Correct disulfide bond formation is essential for proper folding of many proteins, including bacterial virulence factors. The suppressor of copper sensitivity (Scs) proteins have roles in dithiol/disulfide interchange and the bacterial response to copper stress. Encoded in a four-gene cassette (ScsABCD) present in many Gram-negative bacteria, the Scs proteins are enigmatic and poorly characterized. Here, we show that the periplasmic α-domain of the membrane protein ScsB in the Gram-negative bacterium Proteus mirabilis forms a redox relay with the soluble periplasmic protein PmScsC. We also found that the periplasmic α-domain is sufficient to activate the disulfide isomerase activity of PmScsC. The crystal structure of PmScsBα at a resolution of 1.54 Å revealed that it comprises two structurally similar immunoglobulin-like folds, one of which includes a putative redox-active site with the sequence CXXXC. We confirmed the importance of these cysteine residues for PmScsBα function, and in addition, we engineered cysteine variants that produced a stable complex between PmScsC and PmScsBα. Using small-angle X-ray and neutron scattering analyses with contrast variation, we determined a low-resolution structure of the PmScsC–PmScsBα complex. The structural model of this complex suggested that PmScsBα uses both of its immunoglobulin-like folds to interact with PmScsC and revealed that the highly dynamic PmScsC becomes ordered upon PmScsBα binding. These findings add to our understanding of the poorly characterized Scs proteins.

The correct formation of disulfide bonds is an essential component in the folding of many proteins, including bacterial virulence factors. In bacteria, disulfide bond–forming (Dsb)5 proteins are responsible for introducing disulfide bonds into substrate proteins (DsbA and -B) (1, 2) as well as reducing and isomerizing (proofreading and shuffling) disulfide bonds that have been incorrectly introduced (DsbC and -D) (3, 4). Dsb proteins function through the redox action of two catalytic cysteines that are often embedded in a thioredoxin fold. These systems are well characterized in Escherichia coli; however, homologues of Dsb proteins exist in a range of Gram-negative bacteria. A group of related and poorly studied proteins found most commonly in Proteobacteria are the suppressor of copper sensitivity (Scs) proteins (5). The Scs proteins contribute to the bacterial virulence trait of resistance to copper stress (6, 7).

Originally identified in Salmonella enterica serovar Typhimurium (6), the scs locus encodes four proteins, ScsA–D, all of which have predicted catalytic motifs consisting of two cysteines (CXXC or CXXXC). Three of these (ScsB–D) incorporate a predicted thioredoxin fold, and one of the four (ScsA) has a predicted copper-binding motif. ScsD has a predicted N-terminal membrane anchor linked to a predicted periplasmic thioredoxin-fold domain (6) reminiscent of proteins involved in cytochrome c biogenesis, such as DsbE/CcmG (8, 9). The best studied of the four Scs proteins is the soluble periplasmic protein ScsC (5, 7, 10). An extraordinary feature is the variation in structure and function of ScsC proteins across different bacteria. Caulobacter crescentus ScsC (CcScsC) is reported to be a dimeric disulfide isomerase (5), S. enterica serovar Typhimurium ScsC (StScsC) is a monomeric redox protein with no disulfide isomerase activity (7), and Proteus mirabilis ScsC

The abbreviations used are: Dsb, disulfide bond–forming; Scs, suppressor of copper sensitivity; Pm, P. mirabilis; Cc, C. crescentus; St, S. enterica serovar Typhimurium; Ec, E. coli; r.m.s.d., root mean square deviation; SAXS, small-angle X-ray scattering; SANS, small-angle neutron scattering; WAXS, wide-angle X-ray scattering; AMS, 4-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid; SAD, single-wavelength anomalous diffraction; SEC, size exclusion chromatography; PaScsBα C114A, deuterium-labeled PmScsBα C114A; p(0), pair-distance distribution function; I(0), scattering intensity at zero angle; BisTris, 2-(bis[2-hydroxyethyl]aminomethyl)-2-(hydroxyethyl)propane-1,3-diol; scRNase A, scrambled RNase A; Rg, radius of gyration.

1 Present address: Cello Health Consulting, Farnham, Surrey GU9 7DN, United Kingdom.
2 Present address: Bristol–Myers Squibb, Annulstrasse 29, 80636 Munich, Germany.
3 To whom correspondence may be addressed. E-mail: awhi@ansto.gov.au.
4 To whom correspondence may be addressed: Griffith Institute for Drug Discovery, Griffith University, Nathan, Queensland 4111, Australia. E-mail: jlm@griffith.edu.au.
P. mirabilis ScsBα and ScsC form a functional redox relay

Figure 1. Schematic representation of bacterial disulfide isomerases and their redox relay partners. A. EcDsbC is reduced by EcDsbDα, which obtains its reducing power from a reduction cascade that starts with thioredoxin reductase in the bacterial cytoplasm. B. PmScsC and PmScsBα are putative interaction partners, and the source of PmScsB’s reducing power is yet to be determined (represented by the question marks). The catalytic cysteines in each protein are shown as orange spheres.

(PmScsC) is a highly dynamic “shape-shifting” trimeric disulfide isomerase (10).

The present work focuses on the structure and function of P. mirabilis ScsB, a putative redox partner of P. mirabilis ScsC. ScsB resembles E. coli DsbD in many respects (5). EcDsbD is the membrane protein partner of the archetypal disulfide isomerase E. coli DsbC. Like EcDsbD, the ScsB protein is predicted to comprise three domains: an N-terminal periplasmic domain (DsbDα/ScsBα), a transmembrane domain (DsbDβ/ScsBβ) of eight helices, and a periplasmic C-terminal thioredoxin-fold domain (DsbDγ/ScsBγ) (Fig. 1, A and B). Each of the three domains in both EcDsbD and ScsB have two catalytic cysteine residues. In DsbD, these cysteine pairs in each domain form a reduction cascade that originates from thioredoxin reductase and NADPH in the cytoplasm, is passed through thioredoxin to DsbDβ, DsbDγ, and then DsbDα, which reduces the cysteines in specific substrate proteins, such as DsbC (11, 12) (Fig. 1A). The similarity between the predicted architectures of EcDsbD and ScsB suggests the ScsB protein forms a similar redox relay system. Indeed, C. crescentus ScsB has been shown to interact with CcScsC and to maintain CcScsC in its reduced state, which is necessary for disulfide isomerase activity (5). A major difference between EcDsbD and ScsB is the size of their N-terminal α-domains. DsbDα comprises ~165 residues, whereas ScsBα is much larger at ~255 residues, and there is no detectable sequence identity between the α-domains of DsbD and ScsB. Because the α-domain of EcDsbD interacts directly with substrates, such as EcDsbC, the different size and sequence of the ScsB α-domain suggests that the proteins interact with different substrates (5).

In the present study, we investigated the structure and function of P. mirabilis ScsBα, showing that it forms a functional redox relay with the highly dynamic, trimeric disulfide isomerase PmScsC. We also report the first crystal structure of any ScsBα, revealing unexpectedly the presence of tandem immunglobulin folds in PmScsBα and showing that this same arrangement is likely to be shared in ScsBs encoded by other organisms. We created a stable PmScsC–PmScsBα (trimmer–monomer) complex and analyzed small-angle X-ray scattering (SAXS) data and small-angle neutron scattering (SANS) data with contrast variation to produce a low-resolution model of the complex. This model revealed that the highly dynamic PmScsC protein becomes more symmetric and ordered upon interaction with PmScsBα and that both immunoglobulin folds of PmScsBα interact with one protomer of PmScsC.

Results

PmScsC and PmScsBα form a specific redox relay system

From sequence analogy with CcScsB and CcScsC, PmScsBα is predicted to be the redox partner of PmScsC. If this functional relationship holds true, PmScsBα would specifically reduce oxidized PmScsC to convert it to the active reduced form. To confirm that a redox relay occurs between PmScsC and PmScsBα, we performed a redox gel-shift assay. First, we mixed PmScsC with PmScsBα and then analyzed the redox states of the two proteins after an incubation period. The redox state was defined by analyzing the proteins by SDS-PAGE after reaction with 4-acetamido-4’-maleimidyl stilbene-2,2’-disulfonic acid (AMS) (Fig. 2A). When AMS binds to a reduced thiol, the mass is increased by 0.5 kDa, thus allowing discrimination between reduced and oxidized cysteine forms of a protein on mass-based gel separation. The results showed that when oxidized PmScsC was incubated with reduced PmScsBα, both proteins changed their redox states, confirming that disulfide exchange occurs between the two proteins (Fig. 2B). The reverse redox reaction did not occur: reduced PmScsC remained reduced when mixed with oxidized PmScsBα. Moreover, we were able to confirm the residues involved in the change in redox state because addition of the variant PmScsBα C114A to oxidized PmScsC did not catalyze disulfide exchange. However, there was some evidence for formation of a PmScsC–PmScsBα C114A complex (Fig. 2B, band marked X).

Control experiments using PmScsC or PmScsBα alone showed that, under the conditions of the experiment, these proteins did not spontaneously change their redox state. These results support the notion that PmScsC and PmScsBα form a specific redox relay system (Fig. 2B) and that the residues Cys114 and Cys118 of PmScsBα are responsible for the redox interaction.

PmScsBα primes the disulfide isomerase activity of PmScsC

We have previously shown that PmScsC is a disulfide isomerase (10). We tested the hypothesis that the redox interaction between PmScsBα and PmScsC might contribute to this function of PmScsC. We measured the disulfide isomerase activity of PmScsC using scrambled RNase A as substrate (Fig. 2A). Reduced PmScsC, but not oxidized PmScsC, was able to refold scrambled RNase A and thereby activate the enzyme (10) (Fig. 2C). PmScsBα on its own (oxidized or reduced) had no effect on the activity of scrambled RNase A. However, in the presence of reduced PmScsBα, the inactive oxidized PmScsC was able to restore the activity of RNase A. This result supports the hypothesis that PmScsBα reduces the disulfides of oxidized PmScsC,
thereby enabling its disulfide isomerase activity (Fig. 2D). This experiment also confirmed that disulfide exchange in this system does not flow in the reverse direction: reduced PmScsC did not lose its ability to shuffle scrambled RNase A disulfides when it is incubated with oxidized PmScsB. This may suggest that reduced PmScsC is unable to interact with PmScsB. Furthermore, the cysteine variant in which Cys114 is mutated to Ala (PmScsB/C114A) does not activate oxidized PmScsC, showing that this cysteine is required for the redox relay to operate.

Crystal structure of PmScsB

After removal of the periplasmic signal sequence, the predicted PmScsB α-domain comprises 255 residues (residues 21–275 of the reported PmScsB protein sequence, UniProt entry B4EV20). The crystal structure of this domain was solved to a resolution of 1.54 Å by using selenomethionine (SeMet)-labeled protein together with SAD phasing (Protein Data Bank code 6C29; Table 1 and Fig. 3, A–D). The structure revealed a monomer consisting of two immunoglobulin-like folds linked by an α-helix. This arrangement was consistent across all three molecules in the asymmetric unit (r.m.s.d., 0.7–1.0 Å, 247 residues aligned). Curiously, the two subdomains, A and B, are reasonably similar in structure (Fig. 3D; r.m.s.d., 2.5 Å, 81 residues aligned), although the sequence identity for such an alignment is very low (7%). The subdomains each comprise two β-sheets, one with three β-strands and the other with four β-strands (Fig. 3, A and B). Subdomain A has additional features, including the cysteine motif (114CXXXC118), two β-strands forming a β-hairpin that links the two β-sheets of the canonical immunoglobulin fold, and a loop between β2 and β3 located on one side of the catalytic cysteines (Fig. 3D, arrows). The cysteines are present in the reduced form in all three molecules in the asymmetric unit.

Table 1

| Data collection | Protein Data Bank code |
|-----------------|------------------------|
| Wavelength (Å)  | 0.9786                 |
| Resolution (Å)  | 97.06–1.538 (1.544–1.538)* |
| Space group     | P2_1_2_1               |
| Unit cell dimensions | 69.98, 97.06, 110.20 |
| α, β, γ (°)     | 90, 90, 90             |
| No. of reflections | 1,761,650             |
| No. of unique reflections | 111,511               |
| Rmerge (%)      | 0.146 (2.114)          |
| Rpremer (%)     | 0.038 (0.531)          |
| Mean (σ)        | 16.0 (2.1)             |
| Redundancy      | 15.8 (16.3)            |
| Completeness (%)| 99.7 (98.3)            |
| Wilson B        | 15.96                  |

SAD phasing

| No. of sites | 14 |
| Figure of merit | 0.42 |

Refinement

| No. of monomers in a/u | 3 |
| Resolution used in refinement (Å) | 5.10–1.538 |
| Rfree (%) | 111.388 |
| Rwork (%) | 19.8 |
| No. of protein atoms | 5,860 |
| No. of ligand atoms | 0 |
| No. of waters | 669 |
| β factors (Å²) | 25.5 |
| Average Protein atoms | 24.8 |
| Ligands | NA |
| Waters | 31.4 |
| r.m.s.d. bond length (Å) | 0.011 |
| r.m.s.d. bond angles (°) | 1.332 |
| Ramachandran favored/outlier (%) | 97/0 |

* Values in parentheses refer to the highest resolution shell.

The presence of an immunoglobulin-like fold in both PmScsBα subdomains resulted in many hits in a DALI search against the Protein Data Bank (13). Notably, the top hit was the
functionally equivalent *E. coli* DsbD (Protein Data Bank code 1JPE; r.m.s.d., 2.5 Å, 103 residues aligned), although it has only one immunoglobulin-like fold, which can be aligned with subdomain A of *P. mirabilis* ScsBα (Fig. 4A). Like *P. mirabilis* ScsBα subdomain A, *E. coli* DsbD has two catalytic cysteines and a β-hairpin close to the active site, but it does not have the β2-β3 loop of *P. mirabilis* ScsBα. Two other hits in the DALI search were the mouse γ-adaptin appendage domain from clathrin-binding adaptor AP-1 (Protein Data Bank code 2A7B; r.m.s.d., 2.4 Å, 106 residues aligned) and the GAE domain of the clathrin-binding adaptor GGA protein from *Saccharomyces cerevisiae* (Protein Data Bank code 3MNM; r.m.s.d., 2.4 Å, 102 residues aligned) (Fig. 4, B and C). These proteins have typical immunoglobulin-like folds with no catalytic cysteines, and like EcDsbDα they have one immunoglobulin fold that aligns with subdomain A of *P. mirabilis* ScsBα. These three proteins were also hits in a DALI search against *P. mirabilis* ScsBα subdomain B although with much higher r.m.s.d. values (Protein Data Bank code 1JPE: r.m.s.d., 3.8 Å, 75 residues aligned; Protein Data Bank code 2A7B: r.m.s.d., 3.0 Å, 84 residues aligned; Protein Data Bank code 3MNM: r.m.s.d., 2.9 Å, 86 residues aligned). Curiously, there were no high-scoring DALI hits that had a tandem immunoglobulin-fold arrangement like that of *P. mirabilis* ScsBα. We did find lower-scoring DALI hits with two immunoglobulin-like folds (mostly antigen-binding fragments of antibodies; e.g. Protein Data Bank code 5JUE; Z-score, 4.6; r.m.s.d., 3.0 Å, 80 residues aligned), but they lacked the connecting α-helix of *P. mirabilis* ScsBα.

**Structure-based sequence alignment**

The amino acid sequence of *P. mirabilis* ScsBα was aligned with that of EcDsbDα using structure-based alignment and the sequences of CcScsBα and StScsBα (Fig. 5). ScsBα from *S. enterica* Typhimurium and *C. crescentus* were chosen as these are the only other organisms where the interaction partner, ScsC, has been characterized. EcDsbDα was chosen because of the known structural and functional relationship. *P. mirabilis* ScsBα shares 44% sequence identity with StScsBα (280 residues), 21% identity with CcScsBα (291 residues), and just 12% identity with ScsBα from *S. enterica* Typhimurium and *C. crescentus*. The high sequence conservation between EcDsbDα and *P. mirabilis* ScsBα is consistent with the structural similarity observed in the DALI search.
**P. mirabilis ScsBα and ScsC form a functional redox relay**

The interaction between the catalytic cysteines of PmScsC and PmScsBα is transient. For this reason, the catalytic cysteine variants PmScsC C87S and PmScsBα C114A were produced. These variants allowed the capture of a stable PmScsC–PmScsBα complex for structural studies. Size exclusion chromatography (SEC) and subsequent SDS-PAGE analysis suggested that the predominant complex species had a 3:1 ratio of PmScsC to PmScsBα, corresponding to one PmScsC trimer bound to one PmScsBα monomer (Fig. 6). The predicted molecular mass calculated from SAXS and SANS analysis (Table 2) also provided evidence to support this stoichiometry. A peak corresponding to a larger species was seen on the SEC chromatogram (Fig. 6A, peak marked Z), suggesting that a smaller amount of 3:2 and/or 3:3 complex may have also formed.

**SAXS and SANS provide structural insights into the PmScsC–PmScsBα interaction**

SANS contrast variation experiments were performed on the 3:1 PmScsC–PmScsBα complex formed using unlabeled PmScsC C87S and deuterium-labeled PmScsBα C114A (3PmScsBα C114A) (Fig. 7). Deuterium labeling changes the neutron scattering length density of PmScsBα. It is possible to tune the neutron scattering length density of the solvent by changing its deuterium content such that it matches one of the components of the protein complex. This is said to be the match point of that component, and the measured scattering data can then be interpreted as being from the unmatched component of the complex alone. Data collected close to the 3PmScsBα C114A match point (100% D₂O, where PmScsC C87S dominates the scattering) indicate that the highly dynamic PmScsC protein becomes comparatively rigid and adopts a conformation that, at low-resolution, appears symmetrical upon interaction with PmScsBα. Specifically, the pair-distance distribution function (p(r)) is bimodal (Fig. 8A, green curve) with two well defined peaks and similar to the p(r) generated from the extended symmetrical crystal structure (Protein Data Bank code 5ID4) (Fig. 8A, black dashed curve) (10). An asymmetric arrangement of the PmScsC trimer would yield an asymmetrically shaped second peak in the p(r), whereas significant flexibility would see this same peak broaden.

Analysis of the SANS data showed that a plot of the scattering intensity at zero angle (I(0)) normalized by protein concentration versus deuterium content of the solvent was well represented by a parabolic function, indicating that the samples were pure (Fig. 8B). A Stuhrmann plot revealed that the 3PmScsBα C114A molecule was located toward the center of the complex (Fig. 8C). A rigid body model of the complex was optimized simultaneously against the SAXS data set and five SANS contrast variation data sets. The resulting model provided an excellent fit to all of the scattering data (Fig. 9A). The configuration adopted by PmScsBα and PmScsC in the model precludes the simultaneous binding of two (or three) PmScsBα molecules to PmScsC, helping to explain the preferential formation of a trimer–monomer complex (Fig. 6). The model also highlights two potential interfaces between PmScsC and PmScsBα (Fig. 9A). The most obvious interface is that between chain A of PmScsC and subdomain A of PmScsBα for which the intermolecular disulfide bond is modeled. The resolution of the model is too low to be definitive, but the interface includes residues that could form hydrogen bond interactions. The second interface is between chain A of PmScsC and subdomain B (the second immunoglobulin domain) of PmScsBα.

**Discussion**

Suppressor of copper sensitivity proteins play a role in the response to copper stress of the important human pathogens *S. enterica* Typhimurium and *P. mirabilis* (7, 10). At least two of
P. mirabilis ScsBα and ScsC form a functional redox relay

Figure 5. Sequence alignment. The sequence alignment of the PmScsBα crystal structure with StScsBα and CcScsBα sequences and the structure-based sequence alignment with EcDsbDα (Protein Data Bank code 1JPE) are shown. Secondary structure elements of PmScsBα are colored according to the scheme in Figure 3. Residues that are conserved between all four proteins are highlighted in red; those that are conserved only between the ScsBα proteins are colored blue.

the four Scs proteins are homologues of Dsb proteins (6), which are essential for the correct formation of disulfide bonds in virulence factors of many Gram-negative bacteria (14–17). P. mirabilis ScsC was recently shown to be a trimeric, highly dynamic disulfide isomerase (10). The present study aimed to characterize PmScsBα, the predicted interaction partner of PmScsC, and to investigate the nature of the interaction between the two.

The domain architecture of full-length ScsB resembles that of E. coli DsbD, the major difference being the size of the periplasmic N-terminal domain. This difference suggested that ScsBα would be structurally distinct from DsbDα perhaps because they interact with different substrates. We confirmed that PmScsC and PmScsBα are redox partners and are therefore functionally similar to EcDsbC and EcDsbDα. We also reported the structure of PmScsBα, showing that it consists of two immunoglobulin-like folds connected by an α-helix. The structures of the two immunoglobulin-like subdomains of PmScsBα are broadly similar with the exception that subdomain A has a catalytic CXXXC motif as well as a β-hairpin and extended loop that both cover the CXXXC active site. The positioning of the β-hairpin and the loop could potentially shield the active site from nonspecific interactions or facilitate interactions with partner proteins. Unexpectedly, we found that domain A of PmScsBα shares the features of the immunoglobulin fold of EcDsbDα (Fig. 4; r.m.s.d., 2.5 Å, 103 residues aligned) despite a sequence identity of just 12%. The key difference between the structures of subdomain A and EcDsbDα is that EcDsbDα lacks the β2-β3 loop of PmScsBα. The sequence of this β2-β3 loop is conserved in S. enterica Typhimurium and C. crescentus ScsBα, suggesting that it plays a key functional role. A residue that is conserved across the three ScsBα and EcDsbDα is Tyr265 (PmScsBα numbering). In EcDsbDα, the equivalent Tyr62 hydroxyl is thought to be involved in nuclease attack of Cys103, which resolves the intermolecular disulfide formed between EcDsbDα and its substrate (e.g. EcDsbC) (18). The nuclease attack in EcDsbDα is proposed to also require Asp168 (18), a residue that is not conserved in Pm-, Cc-, or StScsBα. It is unclear from the PmScsBα structure which residue, if any, might play a role equivalent to that of Asp168.

The discovery of tandem immunoglobulin-like folds in PmScsBα raises the question as to the function of the second or noncatalytic immunoglobulin-like fold. EcDsbDα has a single immunoglobulin-like fold, which, in the E. coli DsbC-DsbDα crystal structure, spans the central cleft of the V-shaped DsbC substrate to interact with both domains of the disulfide isomerase (Fig. 9B) (19). This binding mode is thought to favor binding to dimeric EcDsbC; EcDsbDα is unable to interact with monomeric EcDsbC (3, 19). By comparison, the SANS-derived model of the PmScsC–PmScsBα complex suggests that PmScsBα subdomain A interacts with just one of the three PmScsC protomers in the trimer. Subdomain B of PmScsBα forms a secondary binding site to PmScsC that may stabilize the primary interaction or add specificity (Fig. 9A). Subdomain B could also be required for interactions with other proteins that have yet to be identified.

EcDsbC–EcDsbDα is the redox relay complex formed between dimeric EcDsbC and monomeric EcDsbDα. The crystal structure revealed a 2:1 (rather than a 2:2) complex between the dimer of EcDsbC and a monomer of EcDsbDα (3, 19). On this basis, we hypothesized that PmScsC–PmScsBα would form a 3:1 complex between trimeric PmScsC and monomeric
P. mirabilis ScsBα and ScsC form a functional redox relay

**Experimental procedures**

**Sequence analysis**

The TMHMM Server (version 2.0) was used to determine the region of the ScsB proteins that formed the N-terminal periplasmic domain. EMBOSS Needle (20, 21) with residues 1–275, 1–279, and 1–290 of UniProt entries B4EV20 (PmScsB), AAL20046 (StScsB), and Q9ABL0 (CcScsB), respectively, was used to calculate the sequence identities reported among these proteins. The DALI sever (13) was used to calculate r.m.s.d. values and structure-based sequence identities. Coot (22) was used to create visuals of the structural alignments. The sequences of all four proteins were aligned using PROMALS3D (23) with the Protein Data Bank files of PmScsBα and EcDsBDα (code 1JPE) instead of the sequence alone. Residue numbers quoted in the text were taken from the deposited Protein Data Bank files for the PmScsC and PmScsBα protein structures, which are numbered based on the tobacco etch virus protease–cleaved protein sequence (starting S4N2A3...).

**Molecular biology**

A codon-optimized gene encoding P. mirabilis ScsBα without the predicted signal peptide (UniProt entry B4EV20, residues 21–275; Integrated DNA Technologies) was inserted into pMCSG7 (24) using ligation-independent cloning. The PmScsC (UniProt entry C2L1E2/B4EV21) construct used throughout this study was created previously (10). The constructs for the variants PmScsC C87S and PmScsBα C114A were created using the QuikChange® Lightning site-directed mutagenesis kit (Agilent Technologies). The WT PmScsC and PmScsBα constructs were used as templates, and primers 5’-ggagtggtccagcctttctataattgcccgta-3’, 5’-tcataattgccgcaggatcttt-3’, and 5’-gtgctgcattgcagtcttttttttttttttttctttt-3’, respectively, were used to introduce the point mutations. PmScsBα was also subcloned into pET24a for deuterated bioreactor expression using restriction enzymes EcoRI and Ndel. All constructs contained an N-terminal His6 tag followed by a tobacco etch virus protease cleavage site and were confirmed by sequencing.

**Protein production**

Except for the selenomethionine-labeled PmScsBα and deuterated PmScsBα C114A, all proteins were expressed and purified as outlined previously (10). The redox state of the cysteines was confirmed spectrophotometrically with 5,5’-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent) as described previously (25). Yields for PmScsC and PmScsBα derivatives were around...
Table 2
SAXS data collection and analysis details for the PmScsC–PmScsBα complex

| Data collection parameters | SAXS | SANS |
|----------------------------|------|------|
| Instrument                 | SAXS-WAXS (Australian Synchrotron) | QUOKKA (ANSTO) |
| Beam geometry              | Point | Point |
| Wavelength (Å)             | 1.033 | 5.00 |
| Sample to detector distance (m) | 2.680 | 2.000 (short); 10.00 (long) |
| q-range (Å⁻¹)              | 0.007–0.35 | 0.03–0.40 (short); 0.01–0.09 (long) |
| Exposure time (s)         | 13 (13 × 1-s exposures) | 0.5%\^, 7.200; 20.6%\^, 10.800; 42.6%\^, 21.600; 80%\^, 7.200; 100%\^, 3.600; 0%\^, 10.800; 100%\^, 7.200 |
| Measurement type          | Concentration series from 96-well plate; 1.0-mm quartz capillary | Neutron contrast variation; Hellma 120-QS |
| Protein concentration range (mg/ml) | 0.56–4.50 | 3.36 (0%); 2.23 (20%); 1.10 (42%); 0.32 (100%) |
| Temperature (K)            | 283 | 289 |
| Absolute intensity calibration | Water | Incident beam intensity |

| Sample details | | |
|----------------|---|---|
| Extinction coefficient (A₀⁻¹, w/v) | 0.979 | 0.979 |
| Partial specific volume (cm³ g⁻¹) | 0.737 | 0.737 |
| Contrast, Δp (10 cm⁻¹) | 2.86 | 3.36 (0%); 2.23 (20%); 1.10 (42%); 0.32 (100%) |
| Molecular mass (from sequence) (kDa) | 105.5 | 105.5 |
| Protein concentration (mg/ml) | 0.56 | 5.30 (0%); 5.20 (20%); 5.10 (42%); 5.00 (80%); 4.90 (100%) |

| Structural parameters | | |
|-----------------------|---|---|
| I(0) (cm⁻¹) (from Guinier) | 0.04050 ± 0.00010 | 0.05020 ± 0.00119 (0%); 0.2217 ± 0.00135 (20%); 0.0357 ± 0.00007 (42%); 0.0561 ± 0.00008 (80%); 0.2211 ± 0.00100 (100%) |
| Rg (Å) (from Guinier) | 39.2 ± 0.2 | 36.3 ± 0.2 (0%); 34.6 ± 0.3 (20%); 24.3 ± 1.1 (42%); 35.6 ± 0.5 (80%); 37.0 ± 0.2 (100%) |
| I(0) (cm⁻¹) (from p(ri)) | 0.04026 ± 0.000010 | 0.0025 ± 0.0001 (0%); 0.0028 ± 0.0002 (20%); 0.0030 ± 0.0004 (42%); 0.0031 ± 0.0005 (80%); 0.0032 ± 0.0006 (100%) |
| Rg (Å) (from p(ri)) | 38.7 ± 0.1 | 0.1 Å, M = 105 ± 5 kDa (4.50 mg/ml). There is a small systematic change in Rg but no systematic change in M; thus, it was deemed that the complex is largely free of concentration-dependent effects up to a concentration of ~5.0 mg/ml. |
| Dmax (Å) | 115 ± 3 | 110 ± 3 |
| Porod volume (Å³) | 131,300 ± 7,000 | 116,000 ± 6,000

| Molecular mass determination | | |
|-----------------------------|---|---|
| Molecular mass from I(0) (kDa) | 97 ± 5 | 95 ± 5 |
| Molecular mass from Porod (kDa) | 108 ± 5 | 95 ± 5 |

\* I(0) = 0.0854 ± 0.0002 cm⁻¹, Rg = 39.0 ± 0.1 Å, M = 105 ± 5 kDa (1.13 mg/ml); I(0) = 0.1779 ± 0.0002 cm⁻¹, Rg = 39.0 ± 0.1 Å, M = 107 ± 5 kDa (2.25 mg/ml); I(0) = 0.3476 ± 0.0003 cm⁻¹, Rg = 38.8 ± 0.1 Å, M = 105 ± 5 kDa (4.50 mg/ml). There is a small systematic change in Rg but no systematic change in M; thus, it was deemed that the complex is largely free of concentration-dependent effects up to a concentration of ~5.0 mg/ml.

\* The Porod volume calculation is not valid for systems where the complex is not homogeneous (such as is the case for the neutron contrast variation experiment reported here). Instead, the composite scattering functions (Fig. 8D) were summed together to give the scattering curve of the protein complex with homogeneous contrast (i.e. homogeneous contrast = I₁₁ + I₂₂ + I₃₃), and the Porod volume was determined from this curve. The molecular mass derived from the I(0) was also taken from this curve.

80 and 40 mg/liter of culture (~15 g cell weight harvested), respectively. SDS-PAGE (NuPAGE® system, 4–12% BisTris gel, Invitrogen) with Coomassie Blue stain was used to assess the protein quality.

**Expression of SeMet-labeled PmScsBα**

To express SeMet-labeled PmScsBα, needed for experimental phasing in crystallographic structure determination, a 10-ml starter culture of _E. coli_ BL21 (DE3) pLysS cells containing pMCSG7-PmScsBα was grown in LB medium supplemented with the appropriate antibiotics at 37 °C for 4–5 h at 220 rpm. This culture was then used to inoculate 1 liter of M9 minimal medium supplemented with antibiotics and amino acids (100 mg of l-methionine, l-lysine, l-threonine, and l-phenylalanine and 50 mg of l-leucine, l-isoleucine, and l-valine) and then incubated overnight at 37 °C at 220 rpm. Cells were harvested from the overnight culture, washed with 1 liter of sterile water, and resuspended in water to an A₆₀₀ of ~1. M9 minimal medium containing antibiotics but no amino acids was then inoculated with the cells to a final A₆₀₀ of 0.1. The cells were cultured at 30 °C at 220 rpm for 6–8 hours until an A₆₀₀ of 0.5 was reached. Amino acids, including 100 mg of l-selenomethionine instead of l-methionine, were then added to the culture flasks, and after 15 min at 30 °C isopropyl-β-D-galactoside was added to a final concentration of 1 mM to induce expression. The cultures were incubated at 30 °C at 220 rpm overnight. Cells were harvested, and the protein was purified as described previously (10) with the addition of 5 mM DTT to all purification buffers. The yield of SeMet-labeled PmScsBα was 5 mg/liter of culture (~5 g of cell pellet).

**Expression of deuterated PmScsBα C114A**

Deuterated PmScsBα C114A was expressed for SAXS analysis of the complex between PmScsC C875 and PmScsBα C114A. The deuterated protein PmScsBα C114A was produced at the National Deuteration Facility, Australian Nuclear Science and Technology Organisation in 1 liter of “ModC1” medium (26) containing 90% (v/v) D₂O and unlabeled glycerol (40 g/liter). Miniprep plasmid DNA “pET24a-PmScsBα C114A” was used to transform 50 μl of Invitrogen competent BL21*(DE3) cells, which were then incubated with 250 μl of SOC medium for 2 h. The 300-μl culture was added to 10 ml of ModC1 medium containing 50% D₂O and 40 μg/ml kanamycin, shaking at 200 rpm at 37 °C. After 16 h (A₆₀₀ 1.2), 9 ml was added to 36 ml of fresh medium with kanamycin at 100% D₂O (i.e. 45 ml at 90% D₂O). After another 5 h (A₆₀₀ 0.9), the culture was diluted with fresh 90% D₂O medium to 102 ml. After another 5 h, 100 ml (A₆₀₀ 0.87) was used to inoculate 900 ml of fresh 90% D₂O medium with kanamycin in a 2-liter Real Time Engineering bioreactor aerated with air at 0.5 liter/min and with pH held to a minimum of 6.2 by addition of 28% NH₄OH in H₂O (Sigma). Dissolved oxygen tension was set to 75% and was
above 60% throughout. At an \( A_{400} \) of 14.2, the temperature was lowered to 20 °C, and expression was induced by the addition of 1 mM isopropyl 1-thio-\( \beta \)-d-galactopyranoside. At 17 h postinduction, with an \( A_{400} \) of 32, the culture was harvested with a wet weight yield of 79.4 g and with excellent expression as evident by SDS-PAGE. The deuteration level was determined by partial trypsin digest MALDI-TOF comparison of unlabeled and labeled samples and was found to be 70.8%. Deuterated PmScsB was purified as described for the unlabeled protein except the His\(_6\) tag was not cleaved as it was needed for the purification of the complex. The yield of purified deuterated PmScsB was \( \sim 240 \) mg/liter of bioreactor culture (\( \sim 40 \) g of cell pellet).

**Determination of protein concentration**

The reported protein concentrations were determined using the \( A_{280} \) of the sample (read using a Thermo Scientific Nano-Drop 2000c spectrophotometer) and calculated extinction coefficients from ProtParam (27).

**Crystal structure of PmScsB**

A number of conditions from commercial screens resulted in PmScsB crystals. These conditions were replicated in 24-well plates, and the best diffracting SeMet-labeled PmScsB crystals were obtained in a drop containing 1 \( \mu l \) of 43 mg/ml protein in 10 mM HEPES, pH 7.4, 5 mM DTT and 1 \( \mu l \) of 0.1 M MES monohydrate, pH 6, 6% (v/v) Tacsimate, pH 6, 24% PEG 4000. A commercial additive screen (Hampton Research) was used to optimize crystals, and those grown over 2 days to \( \sim 400 \) \( \mu \)m with the addition of 4% 2,5-hexanediol or 4% 1,3-butanol resulted in the best diffraction. Crystals were cryoprotected in the crystallization condition plus 20% glycerol and flash frozen in liquid nitrogen.

Because of the lack of a suitable model for molecular replacement, experimental phasing was required to solve the crystal structure of PmScsB. A fluorescence scan was used to determine the absorption edge of SeMet-labeled crystals, and a data set (Table 1) was collected near the peak absorption wavelength (0.9786 Å) at 100 K. Data processing in autoPROC (28), which utilizes XDS (29) and AIMLESS (30), determined the space group to be P2\(_1\),2,2\(_1\), SAD phasing using the SHELX workflow (31) in HKL2MAP (32) found 14 selenium sites with an occupancy >0.2. We expected only 12 selenium sites; the extra two sites were positioned very close to other sites, suggesting alternate conformations, although only one SeMet was modeled with an alternate conformation in the final structure. SAD phasing resulted in an initial incomplete alanine model of the protein structure (figure of merit, 0.42). This initial model was then used for molecular replacement against the same data in Phaser (33) in the CCP4 suite (34). Two rounds of automated building and refinement using Buccaneer (35) with REFMAC (36), as implemented in CCP4 suite (34), completed the structure, revealing three molecules in the asymmetric unit. Then several rounds of manual adjustment in Coot (22) and refinement in PHENIX (37) were performed with reference to the validation program MolProbity (38). Multiple residues in the crystal structure have alternate conformers, and occupancies of the selenium atoms in the SeMet residues were all <1.0. The electron density for some loop regions is poor in chains B and C, particularly residues 79–82, 99–100, and 51–52 (chain B only).

**Redox interaction assay**

To confirm the redox interaction between PmScsC and PmScsB, oxidized PmScsC and reduced PmScsB were incubated together at final concentrations of 10 \( \mu M \) in 100 mM sodium phosphate, pH 7, 1 mM EDTA for 2 h. As controls, oxidized and reduced forms of both proteins, a sample containing reduced PmScsC and oxidized PmScsB, and a sample with oxidized PmScsC with PmScsB C114A were also incubated for 2 h. All samples were then treated with 10% TCA to precipitate the protein, and after centrifugation the pellets were washed with 100% cold acetone and dried. The protein pellets were resuspended in a solution of 200 \( \mu M \) AMS in 1% SDS, 50 mM Tris, pH 7.0. AMS binds to the reduced cysteines of pro-

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**Figure 7. SAXS/SANS scattering curves.** SAXS and SANS data (offset for clarity) collected from the 3:1 PmScsC C87S–DPmScsB C114A complex with the model scattering curves overlaid (solid black line): 0% (\( \chi^2 = 1.16 \); red; on absolute scale), 20% (\( \chi^2 = 1.20 \); gray; offset by a factor of 50), 42% (\( \chi^2 = 1.00 \); blue; offset by a factor of 50), 80% (\( \chi^2 = 1.11 \); cyan; offset by a factor of 50), and X-ray (\( \chi^2 = 1.80 \); orange; offset by a factor of 50). A Guinier plot for the SAXS data is shown in the inset. The match point of PmScsC C87S is \( -42\% D_2O \) (blue) and \( \sim 100\% D_2O \) (green) where PmScsC C87S dominates the scattering. Error bars represent the S.D.
P. mirabilis ScsBα and ScsC form a functional redox relay

Figure 8. Other SAXS/SANS results. A, p(r) derived from the experimental X-ray scattering data (orange) and the 100% scattering data (green), which should approximate the p(r) for PmScsC C87S alone. The p(r) derived from the extended (ext.) crystal structure (Protein Data Bank code 5ID4) is shown as a black dotted line for reference. B, a plot of I(0) normalized by concentration as a function of D2O content of the supporting solvent. The plot is parabolic in shape and reveals that the match point of the entire complex is 59% D2O (vertical dotted line). C, Stuhrmann plot for the 3:1 PmScsC C87S–PmScsB C114A complex, conforming to \( R_m^2 = R_m^2 + n \Delta P \alpha - \beta \Delta P \) where \( R_m \) is the radius of gyration of that object with homogenous contrast and \( \alpha \) and \( \beta \) are related to the contrast variations within the object. The values obtained from a fit to the plot (solid black line) are: \( R_m = 37.5 \pm 0.4, \alpha = -210 \pm 50, \beta = 520 \pm 100 \). The negative value of \( \alpha \) reveals that the region with higher contrast (i.e. PmScsB C114A) lies toward the center of the molecule. Error bars represent the S.E. D, the composite scattering functions determined from the neutron contrast variation data. The \( I_1 \) curve (green; left y axis) corresponds to scattering from PmScsC C87S, the \( I_2 \) curve (blue; left y axis) corresponds to scattering from PmScsB C114A, and the \( I_{12} \) curve (gray; right y axis) is related to the arrangement of PmScsB C114A relative to PmScsC C87S. \( h_{\text{homogeneous}} = I_1 + I_2 + I_{12} \) (red; left y axis) is the scattering curve of an object with the same shape as the PmScsC C87S–PmScsB C114A complex but with homogeneous contrast and is used for estimating the Porod volume and molecular mass from the SANS data.

Figure 9. Binding sites in the PmScsC–PmScsBα SANS model and EcDsbC–EcDsbDα structure (Protein Data Bank code 1JZD). A, model of the 3:1 complex generated from the SAXS/SANS data shown in backbone and surface representation. PmScsBα binds to one PmScsC (green) protomer with both subdomains A (purple; Site 1) and B (magenta; Site 2). B, crystal structure of the EcDsbC–EcDsbDα complex. EcDsbDα (purple) binds to both protomers of EcDsbC (green; binding Sites 1 and 2). In both A and B, the catalytic cysteines involved in the intermolecular disulfide bond between the partner proteins are shown as orange spheres.

RNase A isomerase assay

To determine whether PmScsBα activates the isomerase PmScsC, an isomerase assay utilizing scrambled RNase A (scRNase A) and the RNase A model substrate cCMP was performed. Bovine RNase A (purchased from Sigma-Aldrich) was scrambled as described previously (40). The various oxidized and reduced protein samples described above for the gel-shift redox interaction assay were again prepared but with the addition of 40 \( \mu M \) scRNase A to each sample. After 2 h, 50 \( \mu l \) of the protein/scRNase A reactions was taken and added to 150 \( \mu l \) of 4 \( \mu M \) cCMP in a UV-Star 96-well plate (Greiner Bio-One, Austria). The increase in absorbance at 296 nm was monitored over 3 min with a Synergy H1 multimode plate reader (BioTek) and used to calculate the rate of cCMP hydrolysis. Native RNase A– and scRNase A–only samples were included as controls and used to determine the relative activity of the RNase A in each proteins, increasing the molecular weight and allowing the differentiation between the oxidized and reduced forms of a protein when run on a gel. Therefore, all samples were run on a nonreducing, nondenaturing 12% NuPAGE BisTris gel for 2.5 h at 100 V. The gel was Coomassie-stained and imaged using an Odyssey® Fc imaging system (LI-COR Biosciences) with the 700-nm channel.
sample. The results shown are the average and S.D. of three technical replicates.

**Complex formation**

The PmScsC and PmScsB cysteine variants were designed following the same approach used for the EcDsbC–EcDsbDα interaction (19). Mutation of cysteines in EcDsbC and EcDsbDα resulted in a stable complex between the two proteins (3). Cysteines Cys⁸⁷ of PmScsC and Cys¹¹⁴ of PmScsBα are the least accessible of the two cysteines in each active site, suggesting they facilitate resolution of intermolecular disulfide bonds. Variants PmScsC C87S and PmScsBα C114A were therefore prepared and used in complex formation studies.

The 3:1 PmScsC C87S–PmScsBα C114A complex was formed by mixing the two proteins together in a 3:1 ratio at concentrations of ~200 mg/ml and incubating the mixture at 37 °C for 3 days. To separate the complex and free PmScsBα from PmScsC, PmScsBα was left His-tagged, and the protein mixture was then run over 2 ml of fresh TALON resin twice. Columns were washed with 25 mM Tris, pH 7.4, 150 mM NaCl, and the complex and free PmScsBα were eluted with 25 mM Tris, pH 7.4, 150 mM NaCl, 250 mM imidazole. This was then run over a Superdex 200 10/300 Increase size exclusion column on an AKTA system to separate PmScsBα from complex. A peak on SEC eluted earlier than for ScsC, suggesting that a complex between the two proteins had formed (Fig. 6A). Running the fractions from the peak corresponding to the 3:1 complex on nonreducing SDS-PAGE resulted in three bands (Fig. 6B). The lower bands correlated to free PmScsC C87S (24.8 kDa) and a small amount of PmScsBα C114A (30.3 kDa), and the upper band corresponded to the molecular mass of one PmScsC C87S molecule bound to one PmScsBα C114A molecule (55.1 kDa). The upper band dissociated when reducing agent was added, leaving two bands correlating to monomeric PmScsC C87S and PmScsBα C114A in an approximate 3:1 ratio (Fig. 6B). There was also evidence for the formation of small amounts of complex containing PmScsC C87S and PmScsBα C114A in ratios of 3:3 and 3:2, but only those SEC fractions containing 3:1 complex were collected for further analysis. The complex formation process was repeated multiple times to gain enough sample for SAXS/SANS analysis as the overall yield of 3:1 complex was ~2.5%.

**Small-angle solution scattering**

Small-angle neutron scattering data shown in Table 2 were collected on the QUOKKA instrument at the Australian Nuclear Science and Technology Organisation (Table 2) (41). Two aliquots of PmScsC C87S–DPmScsBα C114A complex at ~5 mg/ml were dialyzed, one against a buffer with a D,O content of 0% and the other against a buffer with a D,O content of ~100%. Samples with D,O contents of 20, 42, and 80% were obtained by mixing the two original solutions at the appropriate ratios. The two-dimensional data were normalized to a common incident neutron count and corrected for sample transmission, background radiation, empty cell scattering, and detector sensitivity and radially averaged to produce I(q) versus q profiles. Scattering data from the two different sample–detector distances were then merged, and buffer scattering data were then subtracted from the protein + buffer data to give the protein scattering profiles (the 20, 42, and 80% buffer scattering curves were taken as a linear combination of the 0 and 100% buffer scattering curves). To correct for the effects of incoherent scattering by ¹H-rich samples, backgrounds levels were adjusted by a small constant such that the high-q scattering displayed q⁻⁴ dependence.

Small-angle X-ray scattering data were collected on the SAXS-WAXS beamline at the Australian Synchrotron (Table 2) (42). Serial dilutions of the stock solution of PmScsC C87S–DPmScsBα C114A complex were made to give four concentrations between 0.55 and 4.5 mg/ml that were centrifuged at 10,000 × g immediately before loading into a 96-well plate. To reduce radiation damage, samples (~100 µl) were drawn into a capillary from the 96-well plate and flowed past the beam. All measured two-dimensional data were averaged and corrected for sample transmission, solvent scattering, and detector sensitivity and radially averaged to produce I(q) versus q profiles using Scatterbrain (version 2.7.1) (43).

The estimated molecular masses were calculated using values for contrast and partial specific volume predicted from the protein sequence using MULCh (version 1.1) (44). Data processing and Guinier analysis were performed using PRIMUS (version 3.2) (45). The p(r) was generated from the experimental data using GNOM (version 4.6) (4) from which I(0), Rg, and Dmax were determined. For the SANS data, analysis of the dependence of Rg upon contrast and calculation of the composite scattering functions were also calculated using MULCh (Fig. 8D). Corefinement of a rigid-body model against SANS and SAXS data was performed using SASREF (version 7.0) (46) where the crystal structure of PmScsC (Protein Data Bank code 4XVV, chain A) was broken into three rigid sections: 1) residues Ala³–Gln⁴³ were fixed to preserve the structure of the trimerization domain observed in the crystal structure, 2) residues Gln⁴⁴–Phe⁴⁷ were defined as a short linker with a restraint between residues Gln⁴³ and Gln⁴⁴ to keep them in close proximity, and 3) residues Arg⁴⁸–Lys²⁷⁸ were defined as the catalytic domain free to move with a restraint between residues Phe⁴⁷ and Arg⁴⁸ to keep them in close proximity. The additional PmScsC chains were generated by symmetry (assuming a C₃ point group). The crystal structure of PmScsBα (residues Ala³–Ala²⁴⁸) was included in the optimization with a distance restraint between Cys⁸⁴ of a PmScsC monomer and Cys¹¹⁸ of PmScsBα of 8 Å. Thus, although the complex overall is not symmetrical, the PmScsC portion of the model possesses 3-fold symmetry. The optimization was run 16 times, and the model that provided the best global fit to the scattering data is reported here; however, all models obtained are similar in structure.

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