Structural and biochemical analyses indicate that a bacterial persulfide dioxygenase–rhodanese fusion protein functions in sulfur assimilation

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Hydrogen sulfide (H₂S) is a signaling molecule that is toxic at elevated concentrations. In eukaryotes, it is cleared via a mitochondrial sulfide oxidation pathway, which comprises sulfide quinone oxidoreductase, persulfide dioxygenase (PDO), rhodanese, and sulfite oxidase and converts H₂S to thiosulfate and sulfate. Natural fusions between the non-heme iron containing PDO and rhodanese, a thiol sulfurtransferase, exist in some bacteria. However, little is known about the role of the PDO–rhodanese fusion (PRF) proteins in sulfur metabolism. Herein, we report the kinetic properties and the crystal structure of a PRF from the Gram-negative endophytic bacterium Burkholderia phytofirmans. The crystal structures of wild-type PRF and a sulfurftransferase-inactivated C314S mutant with and without glutathione were determined at 1.8, 2.4, and 2.7 Å resolution, respectively. We found that the two active sites are distant and do not show evidence of direct communication. The B. phytofirmans PRF exhibited robust PDO activity and preferentially catalyzed sulfur transfer in the direction of thiosulfate to sulfite and glutathione persulfide; sulfur transfer in the reverse direction was detectable only under limited turnover conditions. Together with the kinetic data, our bioinformatics analysis reveals that B. phytofirmans PRF is poised to metabolize thiosulfate to sulfite in a sulfur assimilation pathway rather than in sulfide stress response as seen, for example, with the Staphylococcus aureus PRF or sulfite oxidation and disposal as observed with the homologous mammalian proteins.

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2 The abbreviations used are: H₂S, hydrogen sulfide; PDO, persulfide dioxygenase (also known as ETHE1); GSSH, glutathione persulfide; PRF, PDO–rhodanese fusion; PDB, Protein Data Bank.

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Characterization of a PDO–rhodanese fusion protein

The existence of PRFs in nature suggests that their mitochondrial homologs, which exist as stand-alone proteins, might interact. Structures of two PRFs are available from structural genomic projects. The first is of CstB (PDB code 3R2U) in which density for the catalytic rhodanese domain is missing. The second structure is of the biochemically uncharacterized Alicyclobacillus acidocaldarius PRF (PDB code 3TP9) (referred to hereafter as AadPRF), which contains a PDO domain fused to two rhodanese domains. Interestingly, these structures reveal the presence of a cysteine-containing loop in the PDO-active site that is absent in the human PDO structure and is not predicted in the sequence of BpPRF. This additional loop is speculated to aid in substrate transfer from the PDO domain to the rhodanese domain (23). As CstB is the only PRF that has been characterized biochemically to date (23, 30), there is a paucity of information on the roles of PRFs in sulfur metabolism in bacteria.

In this study, we have characterized the PRF protein from B. phytofirmans (referred to hereafter as BpPRF), a Gram-negative endophyte originally isolated from onion roots (31). In BpPRF, the N-terminal non-heme Fe(II)-containing PDO domain is followed by a rhodanese domain containing the signature CRXGX(T/R) active-site motif (Fig. 1B). The N-terminal PDO domain of BpPRF displays high sequence identity to human PDO (56%) and displays conservation of active-site binding ligands.

We demonstrate that BpPRF is a bifunctional enzyme that uses the rhodanese domain to preferentially catalyze sulfur transfer from thiosulfate to GSH to form sulfite and GSSH and uses the PDO domain to oxidize GSSH to sulfite (Reactions 2 and 3).

\[
S_2O_3^{2−} + GSH \rightarrow SO_3^{2−} + GSSH
\]

Reaction 2

\[
GSSH + O_2 \rightarrow GSH + SO_3^{2−}
\]

Reaction 3

The crystal structures of BpPRF provide insights into the architecture and the relative juxtaposition of its active sites.

Results

Purification and properties of BpPRF

Recombinant wild-type and variants of BpPRF purified using a one-step protocol were obtained in >95% purity as judged by SDS-PAGE analysis (data not shown). The yield for the full-length proteins (wild type and C314S) was ~120 mg of protein/liter of culture. The rhodanese domain was obtained with a purity of 95% as judged by SDS-PAGE analysis (data not shown). The yield for the PDO domain was ~120 mg of protein/liter of culture. BpPRF eluted from a size-exclusion column with a molecular mass corresponding to
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### Table 1

| Data collection | Wild-type PRF | C314S PRF-apo | C314S PRF-GSH |
|-----------------|---------------|---------------|---------------|
| PDB code       | 5VE3          | 5VE4          | 5VE5          |
| Space group     | P2₁,2₁,2₁     | P6₃,2₂       | P6₃,2₂       |
| Cell dimensions |               |               |               |
| a, b, c (Å)     | 63.7, 108.3, 119.6 | 84.5, 84.5, 549.4 | 83.5, 83.5, 547.6 |
| α, β, γ (°)     | 90, 90, 90    | 90, 90, 120  | 90, 90, 120  |
| X-ray source    | APS 23ID-D    | APS 23ID-B   | APS 23ID-B   |
| Wavelength (Å)  | 1.033         | 1.033        | 1.033        |
| d_{min} (Å)     | 1.79 (1.86–1.79)* | 2.65 (2.75–2.65) | 2.35 (2.43–2.35) |
| R-merge         | 0.119 (1.47)  | 0.126 (1.35) | 0.126 (1.56) |
| Inner-shell R-merge | 0.045 (5.3 Å)* | 0.047 (7.8 Å) | 0.050 (7.0 Å) |
| Average I/σ(I) | 11.6 (1.2)    | 11.0 (1.7)   | 11.0 (1.6)   |
| Completeness (%)| 100 (96.0)    | 100 (100)    | 99.0 (99.0)  |
|Multiplicity     | 12.4 (9.0)    | 8.6 (8.5)    | 8.7 (9.2)    |
|Total observations | 966,980 (67,309) | 303,925 (28,966) | 422,379 (43,869) |
|CC₁/₂ | 0.999 (0.537) | 0.997 (0.398) | 0.998 (0.904) |
|CCₙ/ₙ | 1.0 (0.836)   | 0.999 (0.973) | 0.999 (0.975) |

### Refinement

- Data range (Å): 41.26–1.79, 43.94–2.65, 41.75–2.35
- Reflections used in refinement: 77,982 (7448), 35,305 (3392), 48,680 (4773)
- No. of non-hydrogen atoms: 6051, 8265, 8471
- Protein: 5538, 8153, 8154
- Ligands: 2, 24, 74
- Water: 511, 88, 189
- Amino acid residues: 698, 1047, 1048
- Deviation from ideality:
  - Bond lengths (Å): 0.013, 0.003, 0.007
  - Bond angles (°): 1.20, 0.70, 0.97
  - Average B-factor: 38.1, 81.9, 76.6
  - Macromolecules: 37.6, 82.4, 77.0
  - Ligands: 29.6, 59.0, 83.1
  - Solvent: 44.1, 45.6, 58.4

### Ramachandran plot

- Favored (%): 98.0, 96.0, 97.5
- Allowed (%): 2.0, 2.0, 2.0
- Outliers (%): 0, 0, 0.2

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40 kDa, consistent with it being a monomer (predicted mass = 41.5 kDa). The rhodanese domain eluted as a mixture of a monomer and a dimer (data not shown). The monomeric organization of BpPRF is distinct from that of CstB, which is tetrameric (23).

The thermal stability of the BpPRF variants was assessed in a turbidometric assay. The *T_m* value for the isolated rhodanese domain (55 ± 2 °C) was slightly higher than for wild type (50 ± 2 °C) and C314S (51.6 ± 0.8 °C) BpPRF. This result indicates that the stand-alone rhodanese domain is well folded and contrasts with the instability of the excised catalytic rhodanese domain of CstB (23).

### Metal analysis

Plasma emission spectroscopy revealed the presence of 0.54 mol of iron/mol of monomer wild-type BpPRF. The metal content was also assessed by a colorimetric assay (32), which yielded a similar value (0.60 mol of iron/mol of monomer). The iron content of C314S BpPRF was 0.46 mol of iron/mol of monomer. Attempts to fully reconstitute the metal site with FeCl₂ under anaerobic conditions were unsuccessful.

### Structure of BpPRF

The crystal structure of BpPRF co-crystallized with thiosulfate was obtained at 1.79 Å resolution by molecular replacement using the AadPRF structure (PDB code 3TP9) as template (Table 1). The final model contains two chains in the asymmet-
and hydrophobic residues Ala-316, Gly-317, and Gly-318 on the other side (supplemental Fig. S1C).

C314S BpPRF crystallized in a different space group (Table 1), but the two domains were in the same relative orientation as in wild-type BpPRF. Crystals of C314S BpPRF that had been soaked with GSH displayed additional density in two of the three PDO domains present in the asymmetric unit. GSH coordinates to iron in the PDO-active site and displaces one of the iron-coordinated waters. It is within hydrogen bonding distance of Tyr-176, Arg-193, Arg-142, and Lys-216 (Fig. 3A). Notably, in the C314S BpPRF structure, a chloride ion (from the crystallization solution) sits in place of the Cys-314 persulfide modification.

**Structural comparisons of BpPRF and homologous human proteins**

The architecture of the BpPRF PDO-active site is very similar to that of human PDO (Fig. 3B) (15). The substrate-binding pocket is framed by two α-helices on one side and by Tyr-176 on the other side. Key residues that are predicted to be involved in substrate binding in human PDO, Tyr-197, Arg-214, Arg-163, Leu-231, and Pro-234, are conserved in BpPRF (Tyr-176, Arg-193, Arg-142, Leu-212, and Pro-215) and are found in similar orientations. Lys-216, which is not conserved in human PDO, lines the entrance to the active site and might play a role in substrate positioning.

The BpPRF rhodanese domain and the catalytic domain of bovine rhodanese display very similar folds (Fig. 4A). Minor structural differences include the N-terminal β-hairpin extension in BpPRF and two extra loops in bovine rhodanese. The active sites are also very similar with the conserved residues, Cys-314, Arg-315, and Gly-318 (BpPRF numbering), being in similar orientations (Fig. 4B). However, Thr-252 and Lys-249 in bovine rhodanese occupy the same positions as Arg-319 and Ala-316, respectively, in BpPRF. These substitutions indicate differences in charge distribution that might contribute to differences in substrate specificity.

**PDO activity of BpPRF**

The rate of O2 consumption during conversion of GSSH to sulfite (Reaction 3) was monitored using an O2 electrode (Fig. 5) and yielded the following kinetic parameters for wild-type BpPRF, $K_m(GSSH) = 70 \pm 8 \mu M$, and $K_m(O_2) = 130 \pm 30 \mu M$, and $k_{cat} = 143 \text{ s}^{-1}$ at 22°C (Table 2). C314S BpPRF exhibited a 29-fold lower $k_{cat}$ and an ~5-fold higher $K_m$ for GSSH than
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![Diagram A](image1)

Figure 4. Comparison of the BpPRF rhodanese domain with rhodanese. A, structural overlay of the BpPRF rhodanese domain (red) with the catalytic domain of bovine rhodanese (PDB code 1RHD, gray (34)). The active-site cysteine residues, Cys-247 (bovine) and Cys-314 (BpPRF), are shown in stick representation. Structural differences are shown with arrows: a β-hairpin extension is seen only in BpPRF (red), and two loop extensions are seen only in bovine rhodanese (black). B, structural overlay of the rhodanese active-site loops from BpPRF (red) and bovine rhodanese (gray).

![Diagram B](image2)

Figure 5. Kinetics of PDO activity of BpPRF. Dependence of PDO activity on GSH concentration for wild-type BpPRF (solid circles) and C314S BpPRF (open circles). Oxygen consumption by BpPRF in 100 mM sodium phosphate buffer, pH 7.4, was monitored at 22 °C in the presence of varying concentration of GSH. The data are representative of three independent experiments.

Table 3

| Substrate | KM (mM) | kcat (s⁻¹) |
|-----------|---------|------------|
| GSSH      | 0.2     | 5.1        |
| Cys-SH    | 0.7     | 6.2        |
| GSH       | 0.8     | 0.2        |
| S2O3⁻     | 5.8     | 1.6        |

Conversion of cyanide to thiocyanate in the presence of thiosulfate (Reaction 4) was used to monitor the sulfurtransferase activity of BpPRF (Figs. 6A and 7A).

\[
S_2O_3^- + CN^- \rightarrow SO_4^{2-} + SCN^-
\]

Reaction 4

From this analysis, \( K_m (\text{thiosulfate}) \) of 5.4 ± 0.7 mM, \( K_m (\text{CN}^-) \) of 22.7 ± 0.7 mM, and \( k_{cat} \) of 5.1 s⁻¹ at 22 °C were obtained (Table 3). Under the same conditions, the sulfurtransferase activity of the C314S mutant, carrying an inactivating mutation in the rhodanese domain, was not detectable (data not shown).

Because GSSH, a product of the sulfurtransferase reaction (Reaction 2), is a substrate for the PDO domain (Reaction 3), this activity was monitored in a “coupled” assay, i.e. by detecting O₂ consumption (Figs. 6C and 7C). This assay yielded the following parameters: \( K_m (\text{GSSH}) = 4.7 ± 0.7 \text{ mM} \), \( K_m (\text{thiosulfate}) = 3.1 ± 0.2 \text{ mM} \), and \( k_{cat} = 6.2 \text{ s}^{-1} \) (Table 3). The same reaction was also monitored directly, i.e. by detecting sulfite (Reaction 2) colorimetrically, and it yielded essentially the same results (data not shown).

Cysteine was tested as an alternate sulfur acceptor in lieu of GSH, by monitoring \( \text{H}_2\text{S} \) production using the lead sulfide assay (Reactions 5 and 6) (Figs. 6E and 7E).

\[
\text{S}_2\text{O}_3^{2-} + \text{Cys-SH} \rightarrow \text{SO}_4^{2-} + \text{Cys-SSH}
\]

Reaction 5

\[
\text{Cys-SSH} + \text{Cys-SH} \rightarrow \text{Cys-S-S-Cys} + \text{H}_2\text{S}
\]

Reaction 6

This analysis yielded the following values: \( K_m (\text{Cys}) = 5.2 ± 0.4 \text{ mM} \), \( K_m (\text{thiosulfate}) = 5.8 ± 0.8 \text{ mM} \), and \( k_{cat} = 0.2 \text{ s}^{-1} \). Given the relatively low cellular concentration of cysteine versus GSH and the considerably lower \( k_{cat}/K_m (\text{Cys}) \) value (Table 3), cysteine is unlikely to be a physiologically relevant sulfur acceptor for BpPRF.

Next, we attempted to monitor sulfurtransferase activity in the opposite direction (i.e. reverse of Reaction 2) by tracking...
thiosulfate formation under anaerobic conditions (to inhibit GSSH consumption by PDO). However, unlike human rhodanese (18, 20), sulfur transfer activity from GSSH to sulfite was not detected with \( \text{BpPRF} \) under steady-state turnover conditions.

Sulfurtransferase activity of the rhodanese domain

The isolated rhodanese domain is active and exhibits the following kinetic parameters in the cyanide detoxification assay (Figs. 6B and 7B): \( K_m(\text{thiosulfate}) = 9.2 \pm 0.8 \text{ mM} \), \( K_m(\text{CN}^-) = 23 \pm 1 \text{ mM} \), and \( k_{cat} = 4.6 \text{ s}^{-1} \) (Table 4), which are very similar to those for full-length \( \text{BpPRF} \). GSSH synthesis from thiosulfate and GSH (Reaction 2) was monitored by following \( \text{H}_2\text{S} \) formation (Reaction 7) (Fig. 6D and 7D).

The following values were obtained from this analysis: \( K_m(\text{GSSH}) = 14.9 \pm 0.5 \text{ mM} \), \( K_m(\text{thiosulfate}) = 3.8 \pm 0.5 \text{ mM} \), and \( k_{cat} = 9.0 \text{ s}^{-1} \). As with the full-length protein, cysteine was a poorer acceptor (6-fold lower \( k_{cat}/K_m \)) than GSH (Figs. 6F and 7F), and the reverse reaction (i.e. transfer from GSSH to sulfite) was not detectable under steady-state turnover conditions (Table 4). Similar values for the kinetic parameters were obtained when the rate of sulfite production was detected (data not shown).

\[
\text{GSSH} + \text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{S}
\]

Reaction 7

Table 2

| Enzyme   | Iron content | \( V_{max} \) (GSSH) | \( K_m \) (GSSH) | \( K_m \) (O2) | \( k_{cat} \) | \( k_{cat}/K_m \) (GSSH) | \( k_{cat}/K_m \) (O2) |
|----------|--------------|----------------------|------------------|-------------|-------------|------------------------|------------------------|
| Wild type| \( 0.60 \pm 0.03 \) | \( 207 \pm 6 \) | \( 0.070 \pm 0.008 \) | \( 0.13 \pm 0.03 \) | \( 143 \) | \( 2043 \) | \( 1100 \) |
| C314S    | \( 0.46 \pm 0.02 \) | \( 7.3 \pm 0.1 \) | \( 0.37 \pm 0.04 \) | \( 5 \) | \( 14 \) | | |

Figure 6. Kinetics of \( \text{BpPRF} \)-catalyzed sulfur transfer reactions. The sulfurtransferase activity associated with the rhodanese domain in wild-type \( \text{BpPRF} \) (A, C, and E) or with the isolated rhodanese domain (Rhod domain) (B, D, and F) was determined in the presence of a constant thiosulfate concentration (60 mM) and varying concentrations of cyanide (A and B), GSH (C and D), or cysteine (E and F). The data are representative of 3–4 independent experiments. The data were fitted with the Michaelis-Menten, sigmoidal, or Hill equation as described under “Experimental procedures.”

Figure 7. Kinetics of \( \text{BpPRF} \)-catalyzed sulfur transfer reactions. Dependence of sulfurtransferase activity on varying thiosulfate concentrations in the presence of 60 mM cyanide (A and B), 30 mM GSH (C and D), and 50 mM cysteine (E and F) for the wild-type \( \text{BpPRF} \) and the isolated rhodanese domain (Rhod Domain). The reactions were performed as described under “Experimental procedures.” The data are representative of 3–4 independent experiments. Data were fitted with either Michaelis-Menten or Hill equation.

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Table 3
Kinetic parameters for the sulfur transferase activity of BpPRF sulfurtransferase
The kinetic parameters for the sulfur transfer reactions catalyzed by the isolated rhodanese domain were determined as summarized in Table 3 legend and described in detail under “Experimental procedures.” ND is not detected.

| Donor | Acceptor | $V_{max}$ | $K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ |
|-------|----------|-----------|-------|-------|-----------|---------------|---------------|
|       |          | $\mu$mol min$^{-1}$ mg$^{-1}$ | $\mu$mol mg$^{-1}$ | $s^{-1}$ | $s^{-1}$ | $s^{-1}$ | $s^{-1}$ |
| 1) $S_2O_3^{2-}$ | CN$^-$ | 7.4 ± 0.3 | 5.4 ± 0.7 | 227.7 ± 0.7 | 5.1 | 0.9 | 0.2 |
| 2) GSSH | SO$_2^{2-}$ | ND | ND | ND | ND | ND | ND |
| 3) $S_2O_3^{2-}$ | GSH | 8.9 ± 0.2 | 3.1 ± 0.2 | 4.7 ± 0.4 | 6.2 | 2.0 | 1.3 |
| 4) $S_2O_3^{2-}$ | Cys | 0.32 ± 0.01 | 5.8 ± 0.8 | 5.2 ± 0.4 | 0.2 | 0.04 | 0.04 |

Table 4
Kinetic parameters for the isolated rhodanese domain of BpPRF
The kinetic parameters for the isolated rhodanese domain were determined as summarized in Table 3 legend and described in detail under “Experimental procedures.” ND is not detected.

Stoichiometry of PRF reaction
Our kinetic data predict that BpPRF can catalyze a GSH-dependent net oxidation of thiosulfate to sulfite (Reactions 2 and 3). To test this prediction, the stoichiometry of thiosulfate and O$_2$ consumed to sulfite formed was determined. As noted previously, the method used to synthesize GSSH results in an equivalent of GSH being present, which together with unreacted GSSG and an ~3-fold excess of H$_2$S would confound the reaction stoichiometry. To circumvent this problem, GSSH was synthesized in situ using GSH and thiosulfate as substrates (Reaction 2).

Under single-turnover conditions, the stoichiometry of sulfite formed/thiosulfate consumed/oxygen consumed was 2:1:1 (Fig. 8A) as predicted by Reactions 2 and 3. Also, as expected, the reaction stoichiometry of sulfite formed/GSSH consumed/thiosulfate consumed was 1:1:1 in the absence of oxygen (Fig. 8B). However, when the BpPRF reaction was monitored under multiple turnover conditions in the presence of oxygen, the stoichiometry of sulfite formed/thiosulfate consumed/oxygen consumed switched to 1:1:1 after three turnovers (Fig. 8C). This change in stoichiometry indicates inefficient coupling between the PDO and rhodanese-active sites and is likely confounded by side reactions of the reactive products, particularly GSSH (35), as it builds up under these conditions.

Discussion
Natural fusions of PDO and rhodanese are found in bacteria and suggest a functional interaction between them in other organisms, where they are expressed as stand-alone proteins (36). In this study, we provide the first structural and kinetic characterization of a PRF. To our knowledge, the only other PRF that has been biochemically characterized is CstB from S. aureus (23, 30, 37), but the available CstB structure is missing density for the catalytic rhodanese domain. CstB is located in the cst operon (Fig. 1C). Its operonic partners include CstA, which is a rhodanese, and a sulfide quinone oxidoreductase, which oxidizes H$_2$S to polysulfides (30). Expression of the cst operon is regulated by the persulfide/polysulfide-sensing repressor CstR and is induced by exogenous addition of H$_2$S or polysulfides (38). CstB catalyzes the conversion of low molecular weight persulfdies to thiosulfate via the intermediate formation of sulfite (Reaction 8) (23).

$$2RSSH + O_2 + H_2O \rightarrow 2S_2O_3^{2-} + 2RSH + 2H^+$$

Reaction 8

In contrast to CstB, BpPRF preferentially catalyzes sulfur transfer in the reverse direction, i.e. from thiosulfate to sulfite (Reaction 9), which was confirmed under single-turnover conditions (Fig. 8).

$$S_2O_3^{2-} + O_2 + H_2O \rightarrow 2SO_3^{2-} + 2H^+$$

Reaction 9

GSSH serves as the intermediate sulfur carrier, transferring sulfur processed in the rhodanese-active site to the PDO-active site. Unlike CstB, which functions in the sulfide stress response, the cellular context in which BpPRF functions is not known (38). The gene encoding BpPRF does not reside in an operon, and the metabolic context in which it functions is not immediately obvious. The BpPRF-encoding gene is located downstream from a putative peroxidase and a putative LysR-type regulator, which have been shown to be involved in regulating bacterial mobility, virulence, metabolism, and quorum sensing (39) (Fig. 1C). A potential fate of sulfite, which is toxic, is its oxidation by sulfite oxidase to sulfate, which could be utilized in an assimilatory sulfate-reducing pathway. B. phytofirmans encodes a putative sulfite oxidase. Alternatively, BpPRF might be involved in sulfur assimilation from thiosulfate and sulfite could be reduced by sulfite reductase (encoded by B. phytofirmans) to H$_2$S, which in turn can be used for cysteine synthesis. In Burkholderia cenocepacia, CysB and SsuR regulate genes
Several SU defense sites (supplemental Fig. S2) scored as positive motif hits in the BpPRF promoter region (P < 0.005), suggesting a role for BpPRF in sulfur assimilation.

The catalytic efficiency of BpPRF (kcat/km(GSH)) = 2 × 10^6 M^-1 s^-1 at 22 °C) is 14-fold higher than of human PDO (1.4 × 10^5 M^-1 s^-1 at 22 °C (14)) and ~10^2-fold greater than CstB with its preferred substrate, CoA(SH) (18 × 10^5 M^-1 s^-1 at 25 °C) (23). Mutation of the catalytic cysteine residue, Cys-314, in the rhodanese domain decreased iron content 1.3-fold and PDO activity 29-fold. This result is surprising because it indicates communication between the rhodanese- and PDO-active sites, which are, however, distant and oriented 90° away from each other in the crystal structure (supplemental Fig. S1).

The thiosulfate: cyanide sulfurtransferase activity of BpPRF (kcat = 5.1 s^-1 at 22 °C) is comparable with that of CstB (8.5 s^-1 at 25 °C) (23) and is 180–730-fold lower than human rhodanese (20). The thiosulfate:GSH sulfur transfer activity of BpPRF (kcat = 6.2 s^-1 at 37 °C) is 9-fold higher than for human rhodanese (0.67 s^-1 at 37 °C), which preferentially catalyzes the reaction in the reverse GSSH:sulfite sulfur transfer direction (kcat = 389 s^-1 at 25 °C). The isolated rhodanese domain was well folded as evidenced by its high Tm (55 ± 2 °C) like that of the full-length protein (50 ± 2 °C) and its comparable kcat values in the thiosulfate:cyanide and thiosulfate:GSH sulfur transfer reactions (Table 4). The isolated rhodanese domain exhibits a sigmoidal dependence on GSH and thiosulfate concentration (Figs. 6D and 7D), in contrast to the hyperbolic dependence observed with full-length BpPRF (Figs. 6C and 7C). This difference in behavior could be due to the difference in the oligomeric state of the stand-alone rhodanese domain, which exists as a monomer/dimer mixture in solution, in contrast to the full-length protein, which is a monomer.

Unlike human rhodanese, sulfur transfer from GSSH to sulfite was not detectable with either the full-length BpPRF or the stand-alone rhodanese domain (Tables 3 and 4). However, the GSSH:sulfite sulfur transfer activity was observed for both the full-length BpPRF and the stand-alone rhodanese domain under limited turnover conditions, i.e. between 2 and 5 turnovers (data not shown). This observation indicates that under multiple turnover conditions as the concentrations of products (i.e. GSH or thiosulfate) increase, one of them inhibits the enzyme. In fact, we found that thiosulfate, but not GSH, inhibits the GSSH:sulfite sulfur transfer activity of BpPRF (data not shown) explaining the lack of activity under steady-state assay conditions. The inhibitory role of thiosulfate is consistent with studies on TSTD1 and human rhodanese, in which thiosulfate was reported to exhibit substrate inhibition, although at high concentrations, for sulfur transfer from thiosulfate to GSH (18, 21, 43).

It is possible that our structure of BpPRF represents an “open” conformation versus a “closed” conformation in which the two domains are proximal. We therefore attempted to model a putative closed conformation by superimposing the PDO and rhodanese domains on the structure of AaPRF. In the latter, the PDO and rhodanese-active sites face each other and are separated by a distance of 27 Å (Fig. 9A). AaPRF contains an additional non-catalytic rhodanese domain and a cysteine-containing loop (Cys-202, AaPRF numbering) located in the PDO.

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Figure 8. Product analysis and reaction stoichiometry of the BpPRF. The reaction stoichiometry of BpPRF under single-turnover conditions was analyzed in the presence (A) and absence (B) of oxygen. The reactions were performed in the presence of 250 μM GSH, 250 μM thiosulfate and 250 μM enzyme. C, stoichiometry of BpPRF reactions under multiple turnover conditions was analyzed in the presence of 250 μM GSH, 250 μM thiosulfate and decreasing enzyme concentrations as described under “Experimental procedures.” Data are representative of 4–5 independent experiments.

encoding enzymes involved in the aliphatic sulfonate assimilation pathway (25, 40). BLAST analysis reveals that B. phytofirmans possesses two enzymes that have 23 and 26% identity to the CysB and SsuR transcriptional regulators, respectively. Bioinformatic analysis of the sequence 100 bp upstream of the BpPRF gene queried against CysB- and SsuR-binding sites was performed using the FIMO MEME suite database (25, 40, 41).
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Figure 9. Modeling of potential BpPRF domain interaction interface. A, overlay of BpPRF PDO domain (blue) and the BpPRF rhodanese domain (red) with the PDO domain and catalytic rhodanese domain of AaPRF (PDB code 3TP9) (shown in gray). The active-site loop of AaPRF (yellow) with Cys-202 faces the iron center (orange sphere) in the PDO domain. B, electrostatic surface potential of the BpPRF domains in the same orientation as in A but rotated by 35°. The arrows highlight locations of the PDO- and rhodanese (Rhod)-active sites. Positive and negative electrical potential are shown in blue and red, respectively, and represent a range of −5 to +5 kT/e.

domain active site. This loop, although absent in BpPRF, is also present in CstB and postulated to facilitate substrate transfer between the PDO and catalytic rhodanese domains (23). To adopt a similar domain orientation, the linker region of BpPRF, which is maximally ~36 Å long, would have to span 47 Å requiring unfolding of a secondary structure element. Furthermore, the calculated electrostatic surface potential reveals that positively charged residues line the entrance to the PDO and rhodanese-active sites, suggesting that a direct interaction between them would be unfavorable (Fig. 9B). The structural independence of the active sites is also consistent with the high catalytic activity of the isolated rhodanese domain. This result is in contrast to CstB in which the rhodanese domain, once isolated, does not retain activity (23). Fusion of the PDO and rhodanese domains in BpPRF would serve to increase their effective local concentration and promote transfer of substrates between them.

An overlay of five structures, i.e. two per asymmetric unit of wild-type BpPRF and three per asymmetric unit of C314S BpPRF, was used to assess significant conformational differences, if any, between them (supplemental Fig. S3). Because the electron density for eight residues in the linker region was missing (bold font): forward 5'-TTAATTCTATATGGTTGATCTTCCGGCACGATA-TTC-3' and reverse 5'-TTAATTAAGCTTCTACACGCT- TGTTCACACGCACCG-3'. The resulting PCR fragment was cloned into a pET28b vector. The C-terminal truncated BpPRF (ΔC-BpPRF) construct missing the last four residues (ΔC 354–357) was generated for crystalllography only, using the full-length BpPRF expression construct as a template and the following primers containing Ndel and HindIII restriction sites (bold font): forward 5'-TTAATTCTATATGGTTGATCTTCCGGCACGATA-TTC-3' and reverse 5'-TTAATTAAGCTTCTACACGCT- TGTTCACACGCACCG-3'. The resulting PCR fragment was cloned into a pET28b vector. The C314S mutant was generated using the QuickChange kit (Stratagene) and the wild-type BpPRF expression construct as a template. The isolated rhodanese domain was subcloned from the wild-type PRF expression construct using the following primers containing Ndel and HindIII restriction sites (bold font): forward 5'-TTAATTAAGCTTCTACACGCT- TGTTCACACGCACCG-3'. The resulting PCR fragment was cloned into a pET28b vector.

Expression and purification of BpPRF

The recombinant BpPRF protein was expressed in BL21 E. coli. A 200-ml culture in Luria Bertani medium was grown overnight at 37 °C and used to inoculate a 4× 1-liter culture in

Experimental procedures

Materials

Sodium sulfide, sodium sulfite, sodium thiosulfate, GSH, oxidized glutathione, l-cysteine, para-rosaniline hydrochloride, and 2,4,6-tripyridyl-s-triazine were purchased from Sigma. FluoroPure grade monobromobimane was purchased from Life Technologies, Inc.

Expression constructs for BpPRFs

The cDNA encoding BpPRF was amplified from genomic DNA isolated from B. phytofirmans PsjN (purchased from DSMZ, Braunschweig, Germany), using the following primers containing Ndel and HindIII restriction sites (bold font): forward 5'-TTAATTCTATATGGTTGATCTTCCGGCACGATA-TTC-3' and reverse 5'-TTAATTAAGCTTCTACACGCT- TGTTCACACGCACCG-3'. The resulting PCR fragment was cloned into a pET28b vector. The C-terminal truncated BpPRF (ΔC-BpPRF) construct missing the last four residues (ΔC 354–357) was generated for crystalllography only, using the full-length BpPRF expression construct as a template and the following primers containing Ndel and HindIII restriction sites (bold font): forward 5'-TTAATTCTATATGGTTGATCTTCCGGCACGATA-TTC-3' and reverse 5'-TTAATTAAGCTTCTACACGCT- TGTTCACACGCACCG-3'. The resulting PCR fragment was cloned into a pET28b vector.
the same medium. Cultures were grown at 28 °C, and expression was induced with isopropyl-β-D-thiogalactopyranoside (100 μM) when the optical density at 600 nm reached 0.5. Cultures were supplemented with ferrous ammonium sulfate at the time of induction to a final concentration of 250 μM, and growth was continued for an additional 14 h at 28 °C. Cells were harvested by centrifugation at 2683 × g for 20 min at 4 °C.

*Bp*PRF was purified as follows. The cell pellet from a 4-liter culture was resuspended in 500 ml of 50 mM Tris buffer, pH 8, containing 0.5 M NaCl (Buffer A), 1 tablet of protease inhibitor mixture (Roche Applied Science), and 100 mg of lysozyme (Sigma). DNase (50 mg) and MgCl₂ (10 mM final concentration) were added to the cell suspension and stirred at 4 °C for 60 min followed by sonication on ice with the following pulse sequence: 30-s burst, 1-min rest for a total burst time of 5 min at a power output setting of 6. The sonicate was centrifuged at 8217 × g for 15 min at 4 °C. The resulting supernatant was diluted 2-fold with Buffer A and loaded onto a 20-ml nickel-nitrilotriacetic acid column equilibrated with the same buffer. The column was washed with 500 ml of Buffer A containing 30 mM imidazole buffer. PRF was eluted from the column with a nitrilotriacetic acid column equilibrated with the same buffer. Diluted 2-fold with Buffer A and loaded onto a 20-ml nickel-nitrilotriacetic acid column equilibrated with the same buffer. The column was washed with 500 ml of Buffer A containing 30 mM imidazole buffer. PRF was eluted from the column with a nitrilotriacetic acid column equilibrated with the same buffer. Diluted 2-fold with Buffer A and loaded onto a 20-ml nickel-nitrilotriacetic acid column equilibrated with the same buffer.

**Metal analysis**

Plasma emission spectroscopy was used to analyze the total metal content of wild-type *Bp*PRF at the Chemical Analysis Laboratory (University of Georgia, Athens). Twenty metal ions were detected by this method. The iron content of the enzyme was also measured using a colorimetric assay described previously (32). This method allows for the direct measurement of total iron content, the Fe(II) content, and consequently, estimation of Fe(III) content of the enzyme. Briefly, *Bp*PRF (10–50 μM) was denatured with 0.5 N HCl and 5% (w/v) trichloroacetic acid (final volume 1 ml), mixed for 30 s, and subsequently centrifuged for 10 min at 16,000 × g in a microcentrifuge. To determine the total iron content, the supernatant (700 μl) was mixed with 300 μl of a 1:2:1 mixture of 4 mM 2,4,6-tripyridyl-S-triazine, 50% ammonium acetate, and 10% hydroxylamine chloride, and incubated at room temperature for 5 min. Hydroxylamine is present to reduce residual Fe(III) to Fe(II). Absorbance of the resulting Fe(II)–2,4,6-tripyridyl–S-triazine complex was measured at 569 nm. The total iron concentration was calculated using an extinction coefficient of 22,600 M⁻¹ cm⁻¹ for the Fe(II)–2,4,6-tripyridyl–S-triazine complex (32).

**Molecular mass determination**

Purified *Bp*PRF (5 ml of 3.7 mg/ml) was loaded onto a HiLoad™ 16/60 Superdex G-200 column equilibrated with Buffer A at 4 °C and calibrated with protein standards (Bio-Rad). The protein was eluted at a flow rate of 0.5 ml min⁻¹ and was monitored by absorbance at 280 nm.

**Thermal denaturation assay**

The thermal stability of *Bp*PRF and of the C314S rhodanese domain mutant was evaluated by monitoring the increase in absorbance at 600 nm with increasing temperature. For this, enzyme (100 μg) in Buffer A (final volume 200 μl) was placed in a cuvette housed in a Cary 100 Bio spectrophotometer equipped with a heating block connected to a water bath. The temperature was increased from 25 to 70 °C in 5 °C increments.

**Preparation of GSSH**

GSSH was prepared anaerobically as described previously (14) by reacting oxidized glutathione (GSSG) with Na₂S in a Coy anaerobic chamber (atmosphere of 95:5 N₂/H₂) (Reaction 10).

\[
\text{GSSG} + \text{Na}_2\text{S} \rightarrow \text{GSSH} + \text{GSH}
\]

**Reaction 10**

Briefly, solid Na₂S was added in 4-fold excess, to an anaerobic solution of 50 mM GSSG in 350 mM sodium phosphate, pH 7.4 (final volume 5 ml). The reaction vial was immediately sealed to prevent loss of Na₂S and incubated at 37 °C for 25–30 min. The concentration of GSSH was measured using the cold cyanolysis method as described previously (14). Substrate was either used immediately or stored at −20 °C until use. Substrate concentration was measured both before freezing and after thawing prior to use in the enzyme assay. Cysteine persulfide was prepared using the same procedure.

**Oxygen consumption assay**

The PDO activity of *Bp*PRF was measured by monitoring O₂ consumption at room temperature (22 °C). The reaction mixture consisted of 100 mM sodium phosphate, pH 7.4, and 0.5–2 μg of enzyme (final volume 1 ml). The reaction mixture containing enzyme and the GSSH substrate in 100 mM phosphate, pH 7.4, was prepared anaerobically in an inflatable glove bag (Cole-Palmer) filled with N₂ (>99% purity). The reaction was started by injecting a known concentration of dissolved O₂ (generated using a 100% oxygen tank). The final O₂ concentration varied from 1.6 to 250 μM. The O₂ concentration in oxygenated buffer was independently determined using the O₂ probe.

**Thiosulfate cyanide sulfurtransferase assay**

The cyanide detoxification activity of *Bp*PRF was measured in a colorimetric assay as described previously (44). The reaction mixture contained 60 mM thiosulfate, 60 mM potassium cyanide, and 5–10 μg of protein in 200 mM sodium phosphate...
buffer, pH 7.4 (330 μl final volume). The reaction was initiated by addition of enzyme. After incubation for 20 min at 22 °C, the reaction was stopped by addition of 100 μl of 15% (w/v) formaldehyde. Thiocyanate formation was measured by addition of 500 μl of a ferric nitrate solution containing 165 mM ferric nitrate nonahydrate and 13.3% (v/v) nitric acid. The absorbance of the resulting ferric thiocyanate complex was measured at 460 nm. The concentration of thiocyanate was determined using a standard curve. The concentrations of enzyme activity on substrate concentration.

**Thiosulfate:GSH sulfurtransferase activity monitored in a coupled assay**

The GSSH formed via the sulfur transfer activity of BpPRF was measured by coupling to O2 consumption during GSSH utilization by the PDO activity. For this, the reaction mixture contained 30 mM thiosulfate, 30 mM GSH, 25–30 μg enzyme, and 200 mM sodium phosphate buffer, pH 7.4 (final volume 1.6 ml), mixed in a Gilson-type chamber containing a Clark-type oxygen electrode at 22 °C. GSH and thiosulfate concentrations were varied from 0.3 to 30 mM to determine the dependence of enzyme activity on substrate concentration.

**Thiosulfate:GSH sulfurtransferase activity monitored by sulfite formation**

The thiosulfate:GSH sulfurtransferase activity of BpPRF and of the isolated rhodanese domain was monitored by detecting sulfite formation using a modified version of the colorimetric assay described previously (45). The reaction mixture contained 45 mM thiosulfate, 45 mM GSH, 0.7–3 μg enzyme, and 200 mM sodium phosphate buffer, pH 7.4 (final volume of 500 μl). The reaction was initiated by addition of enzyme. After 5 min of incubation at 22 °C, the reaction was stopped by the addition of 500 μl of 0.23 M HgCl2. After centrifugation at 16,000 × g for 1 min, the supernatant (125 μl) was mixed with 1 ml of a p-roxanilin solution containing 2:1 0.04% p-roxanilin prepared in 0.72 M HCl (w/v), 0.2% formaldehyde. The absorbance of the resulting p-roxanilin sulfonic acid complex was measured at 570 nm. The concentration of sulfite formed was determined using a standard curve. The concentrations of thiosulfate and GSH were varied from 0.3 to 50 mM to determine the dependence of enzyme activity on substrate concentration.

**Thiosulfate–thiol sulfurtransferase activity monitored by H2S formation**

The sulfur transfer activities of BpPRF and of the isolated rhodanese domain was measured by detecting H2S formation using the lead acetate assay described previously (18). The reaction mixture contained thiosulfate (0.5–25 mM) and either GSH (2–40 mM) or cysteine (1–50 mM) in 200 mM sodium phosphate buffer, pH 7.4 (final volume of 1 ml), and was preincubated at 37 °C for 10 min. The reaction was initiated by addition of 0.7–15 μg of enzyme. Formation of lead sulfide was measured at 390 nm, and the concentration of H2S formed was calculated using an extinction coefficient of 5500 M⁻¹ cm⁻¹ for lead sulfide (18).

**GSSH:sulfite sulfurtransferase activity**

The reactions were performed in a Coy anaerobic chamber to prevent oxidation of GSSH by the PDO domain. The standard reaction mixture contained 1 mM GSSH and 1 mM sulfite, 0.5–50 μg of enzyme, and 20 mM sodium phosphate buffer, pH 7.4 (200 μl final volume). The derivatization reaction was initiated by the addition of enzyme. After 5 min of incubation, monobromobimane was added to a final concentration of 3 mM and incubated at room temperature for 15 min. The derivatization reaction was terminated by addition of 100 μl of 0.2 M sodium citrate, pH 2.0. Production of thiosulfate from GSSH and sulfite was monitored by HPLC. For this, a 60 mM stock solution of monobromobimane was prepared in DMSO and protected from light during preparation and handling. Bimane adducts of GSSH, Na2S, GSH, sulfite, and thiosulfate were prepared in 20 mM sodium phosphate, pH 7.4. Each standard (final concentration 250 μM) was incubated with a final concentration of 3 mM monobromobimane (200 μl final volume) for 10 min at 22 °C.

The derivatized samples were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatants were separated on a 4.6 × 150-cm C18 reverse-phase HPLC column (3 μM packing, SunFire) using an Agilent 1100 series HPLC system equipped with a multisignal fluorescence detector. The column was equilibrated with the following solution: 80% solvent A (897.5 ml of water, 100 ml of methanol, and 2.5 ml of acetic acid) and 20% solvent B (97.5 ml of water, 900 ml of methanol, and 2.5 ml of acetic acid). Samples (50 μl) were injected onto the column and resolved using the following gradient of solvent B: 20% from 0 to 5 min; 20–45% from 5 to 10 min; 45% isocratic from 10 to 15 min; 45–50% from 15 to 25 min; 50% isocratic from 25 to 28 min; 50–100% from 28 to 30 min; 100% isocratic from 30 to 39 min; 100–20% from 39 to 42 min; and 20% isocratic from 42 to 48 min. The flow rate was 0.75 min/ml. Fluorescence of the bimane adducts was detected using 340 nm excitation and 450 nm emission. The concentration of thiosulfate was determined using the peak areas and a standard curve.

**Stoichiometry of O2 consumption and sulfite formation**

The stoichiometry of coupling between the PDO and rhodanese-active sites was monitored using wild-type and C314S BpPRF as follows. The reaction mixture contained 1 mM GSSH, 75 mM enzyme, and 200 mM sodium phosphate buffer, pH 7.4 (1.6 ml final volume), in a Gilson-type chamber containing a Clark-type oxygen electrode. The reaction was initiated by addition of GSSH, and O2 consumption was recorded. After 5 min at 22 °C, an aliquot of the reaction mix (50 μl) was added to 60 mM monobromobimane (20 μl) to a final concentration of 17 mM. After incubation for 15 min at room temperature, the reaction was quenched with 100 μl of 0.2 M sodium citrate, pH 2.0. The derivatized samples were processed and separated by HPLC as described above to determine the concentrations of sulfite formed and thiosulfate consumed.

**Reaction stoichiometry under single and multiple turnover conditions**

The reaction stoichiometry of wild-type and C314S BpPRF under single-turnover conditions was monitored as described...
above with the following variation. The reaction mixture contained 250 μM thiosulfate, 250 μM GSH, and varying concentrations of enzyme (250, 125, 83, 62.5, 50, and 42 μM). The reaction was initiated by addition of GSH. After incubation for 30 min at 22 °C, an aliquot of the reaction mix (50 μl) was mixed with 20 μl of 60 mM monobromobimane and processed as described above. For analysis of the reaction stoichiometry under single-turnover conditions in the absence of O2, the enzymatic assay and derivatization reactions were performed in a Coy anaerobic chamber (atmosphere of 95:5 N2/H2).

**Protein crystallization**

The C-terminal truncated BpPRF (ΔCBpPRF) and C314S BpPRF were crystallized by sitting drop vapor diffusion method from a 0.75-μl/0.75-μl mixture of protein stock and well solution. ΔCBpPRF (5 mg/ml in 50 mM Tris, pH 8.0, 0.25 mM NaCl, 1% glycerol, 5 mM thiosulfate) crystallized in 3 days at 20 °C using a well solution of 0.2 mM zinc acetate, 0.1 mM MES, pH 6, 10% PEG-8000. A single rhombohedral rod-shaped crystal was harvested directly from the drop and flash-cooled in liquid nitrogen. C314S BpPRF (5 mg/ml in 50 mM Tris, pH 8.0, 0.25 mM NaCl, 1% glycerol) crystallized in 2 days using a well solution of 0.12 mM NaCl, 0.1 mM imidazole, pH 8.0, 25% PEG-8000. To obtain structural data for C314S BpPRF in complex with GSH, 5 mM GSH was added to the drop overnight. The hexagonal rod-shaped crystals were cryo-protected by passing harvested crystals through 2 μl of reservoir solution with an additional 15% glycerol followed by flash cooling in liquid nitrogen.

**Data collection and crystal structure determination**

Diffraction data were collected at the Advanced Photon Source (APS, Argonne National Laboratory) on the GM/CA beamline 23ID-D or 23ID-B (Table 1). For ΔCBpPRF, data were collected on a single crystal to 1.79 Å resolution. For the apo- and GSH-soaked C314S BpPRF crystals, data were collected to 2.65 and 2.35 Å, respectively. For the C314S BpPRF crystals, data quality was limited by high mosaicity along the c axis. All data were processed using XDS (46). The truncated ΔCBpPRF structure was solved by molecular replacement using BALBES (47) with the AaPRF (PDB code 3TP9), which shares 28% sequence identity with BpPRF, as a search model. Phenix AUTOBUILD was used to build ~70% of the model. To complete the model, consecutive rounds of model building and refinement were performed using Coot (48) and Phenix Refine (49). The structures were validated using MolProbity (50). Electron density is complete for all BpPRF residues except for eight residues in the linker region between the PDO and rhodanese domains and the N-terminal histidine tag. The C314S structure was solved via molecular replacement using Phaser within the Phenix software suite with the complete ΔCBpPRF model as a search model. Molecular replacement successfully placed three PDO domains and two rhodanese domains. The third rhodanese domain is poorly ordered and was built manually. Density is poor for this domain compared with the rest of the structure. The GSH-bound structure was solved via rigid body refinement in Phenix Refine. Electrostatic surface potentials were calculated using the APBS program in PyMOL (42, 51, 52). Figures were prepared using PyMOL.

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**Author contributions**—N. M. designed, performed, and analyzed the experiments and wrote the manuscript. N. M. and M. A. S. solved the crystal structures. M. A. S. and J. L. S. assisted with the crystallography experiments, data collection, and analysis. O. K. helped design and perform the O2 consumption experiments. R. B. helped conceive the experiments, analyzed the data, and co-wrote the manuscript. All authors approved the final version of the manuscript.

**References**

1. Beauchamp, R. O., Jr., Bus, J. S., Popp, J. A., Boreiko, C. J., and Andjelkovich, D. A. (1984) A critical review of the literature on hydrogen sulfide toxicity. Crit. Rev. Toxicol. 13, 25–97

2. Kimura, H. (2010) Hydrogen sulfide: from brain to gut. Antioxid. Redox Signal. 12, 1111–1123

3. Kabil, O., and Banerjee, R. (2010) The redox biochemistry of hydrogen sulfide. J. Biol. Chem. 285, 21903–21907

4. Kabil, O., Motl, N., and Banerjee, R. (2014) H2S and its role in redox signaling. Biochim. Biophys. Acta 1844, 1355–1366

5. Zhao, W., Zhang, J., Lu, Y., and Wang, R. (2001) The vasorelaxant effect of H2S as a novel endogenous gaseous KATP channel opener. EMBO J. 20, 6008–6016

6. Eldred, J. W., Calvert, J. W., Morrison, J., Doeller, J. E., Kraus, D. W., Tao, L., Jiao, X., Scala, R., Kiss, L., Szabo, C., Kimura, H., Chow, C. W., and Lefer, D. J. (2007) Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc. Natl. Acad. Sci. U.S.A. 104, 15560–15565

7. Wallace, J. L., Vong, L., McKnight, W., Dicay, M., and Martin, G. R. (2009) Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterology 137, 569–578

8. Gao, X. H., Krokowski, D., Guan, B. J., Bederman, I., Majumder, M., Parisien, M., Diatchenko, L., Kabil, O., Willard, B., Banerjee, R., Wang, B., Bebek, G., Evans, C. R., Fox, P. L., Gerson, S. L., et al. (2015) Quantitative H2S-mediated protein sulphydrylization reveals metabolic reprogramming during the integrated stress response. eLife 4, e10067

9. Vitvitsky, V., Kabil, O., and Banerjee, R. (2012) High turnover rates for hydrogen sulfite allow for rapid regulation of its tissue concentrations. Antioxid. Redox Signal. 17, 22–31

10. Banerjee, R. (2017) Catalytic promiscuity and heme-dependent redox regulation of H2S synthesis. Curr. Opin. Chem. Biol. 37, 115–121

11. Singh, S., and Banerjee, R. (2011) PLP-dependent H2S biogenesis. Biochim. Biophys. Acta 1814, 1518–1527

12. Hildebrandt, T. M., and Grieshaber, M. K. (2008) Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. FEBS J. 275, 3352–3361

13. Mishanina, T. V., Libiadi, M., and Banerjee, R. (2015) Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. Nat. Chem. Biol. 11, 457–464

14. Kabil, O., and Banerjee, R. (2012) Characterization of patient mutations in human persulfide dioxygenase (ETHE1) involved in H2S catabolism. J. Biol. Chem. 287, 44561–44567

15. Pettinati, I., Brem, J., McDonough, M. A., and Schofield, C. J. (2015) Crystal structure of human persulfide dioxygenase: structural basis of ethylmalonic encephalopathy. Hum. Mol. Genet. 24, 2458–2469

16. Tiranti, V., Briem, E., Lamantea, E., Miner, R., Papaleo, E., De Gioia, L., Forlani, F., Rinaldo, P., Dickson, P., Abu-Libdeh, B., Cindro-Hebler, L., Owaidha, M., Jack, R. M., Christensen, E., Burlina, A., and Zeviani, M. (2006) ETHE1 mutations are specific to ethylmalonic encephalopathy. J. Med. Genet. 43, 340–346

17. Tiranti, V., D’Adamo, P., Briem, E., Ferrari, G., Miner, R., Lamantea, E., Mandel, H., Balestri, P., Garcia-Silva, M. T., Vollmer, B., Rinaldo, P., Hahn, S. H., Leonard, J., Rahman, S., Dionisi-Vici, C., et al. (2004) Ethylmalonic encephalopathy is caused by mutations in ETHER1, a gene encoding a mitochondrial matrix protein. Am. J. Hum. Genet. 74, 239–252
Characterization of a PDO–rhodanese fusion protein

18. Libiadi, M., Yadav, P. K., Vitvitsky, V., Martinov, M., and Banerjee, R. (2014) Organization of the human mitochondrial H$_2$S oxidation pathway. J. Biol. Chem. 289, 30901–30910

19. Bordo, D., and Bork, P. (2002) The rhodanese/Cdc25 phosphatase superfAMILY. Sequence-structure-function relations. EMBO Rep. 3, 741–746

20. Libiadi, M., Srijaman, A., and Banerjee, R. (2015) Polymorphic variants of human rhodanese exhibit differences in thermal stability and sulfur transfer kinetics. J. Biol. Chem. 290, 23579–23588

21. Melideo, S. L., Jackson, M. R., and Jorns, M. S. (2014) Biosynthesis of a central intermediate in hydrogen sulfide metabolism by a novel human sulfurtransferase and its yeast ortholog. Biochemistry 53, 4739–4753

22. Spallarossa, A., Forlani, F., Carpen, A., Armirotti, A., Pagani, S., Bolognesi, M., and Bordo, D. (2004) The “rhodanese” fold and catalytic mechanism of 3-mercaptoppyruvate sulfurtransferases: crystal structure of SseA from Escherichia coli. J. Mol. Biol. 335, 583–593

23. Shen, J., Keithly, M. E., Armstrong, R. N., Higgins, K. A., Edmonds, K. A., Iwanicka-Nowicka, R., Zielak, A., Cook, A. M., Thomas, M. S., and Hryniewicz, M. M. (2007) Regulation of sulfur assimilation pathways in Escherichia coli and plant-beneficial bacteria with plant-beneficial properties. Microbiology 154, 3609–3623

24. Luebke, J. L., Shen, J., Bruce, K. E., Kehr-Fie, T. E., Peng, H., Skaar, E. P., and Giedroc, D. P. (2014) The CsoR-like sulfurtransferase repressor (CsrR) is a persulfide sensor in Staphylococcus aureus. Mol. Microbiol. 94, 1343–1360

25. Maddocks, S. E., and Oyston, P. C. (2008) Structure and function of the LysR-type transcriptional regulator (LTTM) family proteins. Microbiology 154, 3609–3623

26. Jarabak, R., and Westley, J. (1974) Human liver rhodanese. Nonlinear kinetic behavior in a double displacement mechanism. Biochemistry 13, 3233–3236

27. Motl, N., Yadav, P. K., and Banerjee, R. (2013) In Hydrogen Sulfide and Its Therapeutic Applications (Kimura, H., ed), pp. 1–36, Springer, New York

28. Czyzewski, B. K., and Wang, D. N. (2012) Identification and characterization of a bacterial hydrosulphide ion channel. Nature 483, 494–497

29. Sattler, S. A., Wang, X., Lewis, K. M., DeHan, P. J., Park, C. M., Yin, Y., Liu, H., Xian, M., Xun, L., and Kang, C. (2015) Characterizations of two bacterial persulfide dioxygenases of the metallo-$\beta$-lactamase superfamily. J. Biol. Chem. 290, 18194–18193

30. Shen, J., Peng, H., Zhang, Y., Trinidad, J. C., and Giedroc, D. P. (2016) Staphylococcus aureus srr encodes a type II sulfide-quinoine oxidoreductase and impacts reactive sulfur speciation in cells. Biochemistry 55, 6524–6534

31. Sessitsch, A., Coeny, T., Sturz, A. V., Vandamme, P., Barka, E. A., Salles, J. F., Van Elsas, J. D., Faure, D., Reiter, B., Glick, B. R., Wang-Pruski, G., and Nowak, J. (2005) Burkholderia phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties. Int. J. Syst. Evol. Microbiol. 55, 1187–1192

32. Fischer, D. S., and Price, D. C. (1964) A simple serum iron method using the new sensitive chromogen tripyridyl-triazine. Acta Crystallogr. D. Biol. Crystallogr. 18, 1137–1145

33. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. I., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D. Biol. Crystallogr. 66, 213–221

34. Chen, V. B., Arendall, W. B., 3rd, Headd, J. I., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D. Biol. Crystallogr. 66, 12–21

35. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl. Acad. Sci. U.S.A. 98, 10037–10041

36. Dolinski, T. J., Czodrowski, P., Li, H., Nielsen, J. E., Jensen, J. H., Klebe, G., and Baker, N. A. (2007) PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. Nucleic Acids Res. 35, W522–W525