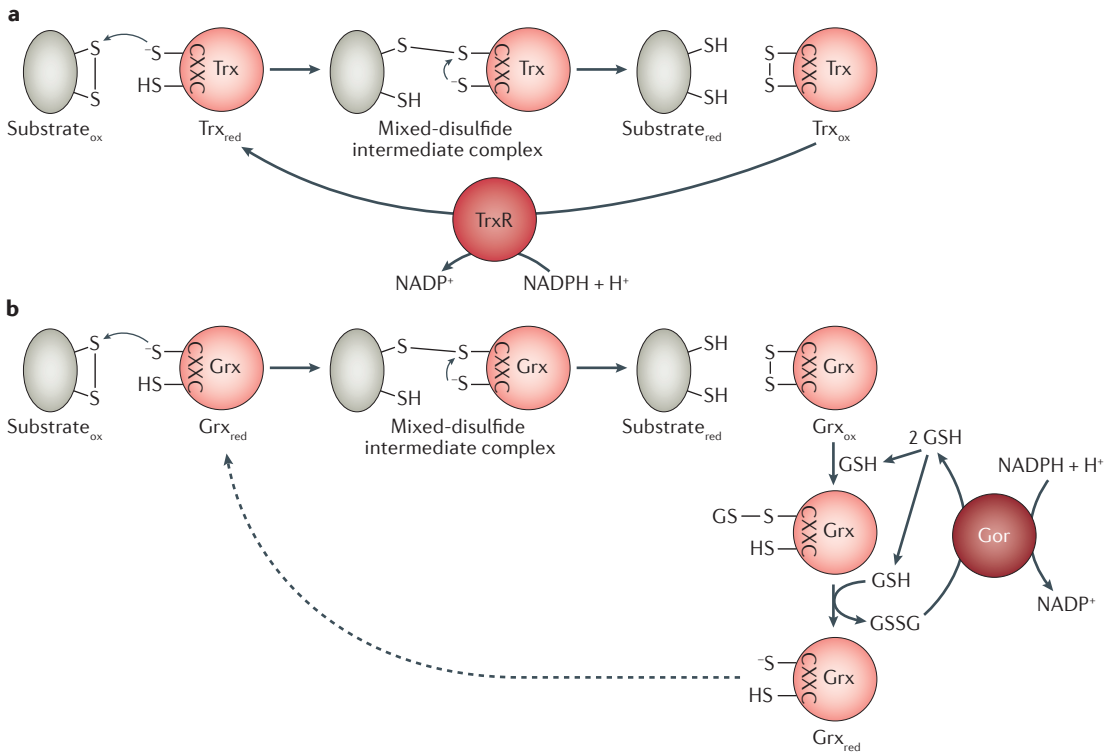
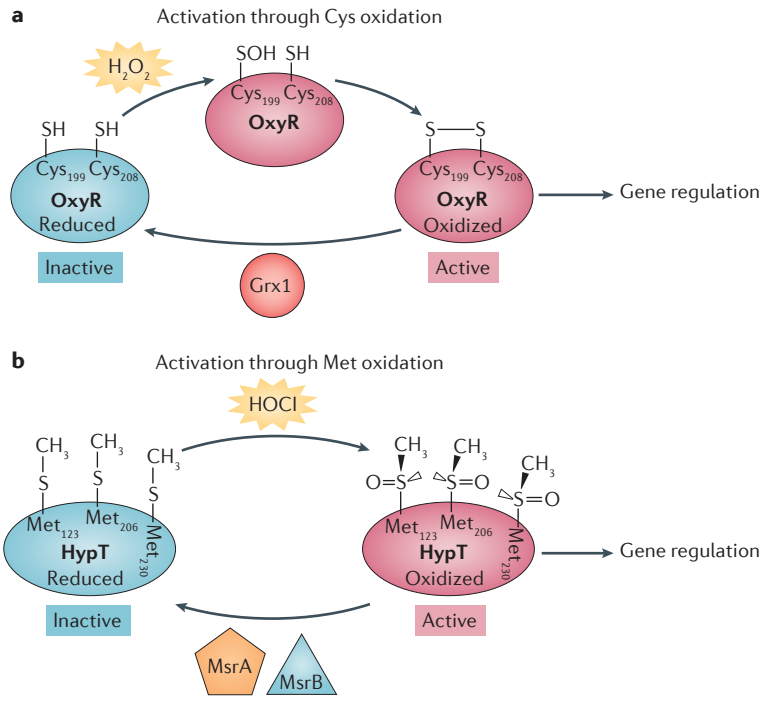


Figure 2 | **Mechanisms of Trx and Grx activity. a** | Reduction of a disulfide bond in a substrate protein by a thioredoxin (Trx). The reaction begins with nucleophilic attack by the amino-terminal cysteine of the CXXC motif on the disulfide bond in the oxidized substrate protein. Consequently, a mixed-disulfide intermediate complex is formed that is reduced through nucleophilic attack by the carboxy-terminal cysteine of the CXXC motif. This action releases a reduced substrate protein and an oxidized Trx enzyme, which is recycled by a thioredoxin reductase (TrxR). **b** | Reduction of a disulfide bond in a substrate protein by a glutaredoxin (Grx). The reaction begins with nucleophilic attack by the N-terminal cysteine of the CXXC motif of dithiol Grxs on a disulfide bond in the oxidized substrate protein. Consequently, a mixed-disulfide intermediate complex is formed between Grx and the substrate protein that is reduced through nucleophilic attack by the C-terminal cysteine of the CXXC motif. This action releases a reduced substrate and an oxidized Grx. The oxidized Grx is then recycled by two glutathione (GSH) molecules, generating an oxidized glutathione molecule (GSSG). Reduction of GSSG to GSH is carried out by a glutathione reductase (Gor). Subscripted 'red' and 'ox' represent reduced and oxidized forms of a molecule, respectively.

Box 1 | **Oxidation of Cys and Met can activate protein functions**

The oxidation of Cys residues to sulfenic acids or disulfides, or the oxidation of Met to methionine sulfoxide (Met-O), can lead to the activation of protein functions (see the figure). Similarly to the phosphorylation and dephosphorylation cycles of proteins, sulfenylation of specific Cys residues modulates signal transduction pathways by modifying the activity and function of cellular proteins. Sulfenylation modulates the activity of several prokaryotic and eukaryotic proteins[136–141], and enables the cell to respond to the redox status of specific cellular environments. A milestone in this field was the discovery of the oxidative stress-activated bacterial transcription factor OxyR, which positively regulates the expression of several antioxidant genes, including those that encode peroxiredoxins and catalases[13,26] (see the figure, part **a**). In *Escherichia coli*, a Cys residue in OxyR (Cys199) can be oxidized to sulfenic acid, which can then react with a second Cys residue (Cys208) to form an intramolecular disulfide bond, resulting in conformational changes that enable OxyR to function as a transcriptional activator. OxyR can now bind to DNA and assist RNA polymerase in initiating transcription[142,143]. Glutaredoxin 1 (Grx1) subsequently deactivates OxyR by reducing the Cys199–Cys208 disulfide bond[143]. Grx1 and glutathione reductase, which indirectly contributes to Grx1 recycling, are induced by OxyR during oxidative stress, resulting in a negative feedback loop that inactivates OxyR. Similarly, protein functions can be activated through the oxidation of Met (see the figure, part **b**). For example, in *E. coli*, hypochlorite-responsive transcription factor (HypT) positively and negatively regulates the expression of several genes in response to HOCl oxidation. Most of the genes that are positively regulated are involved in the biosynthesis of Cys and Met, whereas most of the genes that are negatively regulated are involved in iron acquisition and homeostasis[144]. This could indicate that the intracellular pool of Cys and Met must be replenished in response to oxidative damage, whereas the intracellular concentration of iron, which could enhance the production of reactive oxygen species (ROS), must be kept at low levels. In *E. coli*, for the activation of HypT, three Met residues (Met123, Met206 and Met230) must be oxidized. Substitution of these three Met residues with Met-O mimics (glutamine residues) resulted in a constitutively active HypT variant that enhanced resistance to oxidative stress and decreased intracellular levels of free iron[145]. HypT has different multimeric forms. On binding to DNA, the dodecameric ring-like structure of HypT dissociates into an active tetrameric form that acts as a transcriptional activator. The current model proposes that the oxidation of Met residues promotes the transition of the inactive dodecameric form of HypT to the active tetrameric form[145,146]. Methionine sulfoxide reductase A (MsrA) and MsrB are required to reverse the oxidation state of the oxidized Met residues, thus inactivating the activity of HypT[145].



Whether organisms that lack Msrs experience Met oxidation and use alternative repair systems is unknown. In *E. coli*, four cytoplasmic Msrs have been identified, methionine sulfoxide reductase A (MsrA), MsrB, MsrC and biotin sulfoxide reductase (BisC)[34–38]. MsrA and MsrB can reduce both free Met-O and Met-O in proteins, whereas BisC and MsrC can only reduce free Met-O. MsrA and MsrB share a similar phylogeny that reveals their simultaneous emergence after O_2 built up in the atmosphere[33,39].

Msrs have a strict stereospecificity for their substrates; MsrA reduces Met-*S*-O, whereas MsrB reduces Met-*R*-O[36,40,41]. Consequently, the full repair of an oxidized protein requires the action of both MsrA and MsrB (FIG. 3b). Despite structural and sequence dissimilarities between MsrA and MsrB, they both use a similar catalytic mechanism for the reduction of Met-O[42–45]. In the first step, a nucleophilic Cys residue (Cys_A) attacks a Met-O substrate, which leads to the formation of a sulfenic acid (–SOH) group on Cys_A and the release of reduced Met. In the second step, a nucleophilic Cys residue (Cys_B) attacks Cys_A–SOH, which leads to the formation of an intramolecular disulfide intermediate and the release of a water molecule. In the third step, the intramolecular disulfide intermediate is reduced by a Trx protein and a catalytically active Msr enzyme is regenerated (FIG. 3a). The importance of Trxs in the regeneration of catalytically active Msrs is shown by the absence of Msr activity in *E. coli* mutants that lack Trx1. Indeed, mutants that lack Trx1 cannot use Met-O as a source of Met[38,46].

There are several similar alternative catalytic mechanisms that are used by Msrs to repair oxidized proteins. For example, some MsrA enzymes have an additional recycling Cys residue (Cys_C) that attacks the Cys_A–Cys_B disulfide bond and forms a Cys_B–Cys_C disulfide, which is then reduced by Trx[47]. Alternatively, some MsrB enzymes have just one catalytic Cys[47]. For these MsrB enzymes, it remains unclear how the sulfenic acid group is reduced *in vivo*. Regardless of the catalytic mechanism that is used by Msrs, the rate-limiting step is, for the enzymes for which it has been measured, the Trx-dependent regeneration of a catalytically active enzyme[47]. Notably, some Msrs, mostly found in eukaryotes, have a selenocysteine (Sec) instead of a Cys as the catalytic residue[47]. Some studies suggest that the presence of a catalytic Sec instead of a Cys maximizes the catalytic efficiency of these Msr enzymes[45,48].

Structures of the oxidized and reduced forms of MsrA and MsrB from different bacterial species have been solved, which has provided insights into the molecular mechanisms of their catalytic cycles. The 3D structure of MsrA revealed an α/β roll domain, in which Cys_A is surrounded by strictly conserved amino acids that form a large basin that binds to bulky protein substrates[49,50]. The 3D structure of MsrB revealed two antiparallel β-sheets that form a barrel structure that is surrounded by α-helices. The 3D structure also revealed that the active site, which contains three regions of conserved residues, is located in a surface-exposed pocket

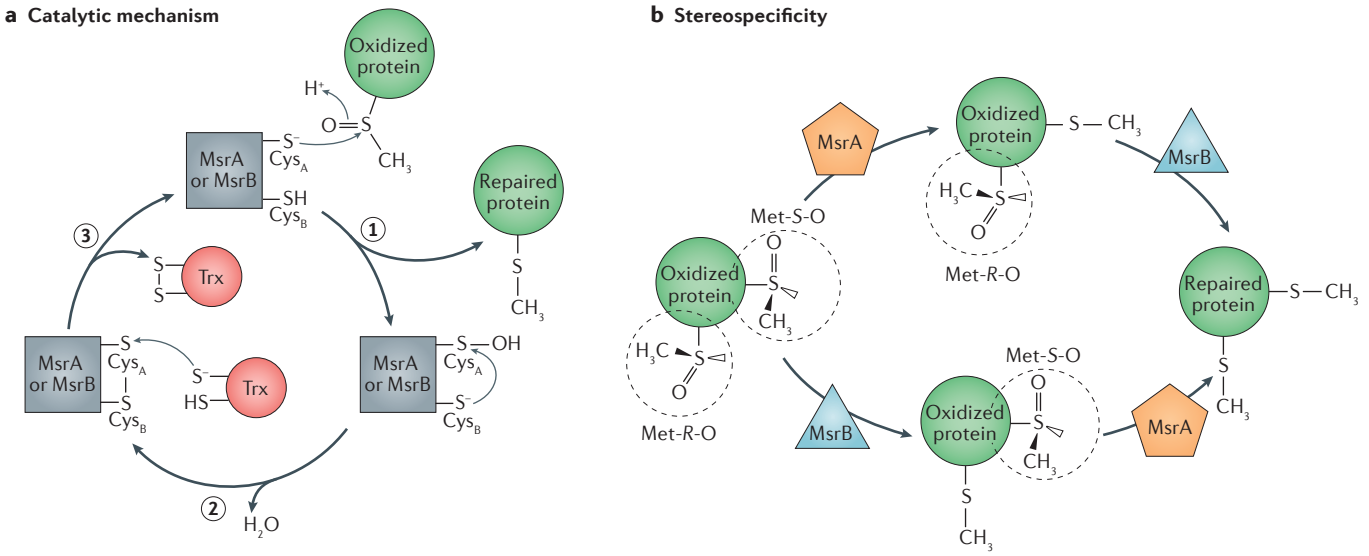


Figure 3 | **Molecular mechanisms of MsrA and MsrB repair activity. a** | Catalytic mechanisms of methionine sulfoxide reductase A (MsrA) and MsrB. The catalytic mechanisms of MsrA and MsrB repair involve a Cys-based three-step mechanism. A three-step catalytic mechanism of MsrA and MsrB with two Cys residues, the catalytic Cys (Cys_A) and the recycling Cys (Cys_B), is shown. MsrA or MsrB forms a complex with methionine sulfoxide (Met-O), followed by nucleophilic attack of Cys_A on the sulfoxide, which results in the simultaneous formation of a sulfenic acid intermediate and the release of reduced, repaired substrate (step 1). Nucleophilic attack on the sulfenic acid intermediate by Cys_B results in the formation of an intramolecular disulfide bond (step 2). The intramolecular disulfide bond is reduced by a thioredoxin (Trx; step 3). **b** | Stereospecificity of MsrA and MsrB and full repair. MsrA and MsrB reduce the (*S*-) and (*R*-) stereoisomers of Met-O, respectively. Full repair of an oxidized protein that has both Met-*S*-O and Met-*R*-O requires the sequential or simultaneous action of both MsrA and MsrB.

on the exterior of the barrel[51,52]. For most MsrB enzymes, the catalytic and recycling Cys residues are located in the surface-exposed pocket. Overall, the architecture of the active site and the conserved residues of MsrA and MsrB explain their ability to act on different diastereoisomer forms of Met-O. Comparison between the active sites from the structures of MsrA and MsrB revealed that they are mirror-like images of each other. Indeed, in both the active sites of MsrA and MsrB, a tryptophan residue, which interacts with the terminal methyl in the epsilon position of Met-O, and hydrogen bonds that form with the oxygen atom are observed, but on opposite sides of the catalytic pockets.

Several proteins have been found to have oxidized Met residues following exposure to oxidants. These proteins include a catalase and a peroxidase from *Helicobacter pylori*[53,54], and molecular chaperones from *E. coli* and *Mycobacterium tuberculosis*[55,56]. Study of the oxidation of glutamine synthetase (GlnA) in *E. coli* led to the hypothesis that proteins can be protected from ROS damage by Met residues. In this hypothesis, surface-exposed Met residues would be the first to get oxidized following the exposure of the protein to ROS, thereby protecting neighbouring functionally important residues from oxidation. The resulting oxidized surface-exposed residues would subsequently be reduced by Msrs[57]. The first biological process that was found to be dependent on Msr activity *in vivo* was the signal-recognition particle (SRP)-dependent protein translocase[58]. The bacterial SRP is a ribonucleoprotein complex of the 4.5S RNA bound to the Met-rich domain of the Ffh protein[59,60]. Oxidation of the Met-rich domain in the Ffh protein inhibits interaction with the 4.5S RNA, which can be reversed by MsrA and MsrB. In agreement with this, when both *msrA* and *msrB* were deleted in *E. coli*, defects in the targeting of SRP-dependent membrane proteins were observed[58].

Repair of cell envelope proteins

The cell envelope of Gram-negative bacteria is a complex multilayered structure that comprises the inner membrane, the outer membrane and the periplasm. The periplasm is a viscous compartment between the inner membrane and the outer membrane that contains a peptidoglycan cell wall. In *E. coli*, approximately 400 proteins are targeted to the periplasm[61], in which they carry out important physiological functions, such as protein folding, stress sensing and envelope assembly[62].

Repair of oxidized Cys residues. The periplasm has a more oxidizing redox potential than the cytoplasm[63]. Therefore, most Cys residues in periplasmic proteins form disulfide bonds that increase protein stability. The formation of disulfide bonds between Cys residues in proteins in the periplasm is catalysed by DsbA, a soluble oxidoreductase that has a Trx-like domain and a CXXC catalytic motif[64]. The catalytic Cys residues of DsbA form an unstable disulfide bond that is transferred to proteins that translocate to the periplasm. Following the transfer of its disulfide to substrate proteins, DsbA

Stereospecificity
A form of enzyme specificity, whereby an enzyme only catalyses its respective reaction if the substrate stereochemistry is correct.

Redox potential
The measure (in volts) of the affinity of a compound for electrons.

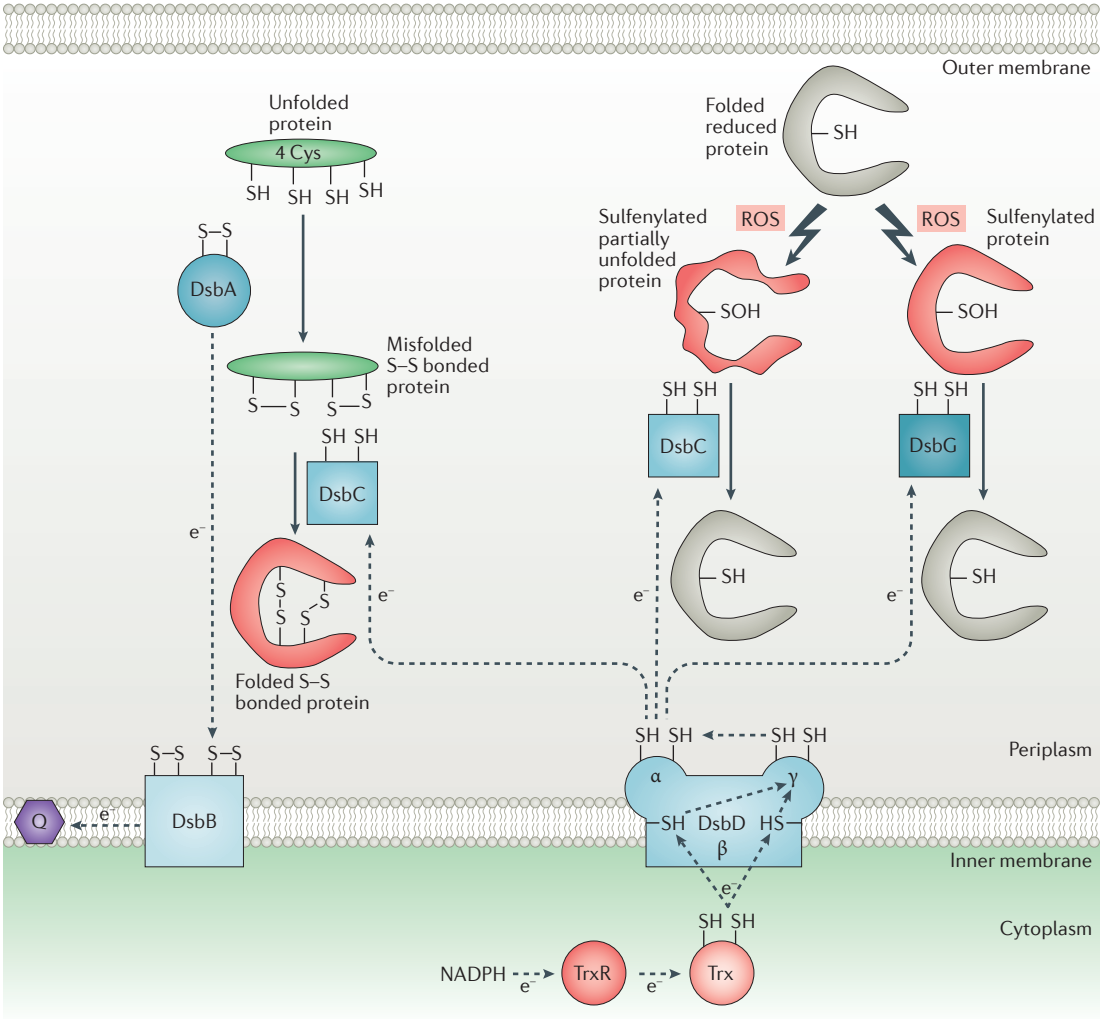


Figure 4 | **Repair of oxidized Cys residues in extracytoplasmic proteins.** A single Cys-containing folded protein is sulfenylated by reactive oxygen species (ROS) and then repaired by DsbG (top right). If the protein is partially unfolded, it is more likely to be repaired by DsbC. Disulfide bond formation is first catalysed by DsbA in Cys-containing proteins and then DsbC catalyses the isomerization of the incorrect disulfide bonds, which results in the release of a correctly folded protein (top left). The inner membrane protein DsbB accepts electrons from DsbA and transfers them to quinones (represented by Q in the figure), thereby recycling DsbA to its disulfide bond-containing form (lower left). The inner membrane multidomain protein DsbD (the α-domain, β-domain and γ-domain are indicated by their respective symbols) transfers electrons from the cytoplasmic thioredoxin (Trx)–thioredoxin reductase (TrxR)– nicotinamide adenine dinucleotide phosphate (NADPH) system to recycle the oxidized DsbC and DsbG proteins (lower right).

becomes reduced and is then catalytically recycled by disulfide bond formation protein B (DsbB), which is an inner membrane protein that transfers electrons from DsbA to the respiratory chain[65]. DsbA preferentially oxidizes consecutive Cys residues[66] (FIG. 4); therefore, DsbA often introduces inappropriate disulfide bonds into proteins that require disulfide bonds between non-consecutive Cys residues for correct folding and function. These inappropriate disulfide bonds can be corrected by DsbC, which is a periplasmic disulfide isomerase[67]. Similar to DsbA, DsbC is a member of the Trx protein family and has a CXXC motif for catalytic activity. DsbD, which is an inner membrane protein, maintains the CXXC motif of DsbC in a reduced state, which is required for DsbC to catalyse disulfide isomerization.

A small but substantial number of periplasmic proteins have Cys residues that do not form disulfide bonds[68] and could therefore be damaged by oxidants. However, DsbG, a periplasmic oxidoreductase that has a Trx-like domain and a CXXC catalytic motif, protects these exposed Cys residues[69] (FIG. 4). Similarly to DsbC, the CXXC motif in DsbG is maintained in a reduced state by DsbD. The first periplasmic protein that was found to be reduced by DsbG was YbiS[69], an enzyme that covalently attaches the major outer membrane lipoprotein Lpp to the bacterial cell wall using a single catalytic Cys residue[70].

Recently, DsbC was found to contribute to oxidative stress defence. Indeed, DsbC controls the redox state of AraF, which is a soluble protein that binds

Oxidative stress
An imbalance between the production of oxidants, such as reactive oxygen species, and antioxidant defences

Cytochrome *b*
A haem-containing membrane protein found in prokaryotic and eukaryotic cells that is involved in electron transport.

Molybdopterin
A class of cofactors found in most molybdenum (Mo) and all tungsten (W) enzymes. Molybdopterin consists of a pyranopterin, which comprises two thiolates that function as ligands in molybdoenzymes and tungstoenzymes.

Two-component system
A molecular system that is used by bacterial cells to sense and respond to signals through a phosphorylation cascade from a membrane receptor to a response regulator.

to L-arabinose in the periplasm and delivers it to an ATP-binding cassette (ABC) transporter in the inner membrane for cytoplasmic import[71]. AraF has a single Cys residue that is first oxidized to a sulfenic acid and can then react with the thiol group of a second AraF molecule to form a disulfide-linked AraF homodimer. This dimer is unable to bind to L-arabinose and deliver it to the transporter. DsbC recycles AraF from its inactive state by reducing the intermolecular disulfide bond[71]. Interestingly, AraF is specifically recognized by DsbC and cannot be reduced by DsbG[71], perhaps owing to differences in the hydrophobicity of the inner surfaces of these proteins; the inner surface of DsbC is hydrophobic, whereas the inner surface of DsbG is more hydrophilic. Therefore, it is tempting to speculate that DsbC substrates partially unfold when oxidized, in contrast to DsbG substrates that have solvent-exposed Cys residues and remain folded[69,72] (FIG. 4).

The catalytic Cys residues of DsbC and DsbG are kept reduced by DsbD[73,74]. DsbD has an N-terminal periplasmic immunoglobulin-like α-domain, a central transmembrane β-domain and a carboxy-terminal periplasmic Trx-like γ-domain. Each of the three domains of DsbD contains a pair of essential redox-active Cys residues that are involved in a series of thiol–disulfide exchange reactions from Trx1 in the cytoplasm to DsbC and DsbG in the periplasm[75]. How the β-domain of DsbD transports electrons across the inner membrane remains unclear; however, recent insight into this mechanism was provided by nuclear magnetic resonance (NMR) analysis of CcdA, a protein that is similar to DsbD but that lacks the periplasmic α-domain and γ-domain and has only six transmembrane segments instead of eight[76]. It remains unclear how Cys residues in the transmembrane domain are able to alternatively interact with oxidoreductases in the cytoplasm and in the periplasm; however, it has been hypothesized that this could be achieved by adopting several conformational states[76].

Repair of oxidized Met residues. Until recently, extracytoplasmic Msrs had only been identified in a limited number of bacterial species, despite the fact that the bacterial envelope is particularly prone to oxidative damage, owing to the high permeability of the outer membrane to hydrophilic low-molecular-weight oxidants. One example, the outer membrane peptide methionine sulfoxide reductase PilB, which is found in *Neisseria* species[52,77,78], has an N-terminal Trx-like domain that is fused to tandem MsrA and MsrB domains (FIG. 5). The activity of PilB depends on the electrons received from DsbD, which are then transferred to the MsrA and MsrB domains through the Trx-like domain[79]. Another example is the surface-exposed fusion protein MsrAB2 in *Streptococcus pneumoniae*[80]. In the case of MsrAB2, electrons are also donated by DsbD homologues to the surface-exposed Trx proteins, which then transfer them to MsrAB2 (REF. 80) (FIG. 5).

Recently, we discovered MsrPQ, a periplasmic Msr system that is widely conserved in Gram-negative bacteria[81]. In the MsrPQ system, MsrP functions as a soluble periplasmic Msr enzyme, whereas the MsrQ subunit is a membrane-bound cytochrome *b*[82] that acts as both a membrane anchor and a redox partner for MsrP (FIG. 5). The MsrPQ system is different from all other Msrs identified thus far, as MsrP is non-stereospecific and is able to reduce both the (*S-*) and (*R-*) diastereoisomers of Met-O. MsrP is unique as it uses a molybdopterin-based reaction mechanism to reduce Met-O in proteins instead of a thiol-based reaction mechanism. MsrPQ is also unique as it uses electrons from the respiratory chain or possibly from the flavin reductase Fre[83], which enables the MsrPQ system to function independently of Trx. The 3D structure of MsrP revealed the presence of 10 β-strands, organized in two β-sheets, and of 12 α-helices that form a single globular domain that holds the molybdenum cofactor[84]. This catalytic cofactor, which consists of a single molybdopterin, is 16 Å from the enzyme surface. Given that the substrates for MsrP are proteins, the buried location of the molybdenum-containing cofactor in the structure raises interesting questions concerning substrate accessibility and folding.

A list of 20 candidate protein substrates of MsrPQ in *E. coli* was obtained using proteomics[81], including several proteins that are involved in metal homeostasis, sugar uptake, molecular chaperones and protein folding catalysts. Studies of the chaperone SurA found that this protein was inactivated by oxidation with HOCl and that MsrPQ was able to restore its chaperone activity[81].

In *E. coli*, expression from the *msrPQ* operon is specifically induced by HOCl through the YedVW two-component system[81]. A mutant that lacked MsrPQ was found to be hypersensitive to treatment with HOCl, in part, because it accumulates oxidized and inactive SurA[81]. Interestingly, MsrPQ was also observed to protect *Azospira oryzae* from HOCl; this organism produces periplasmic RCS as an intermediate of chlorate, or perchlorate, respiration[85]; thus, MsrPQ is thought to have an important role in the defence against RCS.

Reduction in physiology and virulence

Role of the Trxs and Grxs in bacterial physiology and virulence. Trx and Grx reduction pathways have some functional redundancy. However, in numerous bacteria, the presence of a functional Trx-dependent reduction pathway is required to maintain thiol–disulfide homeostasis and for the resistance to oxidative stress. For example, in *E. coli*, mutants that lack Trx1 and Trx2 or thioredoxin reductase show an increased sensitivity to diamide, which is a non-physiological chemical that leads to the rapid formation of disulfide bonds in thiol-containing compounds[25,86,87]. In certain bacteria, genetic inactivation of the Trx system is lethal, despite the presence of low-molecular-weight thiols that act as a redox buffer to protect cells from oxidative damage. Indeed, even under physiological growth conditions, thioredoxin reductase is essential for the growth of *M. tuberculosis*[88], *Staphylococcus aureus*[89] and *Synechocystis* sp. PCC 6803 (REF. 90), whereas homologues of Trx1 are essential for the

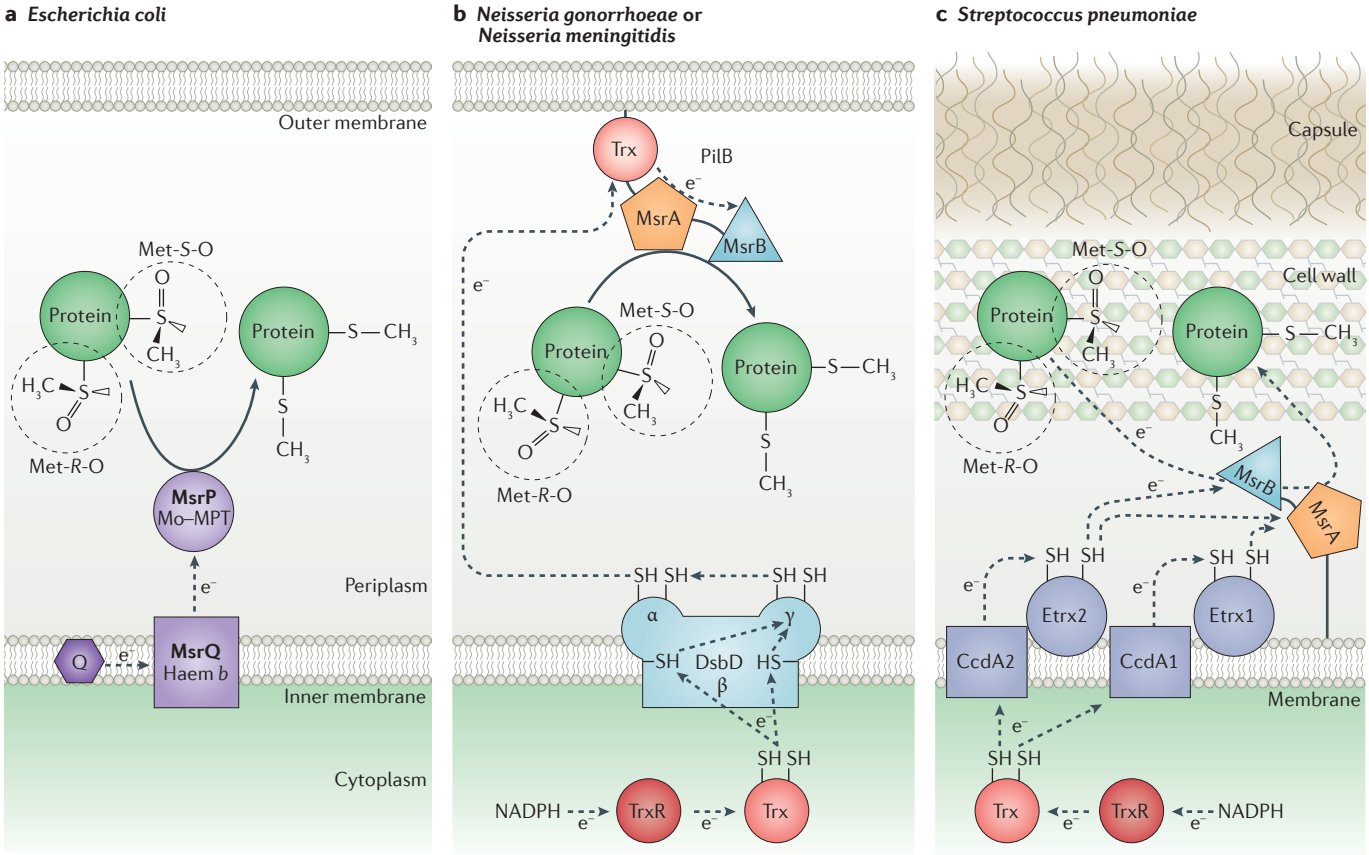


Figure 5 | **Repair of methionine sulfoxide damage in bacterial cell envelope proteins. a** | Methionine sulfoxide (Met-O) reduction in the periplasm of *Escherichia coli*. MsrPQ is composed of a molybdopterin-containing oxidoreductase (MsrP) that is located in the periplasm and a haem-containing membrane-bound protein (MsrQ). MsrP reduces both (*R*-) and (S-) diastereoisomeric forms of Met-O. The MsrPQ system uses electrons from the quinone pool (represented by Q in the figure) to reduce Met-O. **b** | Met-O reduction in the cell envelope of *Neisseria* species. *Neisseria* spp. produce an outer membrane lipoprotein (PilB) that is composed of tandem methionine sulfoxide reductase A (MsrA) and MsrB domains fused to an amino-terminal thioredoxin (Trx) domain. The electrons used by the MsrA and MsrB domains originate from the cytoplasmic thioredoxin (Trx)–thioredoxin reductase (TrxR)–nicotinamide adenine dinucleotide phosphate (NADPH) system through the inner membrane protein DsbD (the α-domain, β-domain and γ-domain are indicated by their respective symbols) and the Trx domain of PilB. **c** | Met-O reduction in *Streptococcus pneumoniae* surface-exposed proteins. The fusion protein MsrAB2 is surface-exposed and anchored to the cell membrane. Electrons used for the reduction by MsrA and MsrB domains originate from the cytoplasmic Trx system through the DsbD homologues CcdA1 and CcdA2 and the surface-exposed thioredoxin-like lipoproteins Etrx1 and Etrx2. Mo–MPT, molybdenum–molybdopterin.

growth of *Rhodobacter sphaeroides*[91], *Bacillus subtilis*[92] and *Synechocystis* sp. PCC 6803 (REF. 93). The Trx system is also necessary for the virulence of certain pathogens, including *H. pylori*, *Neisseria gonorrhoeae*, *Bacteroides fragilis*, *M. tuberculosis* and *Francisella novicida*[88,94–97].

Much less is known about the role of Grxs or analogues in bacillithiol and mycothiol-dependent reduction pathways. However, *E. coli* mutants that lack Grx3, but not mutants that lack Grx1 or Grx2, are killed more efficiently by oxidizing agents[86]. *E. coli* deletion mutants that lack either a Trx protein or a Grx protein are viable owing to the redundancy of the Trx and Grx systems in the recycling of their two main substrates, ribonucleotide reductase and PAPS reductase[27,86,98–100], whereas double mutants are non-viable under aerobic conditions. Notably, the PAPS reductase pathway in *E. coli* is not essential in the presence of exogenous sulfur sources, such as cysteine or GSH[99].

Periplasmic thiol–disulfide interchange proteins and the repair of oxidized proteins. Disruption of the function of DsbD leads to several phenotypes, such as a lack of *c*-type cytochromes in numerous bacteria, including *E. coli*[101–103], *Bordetella pertussis*[104], *Campylobacter jejuni*[105], *Vibrio cholerae*[106] and *Pseudomonas aeruginosa*[107]. In addition, *E. coli* mutants that lack DsbD are killed more efficiently on exposure to copper[103,108], dithiothreitol (DTT) and benzylpenicillin[109]. They also show a partial deficiency in the activation of the Rcs phosphorelay, which makes them unable to activate capsule production under

Dithiothreitol
(DTT). A reducing agent that reduces disulfide bonds through a thiol–disulfide exchange reaction.

Rcs phosphorelay
A complex signalling cascade that is used by enterobacteria to detect stress in the outer membrane and in the peptidoglycan layer.

conditions of envelope stress[110]. Interestingly, the only bacterium in which DsbD has been shown to be essential is *Neisseria meningitidis*, although the reason for this remains unknown[111].

Deletion of *dsbC* exacerbates the phenotypic defects of *E. coli* strains that lack DsbA[112] or the periplasmic chaperone SurA[113], further increasing the permeability of the outer membrane. *E. coli* mutants that lack DsbC are also hypersensitive to DTT, benzylpenicillin[109,114] and to high concentrations of copper. Indeed, copper catalyses the formation of non-native disulfides in periplasmic proteins, and mutants that lack DsbC are unable to rearrange them[108]. Furthermore, the deletion of *dsbC* can also decrease the virulence of pathogens such as *Moraxella osloensis*, *Xanthomonas oryzae* and *Pectobacterium carotovorum* (formerly known as *Erwinia carotovora*)[115–117].

Role of the Msrs. Msrs have been shown to be important for resistance to oxidative stress in almost all bacteria in which their roles have been studied. For example, the inactivation of *msrA* in *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Mycoplasma genitalium*, *Enterococcus faecalis* and *Xanthomonas campestris* pv. *phaseoli* results in more efficient killing by H_2O_2 (REFS 118–121). In *E. coli*, mutants that lack MsrA are also hypersensitive to *S*-nitrosoglutathione (GSNO) and nitrite. However, because GSNO and nitrite cannot oxidize Met residues directly, it was suggested that Met oxidation was due to the generation of $NO^{\bullet}$, which can react with $O_2^{\bullet-}$ or metals to produce $ONOO^-$ or $OH^{\bullet}$, respectively[122]. Moreover, MsrA is important for defence against oxidative damage by HOCl in some bacteria, including *S.* Typhimurium and *P. aeruginosa*[123,124].

Much less is known about the contribution of MsrB to resistance against oxidative stress compared with MsrA; however, the available data indicate that MsrB is more important in some bacteria than in others. For example, the deletion of *msrB* in *S.* Typhimurium and *Mycobacterium smegmatis* did not affect their susceptibility to oxidative damage by peroxides[119,125]. However, MsrB was found to protect *E. coli* from the cadmium-catalysed production of ROS[36], *C. jejuni* from CuOOH and paraquat[126], and *E. faecalis* from H_2O_2 (REF. 118). In some bacteria, MsrB is more important than MsrA for resistance against oxidative stress. For example, in *M. tuberculosis,* mutants that lack MsrB are more sensitive to HOCl than mutants that lack MsrA[127], and, in *P. aeruginosa*, the deletion of *msrB* causes hypersensitivity to HOCl and paraquat[123]. In *S. pneumoniae*, inactivation of the cytoplasmic MsrAB1 protein, but not the surface-exposed MsrAB2 protein, resulted in a higher sensitivity to paraquat[80], possibly due to the activity of pyruvate oxidase (SpxB), which generates high concentrations of H_2O_2 in the cytoplasm[128]. In some bacteria, sensitivity to oxidants is only observed when both *msrA* and *msrB* are deleted, which implies that there is some functional redundancy between these two enzymes. For example, this is the case in *M. tuberculosis* when exposed to acidified nitrite, in *P. aeruginosa* when exposed to H_2O_2 and in *C. jejuni* when exposed to the $NO^{\bullet}$ donor spermine NONOate[123,126,127].

MsrA and MsrB were first identified as virulence factors in the plant pathogen *Dickeya dadantii* (formerly known as *Erwinia chrysanthemi*) when it was found that MsrA-deficient mutants had a non-motile phenotype and were unable to invade plants, whereas MsrB-deficient mutants were not tested[129]. For other pathogens (for example, *E. coli*, *H. pylori*, *S. pneumoniae*, *S.* Typhimurium, *E. faecalis*, *M. genitalium* and *S. aureus*), strains that lack MsrA and/or MsrB have been reported to have a reduced ability to adhere to eukaryotic cells, to form biofilms, and to colonize and survive in their hosts[53,80,118–120,130–134]. Furthermore, a *C. jejuni* mutant that lacked the *msrP* gene was shown to have a decreased ability to colonize chickens[135].

Outlook

Redox versatility of the sulfur-containing amino acids Cys and Met was selected by evolution to increase the repertoire of protein activities. The trade-off of this versatility is that the oxidation of Cys and Met often causes damage to protein structure, which results in impaired protein functions and sometimes in cell death. In this Review, we have discussed examples of how protein structure and activity can change after the oxidation of Cys and Met residues. We have also discussed that the oxidation of Cys and Met is not always detrimental to protein structure and function, and that it can lead to the activation of inherent catalytic activity, to folding modification and enhanced stability or to new regulatory circuits (BOX 1).

Dsb and Msr enzymes have emerged as key participants in protein quality control and homeostasis in the cytoplasm and in the envelope of bacteria. However, several exciting and challenging questions have been raised by recent studies on how bacteria repair oxidatively damaged proteins. Do specific and dedicated degradation pathways exist for proteins that have oxidized Met and Cys residues? If such a pathway exists, is there competition between Msr and Dsb-dependent repair and degradation processes? Do repair proteins, such as Msrs, regulate the activity of molecular chaperones and folding catalysts, and, if so, how is this regulation coordinated? Protein homeostasis and quality control are at the heart of the most pressing health-related matters, such as ageing, cancer and neurological disorders. Studies that are based on bacteria are likely to provide answers and concepts for such issues, as the repair of oxidatively damaged proteins involves Msr-like and Dsb-like enzymes in eukaryotes.

Dsb and Msr enzymes contribute substantially to the virulence of bacterial pathogens, as they are often exposed to host-derived ROS, RNS or RCS. In turn, this implies that the design or identification of compounds that could specifically inhibit bacterial Msr and/or Dsb enzymes, could lead to new treatments that target pathogenic bacteria; however, the similarities between bacterial and host Msr and/or Dsb enzymes could limit the success of this strategy.

Cadmium
A non-redox-active metal that indirectly increases intracellular reactive oxygen species (ROS), primarily by binding to thiol groups, which leads to the inactivation of antioxidant defences, including scavenging enzymes and glutathione (GSH).

Paraquat
A redox-cycling organic compound that is widely used as a source of oxygen radicals in laboratory experiments.

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