The mirrored methionine sulfoxide reductases of *Neisseria gonorrhoeae* pilB

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Methionine sulfoxide reductases (Msr) protect against oxidative damage that can contribute to cell death. The tandem Msr domains (MsrA and MsrB) of the pilB protein from *Neisseria gonorrhoeae* each reduce different epimeric forms of methionine sulfoxide. The overall fold of the MsrB domain revealed by the 1.85 Å crystal structure shows no resemblance to the previously determined MsrA structures from other organisms. Despite the lack of homology, the active sites show approximate mirror symmetry. In each case, conserved amino acid motifs mediate the stereo-specific recognition and reduction of the substrate. Unlike the MsrA domain, the MsrB domain activates the cysteine or selenocysteine nucleophile through a unique Cys-Arg-Asp/Glu catalytic triad. The collapse of the reaction intermediate most likely results in the formation of a sulfenic or selenenic acid moiety. Regeneration of the active site occurs through a series of thiol-disulfide exchange steps involving another active site Cys residue and thioredoxin. These observations have broad implications for modular catalysis, antibiotic drug design and continuing longevity studies in mammals.

Biological catalysis is hallmarked by the stereo-specific recognition of substrates and their subsequent chemical modifications. In modular enzymes, these functions are often separated into distinct globular domains. This division of labor is most apparent in multisubstrate enzymes and multienzyme systems in which each domain either recognizes a particular substrate or channels the reaction intermediate to the next domain[1].

Unlike other known modular enzymes, the pilB protein from *Neisseria gonorrhoeae* has three domains, two of which recognize distinct diastereomeric forms of methionine sulfoxide (Met-(O)). Methionine residues within proteins are modified to Met-(O) by reactive oxygen species generated from normal and aberrant physiological processes[2–4]. For example, the stochastic attack of hydrogen peroxide (H_2O_2) at either lone electron pair of the sulfur atom of methionine results in the formation of alternative epimeric forms of methionine sulfoxide, Met-*R*(O) or Met-*S*(O), with either *R* or *S* stereochemistry, respectively[5]. This oxidative damage has been implicated in a variety of aging-related diseases. The repair or reduction of Met-(O) back to Met is a means of antioxidant defense and may also be used as a mechanism of cellular regulation[2–4,6,7]. This process seems to be a component of the mechanisms used by pathogenic microorganisms to infect host tissues[8,9] and to respond to cell wall-active antibiotics and heavy metals[10,11].

The MsrA (methionine sulfoxide reductase gene A) family of reductases specifically recognizes and reduces the *S*-form of methionine sulfoxide — that is, the epimeric form with L-stereochemistry at the Cα and *S*-chirality at the sulfur[5,12]. Biochemical and structural analyses have shown that catalysis occurs through the formation of a cysteine sulfenic acid intermediate and a series of thiol-disulfide exchange steps involving two additional Cys residues within a Gly-rich C-terminal tail[12–16]. This short tail facilitates the transfer of electrons from the *in vivo* protein partner thioredoxin[15,17]. Primary sequence alignments suggest the existence of at least two classes of MsrAs (Type I and Type II)[15]. In contrast to the Type I enzymes, which have a short C-terminus, the Type II enzymes, such as pilB from *Neisseria gonorrhoeae*, have an appended domain of ~17 kDa (Fig. 1).

Analysis of reductase activity

We postulated that the conserved Cys and His residues within the C-terminal of pilB (residues 375–522) (Fig. 2*a*) might either function in a manner similar to the Cys residues at the C-terminus of the Type I enzymes or modulate the MsrA-like reductase activity. Addition of the purified C-terminal domain of pilB (Fig. 1) to the full-length protein significantly increased the activity toward a racemic mixture of Met-(O) (data not shown). Unexpectedly, a control experiment demonstrated that the C-terminal domain alone had Met-(O) reductase activity. Recently, the homologous, single-domain protein yeaA from *Escherichia coli* (Fig. 2*a*) has been shown to have reductase activity as well[18]. This study also showed that yeaA in combination with MsrA from *E. coli* fully reduces the nine Met-(O) residues of calmodulin after hydrogen peroxide oxidation. This observation led the authors to suggest that yeaA reduces Met-*R*(O) in proteins, in contrast to MsrA, which reduces Met-*S*(O)[5].

To clarify these observations, the substrate specificity of various constructs of pilB and also of two homologous single domain proteins, yeaA and the human protein CBS-1 (ref. 19), were determined with purified Met-*R*(O) and Met-*S*(O) (Fig. 1). Only the constructs containing the C-terminal domain of pilB or its homologs were able to reduce Met-*R*(O). Based on the observed activity, the C-terminal domain, which was originally classified as a domain of unknown function (DUF25, InterPro: IPR002579)[20], has been renamed methionine sulfoxide reductase B, MsrB[18].

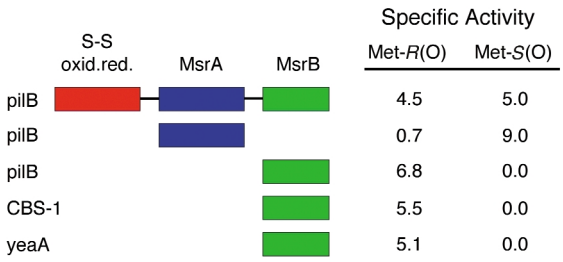

Fig. 1 Domain structure and reductase activity. The activity of various pilB constructs, as well as human (CBS-1) and *E. coli* (yeaA) homologs, toward epimeric forms of Met-(O) are shown. The full-length pilB protein consists of three domains: an N-terminal domain of ~21 kDa, which contains the Trp-X-X-Trp-Cys-X-X-Cys motif found in many disulfide oxidoreductases (such as thioredoxin)[22], a central MsrA domain[13,16] and a C-terminal MsrB domain. The specific activity represents the μmol of Met formed h^{-1} mg^{-1} enzyme. The value of 0.7 for the MsrA domain of pilB with Met-*R*(O) as substrate is a result of a 7% contamination of Met-*S*(O) in the Met-*R*(O) sample, as determined by HPLC. The MsrB domain of pilB shows 42%, 50% and 30% sequence identity to the CBS-1, yeaA and human SelX MsrB proteins, respectively.

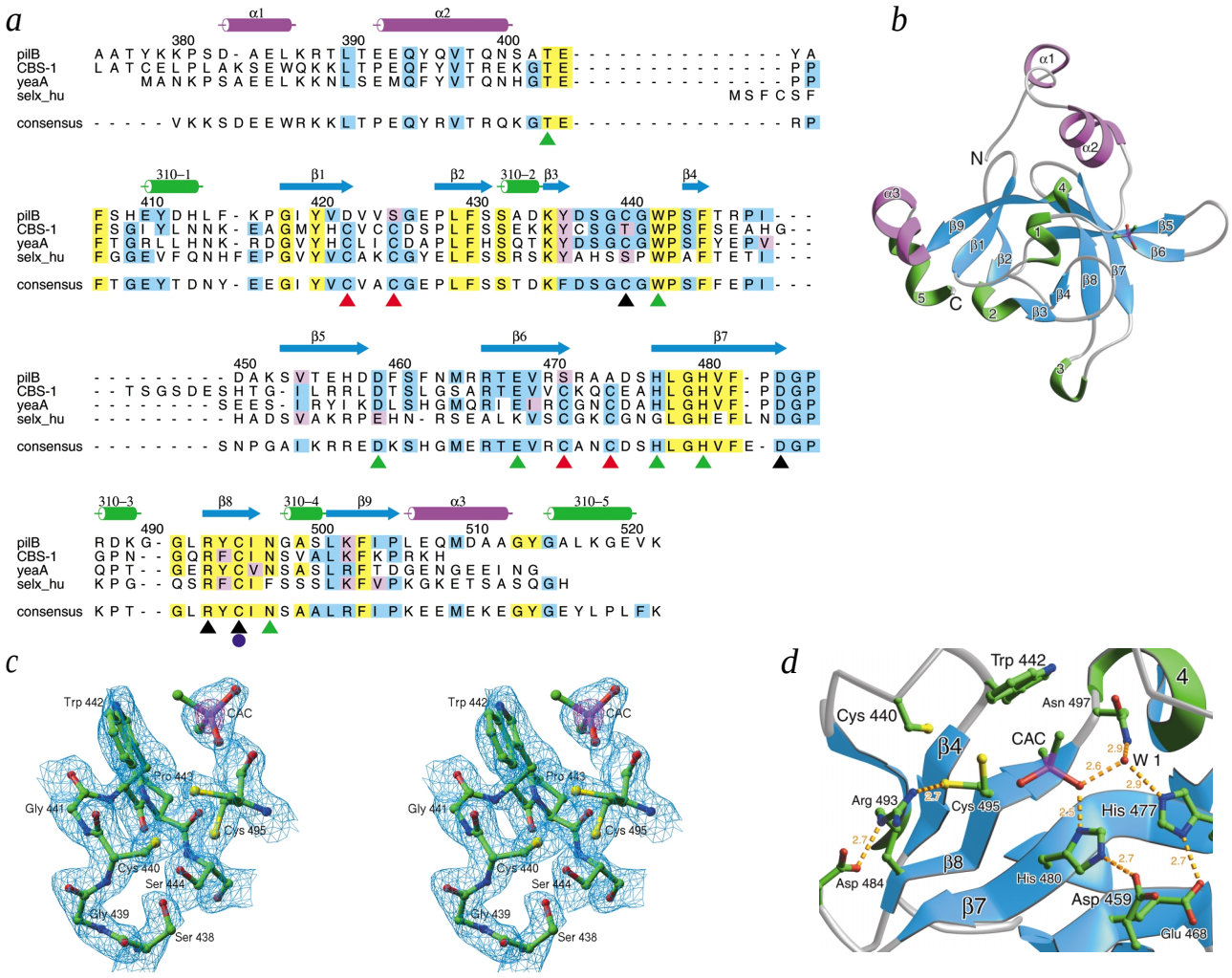

Fig. 2 Conservation and structure of the MsrB domain. ***a***, Primary sequence alignment for the MsrB domain. Representative examples were taken from an alignment of 51 sequences generated by the ProDom 2001.3 database (family accession numbers PD004057 and PD186368)[48]. The residue numbers correspond to the pilB sequence. Gaps, such as that between residues 404 and 405, are inserted to retain the alignment. The consensus sequence based on the full alignment is listed at the bottom. The conservation of amino acids among the 51 sequences is illustrated by shading: yellow, >90% identity; light blue, >50% identity; and purple, chemically similar residues. The secondary structure of pilB is depicted above the alignment. The α-helices (purple), β-strands (blue) and 3_{10}-helices (green) are numbered consecutively based on the primary sequence. Black arrows indicate the residues important for activating Cys 495 as the nucleophile and facilitating the regeneration of the active site. Residues that recognize the oxygen atom of Met-S(O) sulfoxide moiety and stabilize the putative trigonal-bipyramidal intermediate are indicated by green arrows. The SelX protein from human, selx_hu (Swiss-Prot: Q9NZV6), contains a SeCys residue at the position equivalent to Cys 495 (filled blue circle)[25]. Red arrows designate the location of four Cys residues that comprise the zinc site found in the structurally related guanine nucleotide-free chaperone Mss4 (refs 30,31). Alignment generated with ALSCRIPT[49]. ***b***, Overall fold of the MsrB domain in complex with cacodylate. Elements of secondary structure are colored as in panel (*a*). The cacodylate molecule (CAC) is shown in ball-and-stick rendering. ***c***, Stereo view of the MAD-phased, experimental electron density map contoured at 1 σ and 5 σ (blue and purple, respectively) within the active site of the refined model. Cys 495 was observed in two alternative conformations within both molecules in the asymmetric unit. A disulfide bond between Cys 440 and Cys 495 was modeled but does not fit to the observed electron density. The presence of a small amount of the disulfide form cannot be excluded. Atom colors are as follows: carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow; and arsenic, purple. ***d***, Molecular interactions between the enzyme, solvent and cacodylate. Putative hydrogen-bonding interactions (distances in Å) are shown by dashed yellow lines. Cys 495 seems to be activated as the nucleophile of reaction by conserved interactions to Arg 493 and Asp 484. A hydrogen bonding-network, generated by residues Thr 403, Asp 459, Glu 468, His 477, His 480, Asn 497 and one solvent atom (W 1), serves to mediate an interaction with one of the oxygen atoms of the polar face of cacodylate. Thr 403 interacts with W 1 (2.6 Å) but is not shown for clarity. The methyl groups of the hydrophobic face of cacodylate interact with Trp 442. Panels (*b*–*d*) generated with Ribbons[50].

Overall topology and active site

The MsrB domain of pilB (residues 375–522) was produced in *E. coli* with the incorporation of selenomethionine (SeMet) for crystallographic structure determination[21] (Table 1). There are two molecules in the asymmetric unit. The final 1.85 Å model includes residues 378–520 and 375–521 for the respective molecules, as well as three cacodylate buffer molecules and 124 solvent molecules. Cys 495 and Ser 500 of both chains refine in two alternative conformations. Three α-helices and five 3_{10}-helices decorate a core domain composed of two antiparallel β-sheets generated by strands β1, β2 and strands β9 and β3–β7, respectively (Fig. 2*b*).

Three regions of conserved sequence, Ser 438-Gly-Cys-Gly-Trp-Pro-Ser 444, His 477-Leu-Gly-His 480 and Arg 493-Tyr-Cys-Asn 497 (Fig. 2*a*), define a surface-exposed pocket that seems to be the putative active site. One of the MsrB domains in the asymmetric unit has a cacodylate molecule bound within this pocket. The pocket of the second MsrB domain has a crystal

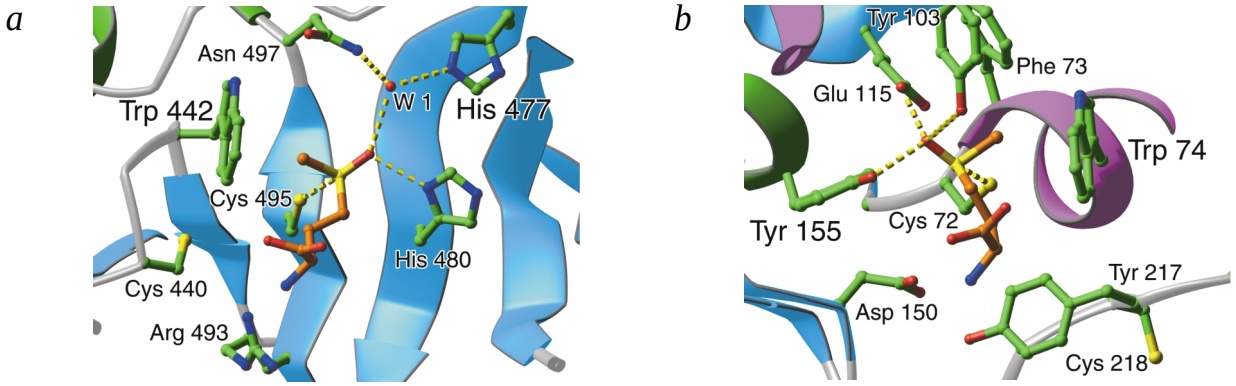

Fig. 3 Mirror-like relationship between the active sites of MsrB and MsrA domains. ***a***, Model for the interactions of Met-*R*(O) (orange carbon atoms) with the active site of the pilB MsrB domain (this work). ***b***, Model for the interaction of Met-*S*(O) with the active site of bovine MsrA[16]. In each case, only small adjustments to the rotamer angle of the presumed nucleophilic Cys residue, Cys 495 or Cys 72, were required to establish a feasible interaction (~2.1 Å) with the sulfur atom of the sulfoxide moiety (dashed yellow lines). The substrates were also docked in a manner to place the oxygen and sulfur atoms at the axial positions of the putative trigonal-bipyramidal intermediate of the reaction (Fig. 4). The MsrA domain of pilB presumably shows interactions similar to those here because the active site residues are conserved and the overall sequence identity is 40% (ref. 15).

contact in the vicinity that seems to prevent ligand binding. The alternation of Gly residues and Pro 443 leads to an unusually open turn between strands β3 and β4, where the hydroxyl side chains of Ser 438 and Ser 444 point inwards (Fig. 2*c*). This turn seems to act as a platform on which the side chains of Cys 440 and Trp 442 rest and form a cavity with which the hydrophobic part of cacodylate interacts. In contrast, His 477 and His 480 are involved in a hydrogen-bonding network and provide contacts to one of the oxygen atoms on the hydrophilic face of the cacodylate (Fig. 2*d*). Cys 495 is located adjacent to Cys 440 between the turn and the His motifs. The hydrogen-bonding interactions between Asp 484, Arg 493 and Cys 495 are conserved except that Glu replaces Asp 484 in some organisms (Fig. 2*a*).

Active site comparisons and putative reaction mechanism

The substrate molecule Met-*R*(O) was modeled into the putative active site of the MsrB domain of pilB (Fig. 3*a*) based on the interactions of the cacodylate molecule. A comparison to Met-*S*(O) modeled into the active site of bovine MsrA (bMsrA) shows that the respective catalytic domains provide a Trp residue to interact with the methyl group of the sulfoxide moiety[16] (Fig. 3*b*). The sulfoxide oxygen atom also interacts with hydrogen-bond partners that are conserved within the respective enzyme families. These functional motifs, however, are located on the opposite sides of the active site. This mirror-like relationship is remarkable because of the distinct three-dimensional folds of the two domains. The relationship is, however, reasonable because the sulfoxide moieties of the two substrates have opposite chirality. The Cα atoms of the respective substrates have the same chirality. Active site side chains that interact with this portion of the substrate, as may be expected, show no suggestion of a mirror relationship.

The correlation between the putative active sites suggests that catalysis by MsrBs (Fig. 4) may occur in a manner similar to MsrAs, although with some differences. For example, the activation of Cys 72 of bMsrA as the presumed nucleophile[14,15] is attributed to its location (Fig. 3*b*) at the positive end of an α-helix macrodipole[13,16] within the Gly 71-Cys-Phe-Trp-Gly 75 motif[12,14,15]. This form of activation has also been described for other oxidoreductases and model peptides[22,23]. In contrast, the deprotonation of Cys 495 in pilB (Fig. 3*a*) is presumably facilitated by its involvement in the novel Cys 495-Arg 493-Asp 484 triad. The substitution of the more reactive selenocysteine (SeCys) in the human MsrB homolog, SelX protein at Cys 495 (Fig. 2*a*) provides further support for its role in catalysis[24,25]. The catalytic Cys of MsrB and the substrate-binding Trp residue are decoupled: they are present in different sequence motifs unlike the MsrA enzymes[12,14,15].

Conserved hydrogen-bond partners within both reductases stabilize the trigonal-bipyramidal intermediate of the reaction (Figs 3, 4). The oxygen atom of the sulfoxide moiety interacts with the His motif in MsrB, whereas MsrA utilizes Glu and Tyr residues. Collapse of the intermediate in bMsrA leads to the

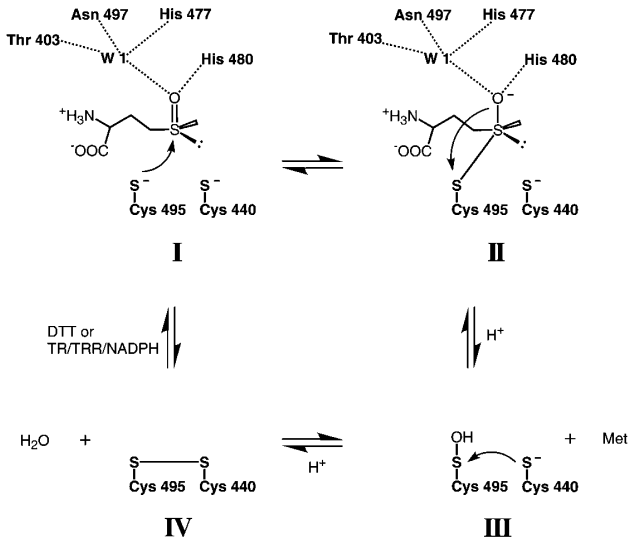

Fig. 4 Proposed reaction mechanism for MsrB catalysis. The active site residues and the solvent molecule, W 1, are indicated in bold. The nucleophilic attack by Cys 495 (I) results in a trigonal-bipyramidal intermediate (II). Collapse of the intermediate leads to the formation of a sulfenic acid derivative of Cys 495 and the release of methionine (III). The analogous derivative within the MsrB homolog SelX probably contains a selenenic acid modification (Fig. 2*a*). A series of thiol-disulfide exchange reactions (III and IV) and the action of reducing thiols, either dithiothreitol (DTT) *in vitro* or the thioredoxin-thioredoxin reductase system (TR/TRR) with its associated cofactor NADPH *in vivo*, return the active site to the fully reduced state. Whether the addition of the proton to the oxygen atom of the sulfoxide moiety occurs between steps I and II or steps II and III, as shown, remains to be determined.

Table 1 Crystallographic data, phasing and refinement

Crystallographic data			
Data set	λ1	λ2	λ3
λ (Å)	0.9792	0.9793	0.9611
d_{min} (Å)	1.85	1.85	1.85
Number of reflections			
Observed	98,562	100,375	100,817
Unique	22,584	22,663	22,706
Completeness (%)[1]	89.5 (89.1)	89.6 (88.7)	89.4 (87.3)
$\langle I \rangle / \langle \sigma(I) \rangle$[1,2]	22.3 (4.6)	22.0 (5.0)	21.2 (3.9)
R_{merge} (%)[1,3]	6.6 (24.5)	6.6 (27.1)	6.8 (29.9)
Phasing			
Phasing $power_{acentric}$			
Iso	2.49	2.96	–
Ano	1.50	1.36	1.11
R_{cullis}	0.57	0.46	
Overall figure of merit[1,4]			
SHARP	0.54 (0.29)		
SOLOMON	0.83 (0.64)		
Refinement			
Resolution (Å)	27.2–1.85		
R_{cryst} (%)[5]	20.7		
R_{free} (%)[6]	23.7		
Δ_{bonds} (Å)[7]	0.005		
Δ_{angles} (°)[8]	1.21		
Average B-factor (Å^2)	20.7		
Ramachandran analysis (%)			
Most favored	91.5		
Additionally allowed	8.1		
Generously allowed	0.4		

[1]The values for the highest resolution shell are given in parentheses.
[2]$\langle I \rangle / \langle \sigma(I) \rangle$ is the r.m.s. value of the intensity measurements divided by their estimated standard deviation.
[3]R_{merge} gives the average agreement between the independently measured intensities.
[4]The values are calculated to 1.85 Å resolution using SHARP[44] and SOLOMON[45].
[5]R_{cryst} is the crystallographic residual following refinement.
[6]R_{free} is the crossvalidated R-factor calculated with 10% of the data omitted from refinement.
[7]Δ_{bonds} and Δ_{angles} give the average departure from ideal values of the bond lengths and angles, respectively.
[8]The average B-factor (or temperature factor) reflects the thermal motion of the molecule.

formation of cysteine sulfenic acid (Cys 72-SOH)[14]. A similar Cys or SeCys (selenenic acid) modification probably occurs on Cys 495 of MsrB and SelX[24,25].

Regeneration of Cys 72 of bMsrA occurs through a series of thiol-disulfide exchange reactions. In particular, Cys 218 of the C-terminal tail forms a disulfide bond with Cys 72, which is subsequently reduced by Cys 227 followed by thioredoxin and its partner thioredoxin reductase[15,17]. By analogy, pilB seems to use Cys 440 for the restoration of the active site by forming a disulfide bond with Cys 495 (Fig. 4). Support for this notion comes from the observed 3.0 Å distance between the sulfur atoms of Cys 440 and Cys 495 (Fig. 2*c*). Because MsrB does not contain another Cys residue in the vicinity similar to Cys 227 of bMsrA, the reduction of the Cys 440–Cys 495 disulfide or selenenylsulfide bond probably occurs through the direct action of thioredoxin on Cys 440 (Fig. 2*d*). Thioredoxin may also act upon Cys 495 of the single-domain MsrBs that have Ser or Thr residues at position 440 (Fig. 2*a*). The surface accessibility of Cys 440 and Cys 495 suggests that such an interaction is feasible, and that MsrBs, similar to MsrAs, may interact with macromolecular protein substrates[16,18].

Biological implications

At present, the only examples of the fusion of the MsrA and MsrB reductase domains come from pathogenic bacteria, including *Neisseria gonorrhoeae* (Genbank accession number: AF482946), *Neisseria meningitidis* (Swiss-Prot: Q9K1N8), *Helicobacter pylori* (Swiss-Prot: O25011), *Haemophilus influenzae* (Swiss-Prot: P45213), *Streptococcus pneumoniae* (Swiss-Prot: P35593) and *Streptococcus gordonii* (Swiss-Prot: Q9LAM9). The *N. gonorhoeae* and *N. meningtidis* proteins are unique in that a thioredoxin-like domain is fused to the N-terminus (Fig. 1). It is tempting to speculate that this domain may function to reduce the active sites of the downstream MsrA/B domains. Moreover, because the pilB-like proteins and those from *H. pylori* and *H. influenzae* contain a signal sequence for secretion[26], the fusion of catalytic domains may be necessary to ensure the repair of Met-(O) residues of protein substrates, possibly including surface receptors of either the bacterium or its host[8]. The design of a bifunctional compound, or an epimeric or racemic mixture of compounds, that would inhibit both the MsrA and MsrB domains could represent an important new class of antimicrobial agents.

Two exceptions to the MsrA/B domain arrangement are known. The protein from *Treponema pallidum* (Swiss-Prot: O83641) has the domain order switched, with the MsrB domain at the N-terminus. One of the many MsrB genes from *Arabidopsis thaliana* (TrEMBL: Q9ZS93) lacks the MsrA domain and has tandem MsrB domains. These different arrangements and the MsrA/B organization for the intracellular enzymes from *S. pneumoniae* and *S. gordonii* may be needed for increased efficiency in as yet unknown repair processes[18].

In most organisms — for example, *E. coli* and human (Figs 1, 2*a*) — the MsrA and MsrB domains are separate proteins. This has potential ramifications for the interpretation of aging studies in rodents[6,7]. For example, the MsrA knock-out mouse shows a reduced lifespan (40%) and the onset of an atypical locomotion phenotype[6]. In spite of lacking the MsrA gene, significant residual Met-(O) reductase activity was observed using racemic substrates. The presence of two mouse homologs nearly identical (81% and 91% identity) to the human proteins CBS-1 and SelX (Fig. 2*a*) strongly suggests that the residual activity could be due to the presence of MsrB activity. Whether mice overexpressing either or both MsrA and MsrB domains have similar increases in longevity, as seen in transgenic flies overexpressing MsrA[27], will be interesting to determine.

The structure of MsrB has also put forward the possibility that the domain may have yet another function. A DALI[28] search for homologous protein folds revealed only two structural homologs, the guanine nucleotide-free chaperones or exchange factors Mss4 and TCTP (6% and 13% sequence identity; Cα atom superposition with an r.m.s. deviation of 2.5 and 3.0 Å, respectively)[29–31]. These proteins bind the unstable, GDP/GTP-free form of the Rab subset of Ras GTPases. These proteins are essential for vesicular transport and have been implicated in a variety of human tumors. A Zn^{2+}-binding site generated by two Cys-X-X-Cys motifs stabilizes the fold of Mss4. Similar Cys motifs, however, are only conserved within the single-domain MsrB proteins (Fig. 2*a*,*b*, between the β1 and β2, and β6 and β7 loops of pilB MsrB). Although inspection of the structures clear-

ly shows that none of the residues essential for reductase activity are present in Mss4 and TCTP, whether the MsrBs have chaperone activity remains to be determined.

Conclusion

The modular protein pilB contains two structurally distinct domains that each reduce different epimers of Met-(O) to Met. The approximate mirror symmetry between the active sites of the MsrA and MsrB domains suggests that catalysis occurs in a related manner. Each domain uses conserved residues for substrate recognition but activates the Cys or SeCys nucleophile in different ways. In the case of MsrB, this seems to be achieved by a unique Cys-Arg-Asp/Glu catalytic triad. The active site in the respective enzymes is primed for another round of catalysis through a series of thiol-disulfide exchange reactions. Although examples are known of single-domain proteins that recognize a specific diastereomer of related substrates through both similar[32–35] and divergent structures[36], pilB represents the first definitive example of such domains occurring in tandem. The multiple β-ketoreductase domains of the polyketide synthases may also show mirror-related active sites, but this remains to be confirmed[1].

Note added in proof: Recent experimental evidence confirms the formation of a sulfenic acid intermediate on Cys 495 of pilB and the incorporation of SeCys within the mouse MsrB homolog[37,38].

Methods

Protein purification and crystallization. Each of the proteins was overexpressed and purified from *E. coli* as N-terminal, poly-His fusions: full-length pilB (residues 3–522)[15], MsrA domain of pilB (residues 181–362), MsrB domain of pilB (residues 375–522), human CBS-1 (residues 45–182, Swiss-Prot: Q9Y3D2) and *E. coli* yeaA (residues 1–137, Swiss-Prot: P39903)[15,18]. The affinity tags were removed by thrombin digestion[15]. Crystals of the MsrB domain of pilB containing SeMet[39] were obtained by the vapor diffusion method. Equal volumes of protein (15 mg ml^{-1} in 20 mM Tris, pH 8.5, and 10% (v/v) glycerol) and well solutions (0.1 M sodium cacodylate, pH 6.5, and 30% (w/v) PEG 4000) were mixed and held at room temperature.

Reductase assay. The two epimers of free methionine sulfoxide, Met-*R*(O) and Met-*S*(O), were generated and purified according to established procedures[12,40]. The specific activity of each protein (μmol of Met formed h^{-1} mg^{-1} enzyme) was determined by measuring Met formation using sodium nitroprusside as described[41]. The 200 μl reactions contained 100 mM KPO_4, pH 6.9, 15 μg thioredoxin, 1 μg thioredoxin reductase, 100 nmol NADPH, 4 μmol glucose-6-phosphate, 1 μg glucose-6-phosphate dehydrogenase, 2 μmol of either Met-R(O) or Met-S(O) and 10–30 μg of purified enzyme. Incubations were for periods up to 2 h at 37 °C.

Data collection and structure determination. A three-wavelength MAD data set was collected on a crystal cryoprotected in paratone (beamline 5.0.2 at the Advanced Light Source, Berkeley, California). The crystal showed $P2_12_12$ symmetry (a = 67.4 Å, b = 68.1 Å and c = 62.8 Å) with two molecules in the asymmetric unit. Data were merged and scaled with DENZO and SCALEPACK[42]. Of the six potential selenium sites, two were found using SOLVE[43]. Refinement of MAD phases with SHARP[44] allowed the identification of three additional sites. The phases were improved by solvent flattening with SOLOMON[45]. The model was built using O[46] and refined with CNS[47] using alternating cycles of simulated-annealing, positional and B-factor refinement. Noncrystallographic symmetry averaging was not used. The Cα atoms of the two chains within the asymmetric unit superimpose with an r.m.s. deviation of 0.42 Å.

Coordinates. Coordinates have been deposited in the Protein Data Bank (accession code 1L1D).

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Competing interests statement

The authors declare that they have no competing financial interests.

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