Rhodaneses minimize the accumulation of cellular sulfane sulfur to avoid disulfide stress during sulfide oxidation in bacteria

Mingxue Ran, Qingbin Li, Yufeng Xin, Shaohua Ma, Rui Zhao, Min Wang, Luying Xun, Yongzhen Xia

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Heterotrophic bacteria and human mitochondria often use sulfide: quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO) to oxidize sulfide to sulfite and thiosulfate. Bioinformatic analysis showed that the genes encoding RHOD domains were widely presented in annotated sqr-pdo operons and grouped into three types: fused with an SQR domain, fused with a PDO domain, and dissociated proteins. Biochemical evidence suggests that RHODs facilitate the formation of thiosulfate and promote the reaction between inorganic polysulfide and glutathione to produce glutathione polysulfide. However, the physiological roles of RHODs during sulfide oxidation by SQR and PDO could only be tested in an RHOD-free host. To test this, 8 genes encoding RHOD domains in Escherichia coli MG1655 were deleted to produce E. coli RHOD-8K. The sqrCp and pdoCp genes from Cupriavidus pinatubonensis JMP134 were cloned into E. coli RHOD-8K. SQRcp contains a fused RHOD domain at the N-terminus. When the fused RHOD domain of SQRcp was inactivated, the cells oxidized sulfide into increased thiosulfate with the accumulation of cellular sulfane sulfur in comparison with cells containing the intact sqrCp and pdoCp. The complementation of dissociated DUF442 minimized the accumulation of cellular sulfane sulfur and reduced the production of thiosulfate. Further analysis showed that the fused DUF442 domain modulated the activity of SQRcp, and prevented it from directly passing the produced sulfane sulfur to GSH. Whereas, the dissociated DUF442 enhanced the PDOcp activity by several folds. Both DUF442 forms minimized the accumulation of cellular sulfane sulfur, which spontaneously reacted with GSH to produce GSSG, causing disulfide stress during sulfide oxidation. Thus, RHODs may play multiple roles during sulfide oxidation.

1. Introduction

H₂S is the most versatile molecule for early life on Earth [1]. Recently, its physiological and signaling roles in plants, animals, and microorganisms have attracted attention [2-5]. However, excess sulfide is toxic to cells [6]. It inhibits cellular respiration by poisoning cytochrome c oxidase or raises oxidative stress with reactive sulfur species [7,8]. We have recently reported that most heterotrophic bacteria are actively producing H₂S during growth, and many of them can oxidize self-produced H₂S by using sulfide: quinone oxidoreductases (SQR) [9] or flavocytochrome c-sulfide dehydrogenases (FCSDs) [10]. SQR, which is more active for sulfide oxidation than FCSDs, often forms an operon with persulfide dioxygenase (PDO) in bacterial phylum [9,11]. Sulfide oxidation by SQR and PDO has been reported in human mitochondria, Staphylococcus aureus, and Cupriavidus pinatubonensis JMP134 as a function of H₂S detoxification [12-16]. SQR oxidizes H₂S to sulfane sulfur (S²⁻). PDO oxidizes S²⁻ in the form of organic persulfide (e.g., GSSH, CoASSH, and BSSH) to sulfite. Sulfite reacts with S²⁻ to form thiosulfate either spontaneously or with the help of RHODs [11,15].

In bacteria, the sqr and pdo genes are often next to each other to form operons on the chromosome (9). Further, rhodanese (RHOD, EC 2.8.1.1) genes are often clustered with sqr and pdo genes with some being even fused with SQR or PDO [8,11,14]. RHODs are also ubiquitous in all three domains of life [17]. They exist as single-domain proteins, such as human TSTD1 [12] and E. coli GlpE [18], or as multiple domain proteins, such as bovine RHOD Rhobov [19] and Azotobacter vinelandii...
RHOD RhdA [20]. They are also present as fusions with other protein domains [21,22].

RHODs catalyze via a ping-pong mechanism, with an active site cysteine residue carrying the transferred sulfate as a covalent intermedium. The catalytic cysteine is the first residue of a loop of six amino acid residues that folds in a cradle-like structure [17,25]. The sulfane sulfur in thiosulfate, polysulfide [11], and GSSH [13] serves as the sulfur donor, and the sulfane acceptors are compounds that can receive sulfane sulfur, such as cyanide, GSH [13], sulfite [12], and thioderioxin [18,24]. Therefore, RHODs transfer sulfane sulfur among various acceptors, maintaining a sulfane sulfur pool in the cytoplasm ([25,26]). RHODs participate in selenium metabolism [27] and the syntheses of Fe–S cluster [23], thiamin [22], and molybdenum cofactor [28,29]. Although the recombinant dissociatively expressed domain (dDUF442) from S. aureus accelerates the reaction of GSH with inorganic sulfur, RHODs in bacteria do not demonstrate whether RHOD enhanced thiosulfate production in vivo [13]. An RHOD domain fused to the C-terminus of persulfide dioxygenase (CstB) from S. aureus promotes thiosulfate formation during sulfide oxidation [15]. Reversely, the RHOD domain of a PDO-RHOD fusion from Burkholderia phytofirmans transfers sulfane sulfur from thiosulfate to GSH, producing GSSH, which is oxidized by PDO to sulfite [34]. CstA is an RHOD-TuSA-TuSd fusion, in S. aureus, and the RHOD domain can transfer sulfane sulfur from thiosulfate to TuSA and TuSd Cys thiols, which may pass the sulfane sulfur to other potential receptors [14]. Most of these studies mainly focus on the activity of RHOD at a biochemical level.

We recently reported that a DUF442 domain fused at the N-terminus of SQR (dDUF442) from C. pinatubonensis JMP134 is also an RHOD domain [11]. Although the recombinant dissociatively expressed DUF442 domain (dDUF442) accelerates the reaction of GSH with inorganic polysulfide (H,Sn ≥ 2) to produce GSSH as well as the reaction of GSSH with sulfite to produce thiosulfate, the inactivation of dDUF442 in SQR did not demonstrate whether RHOD enhanced thiosulfate production by a recombinant Escherichia coli carrying SQR and PDO from C. pinatubonensis JMP134. Since E. coli strain K-12 displays robust RHOD activity and encodes 9 proteins with RHOD domain [35], the effect of DUF442 could be compensated by the host RHODs.

Here we found the RHOD domains were widely distributed in an sqr-pdo operon and could be categorized into three types: fused with an sqr domain, fused with a pdo domain, and dissociated proteins. To identify the functions of these RHOD domains, the sqr and pdo genes were cloned into an E. coli mutant without RHOD activities. The inactivation of the RHOD activity of DUF442 resulted in the accumulation of cellular sulfane sulfur and increased production of thiosulfate, and the changes were minimized by providing dissociated DUF442 (dDUF442). Both prevented sulfane sulfur accumulation but via different mechanisms: dDUF442 modulated the SQR activity, and dDUF442 stimulated the PDO activity. The accumulation of cellular sulfane sulfur causes disulfide stress. These results reveal an important function of RHODs in minimizing cellular sulfane sulfur accumulation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table S1. E. coli strains were cultured in lysogeny broth (LB) at 37 °C or 30 °C, as indicated. C. pinatubonensis JMP134 was cultured in LB medium at 30 °C. Kanamycin (50 µg/mL), spectinomycin (50 µg/mL) or ampicillin (100 µg/mL) were added when required.

2.2. General DNA manipulations

Plasmids used in this study are listed in Table S1. Synthetic primers
agarose (Qiagen, Shanghai, China), according to the supplier’s recommendation. The final buffer was exchanged to 50 mM Tris-HCl (pH = 7.4) buffer by using PD-10 desalting column (GE Healthcare), and then 50% glycerol was added to a final concentration of 10% before storing at −80 °C. The protein concentration was estimated by using the BCA method (Beyotime, China). The purified PDOCp was used to assay PDO activity.

2.6. RHOD activity assays

The RHOD activity was assayed according to a reported method [39]. Briefly, RHOD was added into 50 mM thiosulfate and 50 mM cyanide in 1 mL of 50 mM Tris buffer (pH 7.4) to initiate the reaction at 25 °C for 5 min. Then, 250 μL of 37% formaldehyde was added to stop the reaction, and then 100 μL of the ferric nitrate reagent was added for color formation. After 10 min, the mixture was centrifuged to remove the precipitate, and Fe(SCN)3 in the supernatant was determined at A600. For cell extracts assays, the resting cells disrupted in the Tris buffer at OD600 = 2 were used. The activity detection for purified dDUF442 or the membrane fraction with SQRcp, a defined amount of proteins as indicated in the text was added into the Tris buffer to start the reaction. The membrane fractions with SQRcp were used to represent dDUF442. One unit (U) of RHOD activity was defined to form 1 nmol SCN− per 1 min. The specific activity was expressed as U per mg of protein (nmol min−1 mg−1 of protein).

The GSSH: sulfite sulfurtransferase activity of DUF442 was measured for the production of thiosulfate, as reported [11]. Briefly, the reaction mixture contained 1 mM GSSH, 250 μM sulfite, and 50 μM DTPA in the Tris buffer. DTPA was added to chelate trace transition metals. dDUF442 was added at 0.05 mg per mL to initiate the reaction at 25 °C for 10 min. The membrane fractions containing SQRcp were also tested. The produced thiosulfate was derived with mBB (monobromobimane) and analyzed using HPLC. One unit (U) of RHOD activity was defined to form 1 nmol thiosulfate per 1 min.

2.7. SQR activity assay

The activity of SQRcp relies on the electron transport chain in cell membrane [40,41], and the prepared crude membrane fraction was sufficient to detect SQR activity [42]. In order to maintain the SQRcp activity as in native state, the membrane fractions of SQRcp or SQRcp-C94S proteins overexpression cells were extracted to characterize the enzyme activity in all the biochemical assay in vitro.

The membrane fractions or whole cells containing SQRcp and SQRcp-C94S were prepared as described above. Then, they were used to oxidize sulfide in 50 mM Tris-HCl (pH 7.4) with 50 μM DTPA. The cells were normally at OD600 = 2 and the membrane fractions were used as indicated in the text. One mM sulfide was added into the mixtures to initiate the reaction, and the remained sulfide at different time points was determined by a colorimetric method [43]. One unit (U) of SQR activity was defined to consume 1 nmol sulfide per 1 min.

2.8. PDO activity assay

PDO activity was analyzed by using two methods. The first method was carried out by detecting oxygen consumption, and the other method was by determining the production of sulfite as in our previous report [11,16]. GSSH was prepared by mixing equal volumes of 17 mM glutathione in 50 mM potassium phosphate buffer at pH = 7.4 with a saturated sulfur solution in acetone, containing about 17 mM dissolved elemental sulfur [44]. The reaction was carried out in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM GSSH, 0.1 mg/mL PDO was added to initiate the reaction. Oxygen consumption was directly monitored using an Orion RDO meter (Thermo Scientific, USA). The RDO meter was calibrated with air-saturated water according to the manufacturer’s instructions. One unit (U) of PDO activity was defined to consume 1 nmol oxygen per 1 min. The produced sulfite was derived with mBB (monobromobimane) and detected by HPLC according to a published method [11]. One unit (U) of PDO activity was also defined to generate 1 nmol sulfite per 1 min if indicated in the text.

2.9. Western blotting analysis of recombinant proteins

The western blotting procedure followed a reported method [45]. The proteins from the lysate were separated through a 12% SDS-PAGE gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Sangon, Shanghai, China) at 100 V and 4 °C for 60 min. The recombinant proteins with the His-tag were detected by using anti-His antibody conjugated with horseradish peroxidase (Sangon, Shanghai, China) and the high sensitive ECL luminescence reagent (Sangon, Shanghai, China); fluorescence intensity was detected and quantified by using a transilluminator (Fluor Chem Q; Protein Simple, San Jose, CA).

2.10. Sulfide spiking assay with whole cells and products analysis

The sulfide spiking assay was used to investigate the function of DUF442 inside cells during sulfide oxidation by SQR and PDO. It was done by using our previously established method [9]. Shortly, 15 mL whole cells were prepared and transferred to a 50-mL falcon tube. One mM sulfide was added to initiate the reaction. The mixture was incubated at 30 °C with gently shaking. The sulfide, cellular sulfane sulfur, sulfite, and thiosulfate were analyzed at various time intervals. The sulfide was determined by a colorimetric method [46], and cellular sulfane sulfur was analyzed by a previously used method [47]. Thiosulfate and sulfite were derived with mBB and detected by HPLC according to a reported method [9].

2.11. The determination of activity ratio of SQRcp to PDOcp in native C. pinatubonensis JMP134 or recombinant E. coli strains

The activity ratio is closely related with the enzyme expression levels of SQRcp and PDOcp inside cells. The use of cell lysates and crude membrane fractions would reflect the expression levels of both enzymes. Fresh colonies of C. pinatubonensis JMP134 or recombinant E. coli strains were picked and incubated in LB medium overnight at 30 °C. The cultures were transferred into fresh LB medium with 1:100 dilution and cultured to OD600 = 0.8 at 30 °C. The C. pinatubonensis cells were induced with 50 μM NaHS every 20 min for 5 times, and the E. coli cells were induced with 0.4 mM IPTG for 4–5 h. The cell lysates and membrane fractions were prepared by using procedures as described above. The membrane fractions was used to determine SQRcp activity as described above. After membrane fraction was removed, the residual cell lysate was used to determine PDOcp activity by monitoring oxygen consumption as reported above. The protein concentrations in cell lysate and membrane fractions were determined by using the BCA method (Beyotime, China), and they were used to calculate the specific activities of SQRcp to PDOcp.

2.12. The measurement of glutathione (GSH), glutathione disulfide (GSSG), glutathione polysulfide (GSnH, n ≥ 2)

The detection of cellular GSH and GSSG detection was adopted from a previous report [48]. Briefly, the cell pellet harvested from 1 mL of cell suspension at OD600 = 10 was resuspended and shocked for 2 min in 200 μL of the stock buffer (143 mM sodium phosphate, 6.3 mM EDTA, pH 7.4) and 100 μL of 10% 5-sulfosalicylic acid. Cell debris and precipitated proteins were removed by centrifuging at 12,000 g for 3 min. Then, 5,5′-dithiobis(2-nitrobenzoic) (DTNB)-GSSG reductase recycling method [49] was adopted to measure GSH and GSSG levels by using a commercial kit (Beyotime, China).

GSnH was treated with 50 mM HPE-IAM (β-(4-hydroxyphenyl)ethyl iodoacetamide) in 10 mM phosphate buffer (pH 7.4) at 37 °C for 1 h in
dark. The alkylated adducts were detected by using LC-ESI-MS/MS by the LCMS-8050 Nexera UHPLC system (Shimadzu) with a C18 column (VP-ODS, 150 × 4 mm, Shimadzu) with a reported elution procedure (11). The adducts of GSH (GS-adduct), GSH persulfide (GSS-adduct), GSH trisulfide (GSSS-adduct), GSH tetrasulfide (GSSSS-adduct), and pentasulfide (GSSSSS-adduct) were detected and compared by relative intensity.

2.13. Molecular docking of GS<sub>H</sub> into the catalytic pocket of PpPDO

Autodock Vina was used according to the instructions. Briefly, the structure of PDO<sub>Cp</sub> complexed with GSH (PDB ID = 4YSL) was used as a receptor after the ligand GSH was removed, and GS<sub>H</sub> (n = 2–5) was then used as the ligand. The PDBQT files of the receptor and ligand were generated by adding polar hydrogen and partial charges to them. The grid parameters were delineated (center<sub>x</sub> = -26.75 Å, center<sub>y</sub> = 26.66 Å, center<sub>z</sub> = -17.42 Å, size<sub>x</sub> = 24.5 Å size<sub>y</sub> = 24.5 Å, size<sub>z</sub> = 24.5 Å), H74, D78, H149, D170, F173, Y177, T149, R181, F184, H212, Y214, H236, R250, R253, L257, M259, V261, and L262 in the receptor were selected as flexible residues. The molecular docking was run with these parameters for several rounds by changing the random seeds until a reasonable docking position of GS<sub>H</sub> appeared.

2.14. Bioinformatics

The genes encoding the RHOD domains were searched in the sqr-pdo operons among the 441 strains in our previous report [9]. First, all the corresponding SQR and PDO sequences were collected. Then, proteins with an RHOD domain were identified by using the conserved domain function from NCBI website. 460 proteins had a potential RHOD domain. The RHOD domains that did not have the conserved cysteine at the active site or did not have complete sequences were excluded. 126 representative proteins included 37 PDO-RHOD fusion proteins, 50 SQR-RHOD fusion proteins, and 39 dissociated RHODs were collected by using CD-Hit of 90% similarity as the threshold. These annotated RHOD domains were aligned using ClustalW 2.1 with known RHOD domain sequences in GlpE, PspE, YgaP from *Saccharomyces cerevisiae* and TSTD1, TSTD2, TSTD3 from human were used as standards. A phylogenetic tree was constructed by a neighbor-joining method using MEGA 7.0, with a partial deletion, p-distance distribution, and bootstrap at 1000 repeats.

16 and 72 protein sequences of known PDO were collected from our previously published two papers with known outliers [9,11]. Five protein sequences with published structures were also collected and used for phylogeny analysis with similar parameters as that used for RHOD (vide supra). Representative PDO sequences from distant clades in the phylogenetic tree were collected and used for multiple sequence alignment by using Clustal Omega [50].

2.15. Statistical analysis

Statistical analysis was done using GraphPad Prism 9.0. Data in more than two groups were analyzed using independent one-way analysis of variance (ANOVA) to calculate the adjusted p values; the p-value < 0.033 indicated statistical significance. The significant difference between the two groups was analyzed using an independent student’s t-test; the p-value < 0.05 indicated statistical significance.

3. Results

3.1. Optimizing the expression ratio of SQR<sub>Cp</sub> and PDO<sub>Cp</sub> in RHOD-free *E. coli* to imitate their expression levels in *C. pinatubonensis* JMP134

Nine native genes coding for RHODs or proteins with RHOD domains were sequentially deleted in *E. coli* MG1655 (Fig. S1). The strain with all 9 genes deleted (9K) grew slowly, but the strain with 8 RHOD genes except thiF being deleted (8K) grew equally well as the wild type (Fig. S3A). ThiF is involved in the biosynthesis of both thiamin and 4-thiouridine that is present in tRNA [22]. The RHOD activity in cell extracts of *E. coli* 8K was significantly decreased in comparison with the wild type strain (Fig. S3B). When the nine genes were individually overexpressed in *E. coli* 8K, the cell extracts containing overexpressed GlpE, PspE, or YgaP showed increased RHOD activity (Fig. S3B), but the others did not (Data not shown). *E. coli* 8K was selected as the host to investigate the physiological function of the RHOD or RHOD domain during sulfide oxidation.

To investigate the function of the DUF442 domain (dUF442) in SQR<sub>Cp</sub>, the activity ratio of SQR and PDO in recombinant *E. coli* should be similar to that in *C. pinatubonensis* JMP134. In our first design, the pdo and sgr genes from *C. pinatubonensis* JMP134 were cloned into vector pBBR1MCS-2 as pBBR-nPDO<sub>Cp</sub>-nSQR<sub>Cp</sub> in which the two genes were under the control of lac promoter (Plac) and the native ribosome binding sites (RBSs) (Fig. S2). When tested, the ratio of the specific activities of SQR and PDO in cell extracts of *E. coli* 8K was 3.7 fold higher than that in its native strain *C. pinatubonensis* JMP134 (Table 1). Three artificial RBS sequences with defined strength were applied to reduce the expression level of SQR<sub>Cp</sub> (Fig. S2). When RBS a3 was used, the SQR<sub>Cp</sub> activity was decreased and the ratio of SQR and PDO activities in cell extracts was 1.0, similar to that in *C. pinatubonensis* JMP134 (Table 1). Hence, this new expression cassette Plac-nPDO<sub>Cp</sub>-a3SQR<sub>Cp</sub> in *E. coli* 8K (E. coli 8K-nPDO<sub>Cp</sub>-a3SQR<sub>Cp</sub>) was used to characterize the function of dUF442.

3.2. The DUF442 domain of SQR<sub>Cp</sub> minimised the accumulation of sulfane sulfur during sulfide oxidation by SQR<sub>Cp</sub> and PDO<sub>Cp</sub> in *E. coli*

The DUF442 domain of SQR<sub>Cp</sub> (dUF442) is a functional rhodanese when produced as a dissociated protein (dUF442) [11]. The rhodanese function of dUF442 in SQR<sub>Cp</sub> was inactivated by mutating Cys<sup>94</sup> to Ser (C94S). Sulfide oxidation by *E. coli* 8K-nPDO<sub>Cp</sub>-a3SQR<sub>Cp</sub> and *E. coli* 8K-nPDO<sub>Cp</sub>-a3SQR<sub>Cp</sub>-C94S cells was tested. E. coli 8K-nPDO<sub>Cp</sub>, p-a3SQR<sup>Cp</sup>-C94S cells had an increased rate of sulfide oxidation with increased levels of sulfane sulfur and thiosulfate but reduced sulfite production in comparison with *E. coli* 8K-nPDO<sub>Cp</sub>-a3SQR<sub>Cp</sub> (Fig. 1A–D). By contrast, the mutation (C94S) effect was not apparent when the wild-type *E. coli* MG1655 was used as the host (Fig. S4), suggesting RHOD activity in *E. coli* MG1655 compensates the DUF442 activity loss in SQR<sub>Cp</sub>-C94S (Fig. 1A&B). The dUF442 protein from another vector partially compensated the C94S mutation in *E. coli* 8K-nPDO<sub>Cp</sub>, p-a3SQR<sup>Cp</sup>-C94S/dUF442 during sulfide oxidation with reduced sulfane sulfur accumulation and decreased thiosulfate production as well as increased sulfite production (Fig. 1A–D). Further, *E. coli* rhodanese GlpE and YgaP on the second plasmid partially complemented the C94S mutation in *E. coli* 8K-nPDO<sub>Cp</sub>-a3SQR<sub>Cp</sub>-C94S, but PspE did not (Fig. S5). PspE is a periplasmic rhodanese [51], and its location is likely the cause for the lack of complementation, as the active site of SQR<sub>Cp</sub> is on the cytoplasmic side of the membrane [42]. dUF442 had strong RHOD activity and catalyzed the reaction between GSSH and sulfite to produce sulfane sulfur.

![Image](image.png)

### Table 1

|                | SQR<sub>Cp</sub> | PDO<sub>Cp</sub> | C. pinatubonensis JMP134 |
|----------------|-----------------|-----------------|--------------------------|
| nSQR<sub>Cp</sub> | 93.3 ± 2.4      | 43.3 ± 4.8      | 9.3 ± 1.2                |
| nPDO<sub>Cp</sub> | 44.4 ± 3.3      | 36.3 ± 3.6      | 15.0 ± 0.1               |
| SQR<sub>Cp</sub>/ PDO<sub>Cp</sub> | 2.2             | 1.0             | 0.6                      |

* E. coli strains were cultured in LB and induced by IPTG; *C. pinatubonensis* JMP134 was cultured in LB and induced by H2S. Cells were harvested and lysed. After ultracentrifugation, SQR was in the pellet with the membrane fraction, and PDO was in the supernatant. Their activities (μmol/min/mg of protein) were determined.
thiosulfate (Fig. S6), but the membrane fraction containing SQRCP had very low rhodanese activity and did not catalyze the reaction of GSSH with sulfite to produce thiosulfate, suggesting that dDUF442 and fDUF442 act differently during sulfide oxidation by SQRCP and PDOCP.

When cellular GSH and GSSG during sulfide oxidation were analyzed, GSH levels were gradually decreased in E. coli 8K-nPDOCP-a3SQRCp, but rapidly reduced in E. coli 8K-nPDOCP-a3SQRCp-C94S during the initial phase of sulfide oxidation (Fig. 2). When dDUF442 was supplied via another plasmid, the rapid oxidation of GSH was partially alleviated (Fig. 2). The rapid oxidation of GSH to GSSG causes disulfide stress to cells (S2).

3.3. fDUF442 modulated SQRCp activity to prevent cellular sulfane sulfur accumulation

E. coli 8K carrying pBBR1MCS-2 with pBpp-nsqRCP (E. coli 8K-nSQRCp) and E. coli 8K-nSQRCp-C94S were induced to express SQRCp and SQRCp-C94S, respectively. The expression levels of SQRCp and SQRCp-C94S in the cells were similar according to the western blotting analysis (Fig. S7). At the same cell density, E. coli 8K-nSQRCp-C94S had a higher sulfide oxidation activity than E. coli 8K-nSQRCp, and the complementation of dDUF442 via another plasmid did not slow down sulfide oxidation by E. coli 8K-nSQRCp-C94S/dDFU442 (Fig. 3A). The membrane fractions containing SQRCp-C94S also oxidized sulfide faster than with SQRCp, but the difference was small (Fig. 3B). The kinetic parameters of SQRCp and SQRCp-C94S were determined by using the corresponding membrane fractions (Table S3). GSH greatly increased sulfide oxidation by SQRCp-C94S, but not by SQRCp (Fig. 3B). Sulfite stimulated sulfide oxidation by the membrane fractions containing SQRCp and SQRCp-C94S, but the effect was smaller than GSH (Fig. S8A). dDUF442 did not affect sulfide oxidation by SQRCp-C94S with or without GSH or sulfite (Fig. S8B).

The structure of SQRCp was built via homologous modeling by using AlphaFold2 (Fig. 4), and the active site (Cys310 and Cys490) of the SQ domain and the active site Cys94 of the DUF442 domain were facing each other with a distance between the Cys94 thiol to the Cys310 and Cys490 thiols at 33.7 Å and 30.4 Å, respectively. The distance between the Cys310 and Cys490 thiols was 3.3 Å. The linker of the SQ domain and the active site Cys94 of the DUF442 domain were facing each other with a distance between the Cys94 thiol to the Cys310 and Cys490 thiols at 33.7 Å and 30.4 Å, respectively. The distance between the Cys310 and Cys490 thiols was 3.3 Å. The linker of the SQ and DUF442 domains predicted by AlphaFold2 had a low prediction score, suggesting a flexible region (Fig. 4A). dDUF442 could swing around the SQ domain and move into the above position of the active pocket. Hence, Cys94 might receive sulfane sulfur from the active site (Cys310 and Cys490) and then pass it to GSH. The rate of sulfur transfer was modulated by dDUF442. When Cys94 was mutated to Ser94, the mutated fDUF442 was no longer attracted by the active site (Cys310 and Cys490). A slight move of dDUF442 could create room for GSH to have...
difference between 8K-nPDO−a3SQR−C94S and 8K-nPDO−a3SQR−Cp, and character ‘b’ represents the difference between 8K-nPDO−a3SQR−C94S/dfUF442 and 8K-nPDO−a3SQR−Cp. For B), character ‘a’ was used to show the significant difference between SQR−C94S and SQR−Cp, and character ‘b’ represented the difference between SQR−C94S + GSH and SQR−Cp, and character ‘c’ represented the difference between SQR−Cp−GSH and SQR−Cp−C94S.

3.4. dfUF442 enhanced PDO−Cp activity to avoid sulfane sulfur accumulation

The effect of dfUF442 on PDO−Cp activity was tested. Total sulfite content (TSC), the sum of thiosulfite and sulfate, was used to calculate PDO activity because sulfite spontaneously reacts with sulfane sulfur to produce thiosulfate [11]. PDO−Cp and dfUF442 oxidized GSSH to TSC much more quickly than PDO−Cp did (Fig. 5A). With dfUF442 at M ratios (dfUF442: PDO−Cp) of 0.1:1 and 1:1, the specific PDO activity increased 1.7 and 4.5 folds. At a molar ratio of PDO−Cp and dfUF442 of 1:1, dfUF442 significantly increased the Vmax value of PDO−Cp and slightly increased its Km value for GSSH with overlapping error bars (Table 2). Sulfite was gradually accumulated to 40 μM during GSSH oxidation by PDO−Cp and PDO−Cp with dfUF442 (Fig. 5A). The effect of sulfite on PDO−Cp was further tested, and the addition of 1 and 10 mM sulfite did not affect PDO−Cp activity (Fig. 5B), suggesting that sulfite should not affect PDO−Cp activity with or without dfUF442. We then tested sulfide oxidation by SQRCp−C94S and PDO−Cp with or without dfUF442. As expected, sulfane sulfur was not accumulated and more TSC was produced when dfUF442 was present (Fig. 5B).

When the sulfane sulfur produced by SQRCp−C94S in the presence of GSH was tested, and longer chain GS3H, GS6S, and H2S were detected with dfUF442 than without dfUF442 (Fig. 6 & Table S4). Further, dfUF442 decreased GSSG production (Fig. 6A). Thus, we suspected that PDO might prefer to use long-chain GS3H to short-chain GS2H, as

Fig. 3. The effects of dfUF442 and dfUF442 on sulfide oxidation by SQRCp and SQRCp−C94S, respectively. The induced E. coli 8K-nSQRCp and E. coli 8K-nSQRCp−C94S cells were used to obtain resting cells and membrane fractions. A) Sulfide oxidation by whole cells at OD600 of 2. B) Sulfide oxidation by membrane fractions at 0.3 mg of protein per ml in Tris buffer with or without 1 mM GSH. Adding dfUF442 did not affect sulfide oxidation with or without GSH (Fig. S8). The Tris-HCl buffer was used as the blank. Data are averages and standard deviations of three parallel experiments. The one-way ANOVA method was used to calculate the p-value (*aa*/bb*/cc* = p < 0.002, *aaa*/bb*/ccc* < p < 0.01, and *aaa*/bns*/cns* = no significance) of two groups of data adjusted by the Dunnett method (A) and Sidak method (B) as statistical hypothesis testing. For A), character ‘a’ was used to show the significant

Table 2

| PDO−Cp                  | Vmax μM/ (min mg) | Km μM | Kcat | Kcat/Km mM−1 S−1 | P |
|-------------------------|------------------|-------|------|------------------|--|
| PDO−Cp                  | 67.6 ± 20.1       | 288.9 | 37.1 | 128.4            | NS |
| PDO−Cp + dfUF442        | 375.3 ± 103.6     | 84.9  | 205.7| 534.4            | ** |

The assays were done with changing GSSH concentrations in 50 mM Tris-HCl (pH = 7.4) buffer containing 50 μM DTPA. GSSH was freshly prepared under anoxic conditions. Data are averages of three parallel experiments with standard deviations. Unpaired Student’s t-tests were performed to determine the difference between the two samples (** = p < 0.05, *** = p < 0.01, and NS = no significance).
dDUF442 stimulated the activity of PDO and the formation of longer chain GS\textsubscript{n}H.

### 3.5. The pocket in PDO could accept long-chain GS\textsubscript{n}H as the substrate

We speculated that long-chain GS\textsubscript{n}H may be a better substrate for PDO\textsubscript{Cp}. Since the structure of PDO\textsubscript{Cp} had not been determined, we simulated the structure of PDO\textsubscript{Cp} from that of Pseudomonas putida PDO\textsubscript{Pp} \cite{53} by using the Swiss model. The sequence similarity between these two proteins is 81.9%. The structures of PDO\textsubscript{Cp} and PDO\textsubscript{Pp} were essentially the same (Fig. S10A). PDO\textsubscript{Pp} has a deep catalytic pocket (Fig. S10B), which may relieve the steric hindrance of binding long-chain GS\textsubscript{n}H. The structure of PDO\textsubscript{Pp} complexed with GSH was then used to dock GS\textsubscript{n}H (Fig. 7), and the spatial conformation of the GS\textsubscript{n}H carbon skeleton is quite similar to that of GSH (Fig. S7), and the affinity was shown in Table S5. This result showed that GS\textsubscript{n}H (n > 2) is also possible to enter this deep pocket to become a potential substrate of PDO\textsubscript{Pp}.

PDOs are divided into three types \cite{16}; both PDO\textsubscript{Cp} and PDO\textsubscript{Pp} belong to type II. The result of multiple sequence alignment showed that the amino acid residues composing the catalytic pocket of PDO\textsubscript{Pp} are relatively conserved in both Type I and Type II PDOs (Fig. S11), but not in type III PDOs that do not use GSSH as a substrate. Five protein structures of type I and type II PDOs are available, and they were diversely distributed in the PDO phylogenetic tree (Fig. S12). The type I PDO from human mitochondria (PDO\textsubscript{Hm}) and PDO\textsubscript{Pp} are distantly related with a sequence similarity lower than 40%. However, their tertiary structures were similar (Fig. S13A). The position of the Fe\textsuperscript{2+} atom in the pocket of PDO\textsubscript{Hm} is even deeper than that in PDO\textsubscript{Pp} (Figs. S13A and B). The conformation of the key residues composing the catalytic pockets and the vacuum electrostatics in the pockets are also similar (Figs. S13B–D). The results suggest that type I and II PDOs could use long-chain GS\textsubscript{n}H as the substrate.

### 3.6. The types and distribution of RHOD domains in the SQR-PDO pathway

Previously, we have reported 454 sqr-pdo operons in 441 genomes from 4929 sequenced bacterial genomes (9). Here, we further analyzed genes encoding RHOD domains associated with the operons. Of the 454 operons and linked genes within 3 loci, 385 gene clusters contained 458

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**Fig. 6.** dDUF442 promoted long-chain GS\textsubscript{n}H formation during sulfide oxidation by SQR\textsubscript{Cp}-C94S. The species of GS\textsubscript{n}H produced from 1 mM sulfide oxidated by SQR\textsubscript{Cp}-C94S at 0.15 mg of membrane protein per mL in the presence of 1 mM GSH with or without 0.05 mg per mL were alkylated by HPE-IAM and determined by LC-MS/MS. A) Sulfide; B) Sulfane sulfur; C–E) GS\textsubscript{n}H (n = 1–3); F) GSSG. Dates were detected at defined time intervals. Data in A) and B) are averages and standard deviations of three parallel experiments. Unpaired Student’s t-tests were performed to determine the difference between the two groups (NS = no significance).

**Fig. 7.** The docking of GS\textsubscript{n}H into the catalytic pocket of PDO\textsubscript{Pp}. The flexible docking of GS\textsubscript{2}H (A), GS\textsubscript{3}H (B), GS\textsubscript{4}H (C), GS\textsubscript{5}H (D) into the catalytic pocket of PDO\textsubscript{Pp}, aligned with the original pocket of PDO\textsubscript{Pp} complexed with GSH. The carbon skeleton of the reported GSH was colored in green, and those of GS\textsubscript{n}H (n ≤ 5) were colored in red. The flexible residues in PDO\textsubscript{Pp} were shown in a dash-lined cartoon mode. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
genes coding for 460 RHOD domains (Table S6). 204 SQRs and 166 PDOs were individually fused with one RHOD domain. The remaining 90 RHOD domains were encoded by 88 genes, as dissociated RHODs. (Table S7).

126 representative sequences of these RHOD domains were selected for phylogenetic tree analysis with known RHODs as references (Fig. S14). RHOD domains in SQRs were conserved in the same clade, and the associated SQRs were all distributed in Proteobacteria within five genera: Acinetobacter, Burkholderia, Gluconobacter, Pseudomonas, Cupriavidus (Table S7). Their active sites are highly similar (Fig. 8A); their associated gene clusters were also organized in the same way (Fig. S15). The Rhodanese domains that were either fused with PDO or as dissociated proteins did not form a clear clade (Fig. S14). The three E. coli rhodanases GlpE, YgaP, and PspE were distributed in the different clades (Fig. S14). The sequences of the active sites of the RHOD domains fused with PDO and dissociated RHODs were relatively conserved (Fig. 8B&C). The PDOs fused with RHOD all belong to type III PDO (Table S7) [9].

4. Discussion

With a rhodanese-free E. coli strain, our results show that rhodanese prevents the accumulation of cellular sulfane sulfur during sulfide oxidation by SQR and PDO, explaining why rhodanases or rhodanese domains are often associated with sqr and pdo genes. When a rhodanese domain is fused with SQR, such as SQR_{E. coli}, it modulates SQR activity to prevent rapid production of cellular sulfane sulfur (Figs. 1 and 9A). The dissociated rhodanases work differently by stimulating PDO activity to prevent the accumulation of cellular sulfane sulfur (Figs. 5 and 9B). The presence of rhodanese genes in sqr and pdo gene clusters is an indication of the necessity of keeping cellular sulfane sulfur low.

Our results indicate that RHOD is not necessary for thiosulfate production (Fig. 1). The results may seem to contradict previous reports. In vitro tests have shown that dDUF442 and the human mitochondrial rhodanase catalyze the reaction between sulfite and GSSH to produce thiosulfate [13,54], and the RHOD domain of CstB converts CoA-SSH and sulfite to thiosulfate [15]. We believe that RHOD may play several roles during sulfide oxidation by SQR and PDO. Without RHOD, sulfane sulfur, including GSnH and HSnH, is accumulated (Fig. 1). The produced sulfite spontaneously reacts with HSnH to produce thiosulfate, and this reaction is not catalyzed by RHOD (11). Further, sulfite may function as an acceptor to directly receive a sulfane sulfur from SQR (Fig. S8A) [41]. As a result, sulfite is not accumulated (Fig. 1C), and thiosulfate production is increased (Fig. 1B). In the presence of RHOD, it stabilizes long chain GSnH (Fig. 6C-F), converts HSnH to GSnH [11], and catalyzes the formation of thiosulfate between GSnH and sulfite [11, 13,15]. From a physiological perspective, the lack of RHOD activities leads to the accumulation of sulfane sulfur, which is detrimental to the cells (Fig. 9).

Excessive cellular sulfane sulfur is toxic, causing rapid GSH oxidation (Fig. 2). GSSH reacts with GSH to produce GSSG and H2S, and glutathione reductase reduces GSSG back to GSH at the expense of NADPH [55]. This GSH and glutathione reductase mediates sulfur reduction is proposed as a detoxification mechanism for fungi to resist elemental sulfur, a common fungicide. When glutathione reductase is inactivated in fungi, the mutants are more sensitive to elemental sulfur [55]. OxyR is known to respond to H2O2 stress [38], and exposure to polynsulfide also activates OxyR that upregulates the expression of genes coding for the removal of cellular sulfane sulfur in E. coli [56].

Thus, accumulating GSSG or other disulfides is harmful to microorganisms, and it causes sulfide stress [52]. Disulfide formation inside cells is often triggered by exposure to the synthetic diamide [diazenedicarboxylic acid bis(N, N-dimethylamide)] [57,58]. HOCI that is produced by neutrophils is another agent to induce disulfide formation [59,60]. H2O2 is also an inducer for disulfide formation, but the rate is slow [61]. Further, cystine uptake is likely a common situation that can quickly evoke disulfide stress [62]. Here, we found that the accumulation of cellular sulfane sulfur is new stress that triggers disulfide formation. The disulfide stress leads to the formation of proteins with disulfide bonds that may inactivate their functions [62]. OxyR senses increased levels of cellular sulfane sulfur and turns on the expression of genes encoding enzymes, such as thioredoxin, glutaredoxin, and catalase, for the removal of sulfane sulfur [56]. OxyR also senses disulfide formation induced by cystine uptake [62]. Thus, sulfane sulfur is an inducer of disulfide stress and bacteria like E. coli have defense mechanisms to cope with it.

The function of dDUF442 is to modulate SQR_{E. coli} activity (Fig. 3A). Our
mutational analysis showed that the inactivation of the active site of the dUF442 domain enhanced the SQR activity and allowed direct access of GSH to the active site of the SQR domain (Fig. 3B). The results suggest that dUF442 modulates the release of sulfane sulfur from the active site of the SQR domain to avoid the production of excessive GSSH that spontaneously reacts with GSH to generate H$_2$S and GSSG. Considering sulfane sulfur was mainly produced in cells, the cellular concentration of GSH would become much lower along with sulfide oxidation. Although the nonenzymatic reduction of cellular sulfane sulfur back to H$_2$S by GSH is fast, the cellular GSH content is quite limited in comparison with the concentrated sulfane sulfur in cells. Hence, without PDO, E. coli (SQR$_{Cp}$) oxidized H$_2$S finally to cellular sulfane sulfur, including polysulfide and GSSH (Fig. 6 & Table S4) [11]. In the presence of PDO, the regulated production of GSSH is consumed by PDO to avoid the accumulation of cellular sulfane sulfur (Fig. 1).

The dUF442 also prevents the accumulation of cellular sulfane sulfur via a different mechanism (Fig. 9B). It speeds up PDO$_{Cp}$ activity by several folds so that excessive cellular sulfane sulfur is not accumulated (Fig. 5 & Table 2). Given the binding pockets of type I and type II PDOs, including PDO$_{Cp}$ and PDO$_{Pp}$, are similar and can accommodate GSH as well as long-chain GS$_n$H (Fig. 7), we speculate these PDOs bind GS$_n$H and successively oxidize the terminal sulfur to sulfite until GSH is produced. Since the affinities of GSH and GS$_n$H to PDO$_{Cp}$ are similar (Table S5), the release of GSH may be a limiting step. When GSH is released, another GS$_n$H enters the active site of PDO$_{Cp}$ to start another round of oxidation. Thus, each cycle of substrate binding will lead to more sulfur oxidation for a long-chain GS$_n$H than GSSH. This hypothesis is consistent with our results that dUF442 mainly affects the $K_a$ of PDO$_{Cp}$, but not its $K_a$ for GS$_n$H (Table 2). Thus, type I and II PDOs may prefer to use long-chain GS$_n$H as the substrate.

The mechanism of SQR has been elucidated with the human mitochondrial SQR (SQR$_{mit}$) [63]. The active site of SQR$_{mit}$ consists of two cysteine residues that form a trisulfide linkage; the addition of H$_2$S produces 2 persulfides (2xCys-SSSH); one Cys-SSH reduces FAD, and the other passes the sulfane sulfur to a sulfane sulfur acceptor. Various compounds, such as sulfide, GSH, and sulfite, can receive the sulfane sulfur to produce HSSH, GSSH, and thiosulfate, respectively [13,41,54, 64]. Since GSH enhances SQR$_{mit}$ activity, GSH is likely the preferred sulfane sulfur receptor for the enzyme (13, 23). Since GSH does not speed up SQR$_{Cp}$ activity (Fig. 3B), GSH may not be a preferred sulfane sulfur acceptor. SQR$_{Cp}$ may pass the sulfane sulfur to H$_2$S or sulfite (Fig. 8). We speculate that dUF442 may prevent GSH from accessing the Cys-SSH at the active site of SQR$_{Cp}$ (Fig. 9), but it does not affect the access of sulfite (Fig. 8). When the active Cys residue of dUF442 is mutated to Ser, the mutant protein SQR$_{Cp}$-C94S favors GSH as the sulfane acceptor (Fig. 3B). Further, SQR$_{Cp}$-C94S has increased Vmax and decreased Km over SQR$_{Cp}$, which leads to a significantly increased catalytic efficiency for SQR$_{Cp}$-C94S (Table S3). The results suggest that dUF442 modulates the release of sulfane sulfur and slows down sulfide oxidation. Considering the sequences of the RHOD domains fused with SQRs are conserved (Fig. 8 & S14), they may also modulate the activity of the fused SQRs.

Genes coding for dissociated rhodaneses is quite common in the sqr and pdo gene clusters (Table S6). They are expected to play a role during sulfide oxidation by SQR and PDO, and the human mitochondrial rhodanese TSTD1 is speculated to link the activity of SQR and PDO [12]. Our results show that dUF442 and several E. coli rhodaneses enhance the production of sulfane sulfur (Fig. 5). This finding is similar to that of the RHOD domains fused with PDOs. Both the PDOs from Staphylococcus aureus and Burkholderia phytofirmans have a C-terminal RHOD domain, whose loss would severely weaken the PDO activities [15,34].

There are sqr-pdo gene clusters that do not contain RHOD. Our results indicate that E. coli MG1655 has several dissociated RHODs and their activity is sufficient to support sulfane oxidation by cloned SQR and PDO, preventing the accumulation of cellular sulfane sulfur (Fig. S4). Since rhodanese genes are widely present in bacterial genomes [17], the bacteria should possess other rhodaneses to work with SQR and PDO if a rhodanese gene is not next to sqr and pdo genes. Hence, the role of RHOD in keeping cellular redox homeostasis could be a prevalent mode during the sulfide oxidation process.

In summary, genes coding for fused RHOD domains or dissociated RHODs are widely present in bacterial sqr and pdo gene clusters (Table S6). The function of RHOD during sulfide oxidation by SQR and PDO is to prevent the accumulation of cellular sulfane sulfur (Fig. 9). The dUF442 domain of SQR$_{Cp}$ modulates the SQR activity to prevent the production of excessive sulfane sulfur (Fig. 9A), and dissociated RHODs enhance the PDO activity to expedite the consumption of sulfane sulfur (Fig. 9B). The accumulation of cellular sulfane sulfur causes GSH oxidation into GSSG, an indication of disulfide stress. Thus, RHODs and RHOD domains prevent the accumulation of cellular sulfane sulfur to prevent disulfide stress during sulfide oxidation.

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Data availability

All data are reported in the main text or Supplementary Data.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102345.

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