Rhodanese Functions as Sulfur Supplier for Key Enzymes in Sulfur Energy Metabolism

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Background: The function of SbdP, a cytoplasmic rhodanese from Aquifex aeolicus, is unknown.
Results: SbdP is involved in sulfur energy metabolism via its interaction with key redox enzymes.
Conclusion: SbdP supplies long sulfur chains to enzyme-active sites.
Significance: Rhodanases are part of the substrate traffic in sulfur energy metabolism.

How microorganisms obtain energy is a challenging topic, and there have been numerous studies on the mechanisms involved. Here, we focus on the energy substrate traffic in the hyperthermophilic bacterium Aquifex aeolicus. This bacterium can use insoluble sulfur as an energy substrate and has an intricate sulfur energy metabolism involving several sulfur-reducing and oxidizing supercomplexes and enzymes. We demonstrate that the cytoplasmic rhodanese SbdP participates in this sulfur energy metabolism. Rhodanases are a widespread family of proteins known to transfer sulfur atoms. We show that SbdP has also some unusual characteristics compared with other rhodanases; it can load a long sulfur chain, and it can interact with more than one partner. Its partners (sulfur reductase and sulfur oxygenase reductase) are key enzymes of the sulfur energy metabolism of Aquifex aeolicus and share the capacity to use long sulfur chains as substrate. We demonstrate a positive effect of SbdP, once loaded with sulfur chains, on sulfur reductase activity, most likely by optimizing substrate uptake. Taken together, these results lead us to propose a physiological role for SbdP as a carrier and sulfur chain donor to these key enzymes, therefore enabling channeling of sulfur substrate in the cell as well as greater efficiency of the sulfur energy metabolism of Aquifex aeolicus.

Sulfur is a ubiquitous element in the earth’s crust, well known as an essential component of life. It has come even closer to the center of attention in the light of current views of the origin of life (1, 2). In nature, elemental sulfur (S0, a cyclic chain of sulfur) and sulfur compounds are found in particularly high concentrations in extreme environments, usually volcanic or geothermically active (deep-sea vents and hot springs) (3). In these environments, the reduction and oxidation of these compounds are vital processes for many microorganisms that use sulfur as a basis for their energy metabolism (4, 5). Despite its central importance, however, the metabolism of sulfur is still not fully understood, probably because of the high reactivity of its compounds.

Aquifex aeolicus, isolated from a hydrothermal system at Vulcano in Italy, is one of the most hyperthermophilic bacteria known, with an optimum growth temperature of 85 °C (6, 7). It also represents the earliest branching order in the bacterial domain (8). A. aeolicus is able to grow chemolithoautotrophically and obtains energy for growth in the presence of H2, O2, and CO2, but it also requires the presence of a sulfur compound such as S0, sodium sulfide (Na2S), or sodium thiosulfate (Na2S2O3) (9). After several years of study of the enzymes involved in the respiratory pathways of A. aeolicus growing in presence of S0, we have a better overall picture of its complex energy metabolism. Under these growth conditions, we have demonstrated the existence of three different respiratory pathways as follows: two aerobic from H2 to O2 and from H2S to O2 and one anaerobic from H2 to S0. Two of them (H2/S0 and H2S/O2) have been shown to be organized into supramolecular structures (9–11) that contain enzymes known to be involved in sulfur energy metabolism as follows: a sulfur reductase (SR)4 in H2/S0 pathway and a sulfide quinone reductase (Sqr) in H2S/O2 pathway. A. aeolicus is the sole organism known to date that possesses simultaneously these two complementary energy pathways (both reduction and oxidation of sulfur compounds), and we have proposed an energy coupling between these two pathways (11). A cytoplasmic sulfur oxygenase reductase (SOR), which catalyzes, in the presence of oxygen, the simultaneous oxidation and reduction of elemental sulfur into oxygen sulfur species and H2S, is also present in A. aeolicus (12). In addition, the presence of cytoplasmic sulfur globules of as yet unknown function has been observed in whole cells of A. aeolicus grown on S0 (9). The simultaneous presence of all these sulfur enzymes, sulfur compounds and sulfur-containing struc-

4 The abbreviations used are: SR, sulfur reductase; Sqr, sulfide quinone reductase; SOR, sulfur oxygenase reductase; Psr, polysulfide reductase; SPR, surface plasmon resonance; RU, resonance unit; EDC, 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide.

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Analysis of the subcellular localization of Aq-477 as well as the characterization of its partners allow us to propose a potential role for this protein in the sulfur energy metabolism of *A. aeolicus*. A clear effect of Aq-477, which we rename SbdP (for sulfur-binding-donating protein), on the SR activity supports this model. SbdP might represent a prototype of rhodanese that uses its sulfurtransferase activity to transfer substrates to corresponding enzymes such as SR and SOR. A proposed mechanism for SbdP as a sulfur shuttle in the sulfur energy pathway is further discussed.

EXPERIMENTAL PROCEDURES

Organisms and Growth Conditions

*Agrobacterium* *aeolicus* VF5 was grown at 85 °C in 2-liter bottles containing 400 ml of modified SME medium (29) at pH 6.8 in the presence of flowers of sulfur (7.5 g-liter⁻¹) (which will be referred to as “S²⁻” in this paper) and under 1.4 bar of H₂/CO₂ (80:20) as described previously (9). Cells were harvested in the late exponential growth phase and stored at −80 °C.

*Escherichia coli* BL21-CodonPlus (DE3)-RIPL, transformed with the pET22a*q*477 or pET22SOR plasmids, was grown in LB medium at 37 °C supplemented with 0.5 mM ampicillin (12, 26).

Cell Fractionation

Periplasmic fraction was obtained as described previously (30). After periplasmic extraction, cell material (spheroplasts) was broken. Debris and unbroken cells were removed by centrifugation (10,000 × g, 15 min). Membrane and soluble proteins were separated by ultracentrifugation at 170,000 × g. The membrane fraction was then washed with 50 mM Tris–HCl, pH 7.6, 5% (v/v) glycerol, 0.5% (w/v) aminocaproic acid (buffer A) to avoid cytoplasmic contamination. To dissociate poorly membrane-associated proteins from anchored membrane proteins, the resulting membrane pellet was washed with the same buffer plus 1.5 M NaBr for 30 min at room temperature under gentle shaking and ultracentrifuged as above. The supernatant was kept to identify poorly associated membrane proteins and is named “NaBr supernatant” in this study. NaBr-washed membranes (resulting pellet) were resuspended in buffer A to give a protein concentration of 10 mg/ml and solubilized with 2% deoxycholic acid sodium salt (w/v) for a final concentration (37 °C for 1 h under gentle shaking). After solubilization of the membrane proteins, the supernatant called “solubilized NaBr-washed membranes” was recovered after ultracentrifugation. The fraction called “solubilized membranes” was obtained by the same procedure, with the exception of NaBr washing.

Protein Purification

Hydrogenase-Sulfur Reductase Complex Enrichment (“SR Complex”)—Solubilized membranes from 50 g of cells were obtained as described above but with the detergent *n*-dodecyl-β-D-maltoside (DDM) at 1% (w/v) used instead of sodium deoxycholate and without the NaBr washing step. Membranes were then applied on a DEAE-52 column (Whatman) equilibrated with buffer A with 0.05% DDM (buffer B). Proteins were eluted with a 50 mM step gradient of 0–500 mM NaCl in buffer B. The 100–200 mM NaCl fractions that contained sulfur
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reductase activity were dialyzed and loaded onto a hydroxyapatite column equilibrated with buffer B. Sulfur reductase activity was eluted with 500 mM potassium phosphate in the same buffer. After removal of phosphate and concentration, the SR complex was frozen with liquid nitrogen and stored at −80 °C. We obtained an enriched fraction containing SR clearly identified by mass spectrometry that we will call the SR complex for reasons of clarity.

Sqr Purification—Free Sqr was also purified from solubilized membranes of A. aeolicus as described previously (11).

SbdP Purification—Recombinant SbdP was obtained from E. coli as described previously (26). Previous studies showed that recombinant SbdP behaves similarly to SbdP from A. aeolicus (26).

SOR Purification—Recombinant SOR was obtained from E. coli as described previously (12).

Activity Measurements

Rhodanese (Thiosulfate Cyanide Sulfurtransferase) Activity—Rhodanese activity consists of the transfer of a persulfide group from thiosulfate to cyanide, leading to the formation of sulfite and thiocyanate (see Reactions 1). In vitro, this activity is followed by quantifying the product of the reaction, the thiocyanate (SCN), as described previously (26), in the absence of reducing agent.

\[ \text{S}_2\text{O}_3^{2-} + \text{Rhod} \rightarrow \text{SO}_3^{2-} + \text{Rhod-S} \]

\[ \text{Rhod-S} + \text{CN}^- \rightarrow \text{Rhod} + \text{SCN}^- \]

REACTION 1

Sulfur Reductase Activity—Sulfur reductase activity was determined by quantifying the H₂S formed by the reduction of S⁰ with H₂, as described previously (9), but performed in 50 mM Hepes, pH 7.6. To study the influence of SbdP on SR activity, SbdP (1, 5, 10, 15, and 20 mM, final concentrations) was added to the assay before the incubation at 85 °C. Measurements were made at different reaction times, with constant amounts of SR complex or solubilized NaBr-washed membranes (50 μg/ml each, final concentration). Controls were done without SbdP, with SbdP alone and no SR, or with SbdP inactivated by cysteine alkylation treatment (0.3 mg/ml). SbdP was incubated with 0.5 mM iodoacetic acid overnight in 50 mM Tris-HCl, pH 7.6, 100 mM NaCl).

Analytical Procedures

Native Gel Electrophoresis—Proteins were loaded on 3–8% polyacrylamide gels (Mini-Protein II; Bio-Rad). After migration, proteins were either stained with PageBlue (Fermentas) or detected with specific antibodies, or the gels were used to determine hydorgenase or rhodanese activity.

Denaturing Gel Electrophoresis—Proteins were incubated at 95 °C for 5 min in the denaturing Laemmli buffer, and loaded on a 4% stacking, 5–20% running polyacrylamide gel (Mini-Protein II; Bio-Rad). After migration, gels were stained with PageBlue or used for Western blotting.

Western Blotting—After protein migration in gels, Western blot was performed using standard procedures (11). Anti-SbdP antibodies, anti-Sqr antibodies, and anti-SOR antibodies were used.

In Gel Enzymatic Activities—Hydrogenase activity on native gel was detected as described previously (31). Rhodanese activity was revealed at 85 °C by nitro blue tetrazolium chloride reduction by sulfite (a by-product of rhodanese, see Reaction 1) with phenazine ethosulfate as intermediate electron carrier. The reduction of nitro blue tetrazolium chloride allows the formation of a purple formazan precipitate visible on gel (32). The assay mixture contained 100 mM Tris-HCl, pH 9.0, 2 mM nitro blue tetrazolium chloride, 0.5 mM phenazine ethosulfate, 10 mM Na₂S₂O₃, and 10 mM KCN.

Gel Shift Assay—SR complex (0.5 mg/ml) or Sqr (0.25 mg/ml) was incubated with SbdP (2.5 or 50 μg/ml, respectively) in 50 mM Tris-HCl, pH 7.6, for 30 min at 85 °C and loaded on a native gel.

Protein Concentrations—Protein Concentrations were determined with the modified Lowry method (33) and are expressed in milligrams/ml for most samples. Molar concentrations cannot be expressed because stoichiometry of the active proteins/supercomplexes is yet unknown.

Cross-linking

In Vitro Cross-linking—After dialysis in 40 mM sodium phosphate, pH 7.4, to avoid cross-reaction with Tris, SbdP (2.5 μg/ml) and SQR (0.25 mg/ml) were incubated in 20 mM phosphate buffer, pH 5.7, in the presence of 15 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) at 37 °C for 1 h under gentle shaking. The reaction was stopped by addition of Tris-HCl, pH 6.8, at a final concentration of 50 mM and then loaded on a native gel.

In Vivo Cross-linking—A. aeolicus cells grown on S⁰ were resuspended in 50 mM Hepes, pH 7.0, and were incubated with 1 mM dithiobis(succinimidyl propionate) for 30 min at 37 °C. The reaction was stopped with 100 mM Tris-HCl, pH 7.6. Cells were broken, and membranes were solubilized as described above. Membranes were loaded onto a 20–40% sucrose density gradient in 50 mM Tris-HCl, pH 7.6, and ultracentrifuged overnight at 185,000 × g (50.2 Ti rotor, Beckman-Coulter). The gradient was separated into 11 fractions. The fraction containing most of the rhodanese activity was dialyzed and loaded onto native gel. The band corresponding to SbdP was sliced and analyzed by ion trap mass spectrometry.

Surface Plasmon Resonance (SPR) Binding Experiments

A BIACore T100 apparatus was used to investigate interaction between SbdP and SR complex, Sqr or SOR. SbdP and SR complexes were immobilized on a CM5 sensor chip (BIACore) in 10 mM sodium acetate, pH 5.0, by amine coupling using a kit supplied by BIACore. Running buffer, used when interactions were tested, contained 50 mM Hepes, pH 7.6, 50 mM NaCl, and 0.05% Tween 20 with a flow rate of 50 μl/min. Samples were diluted in the same buffer with 100 mM NaCl. Control experiments were done on sensor chips in the absence of immobilized proteins. Because of the difference between the index of refraction of the running buffer and the sample buffer, a burst phase could be observed at the beginning and the end of sample injection.
Mass Spectrometry (MS)

**Protein Identification**—Tryptic digestion of excised gel plugs, ion trap, and MALDI-TOF MS protein identification were done as described previously (10).

**MS Analysis of Nondigested Proteins**—To determine the binding of sulfur atoms on SbdP, the protein (2 mg·ml⁻¹) was incubated in 100 mM Tris-HCl, pH 9.0, with thiosulfate (10 mM) or with flowers of sulfur (5% w/v) or without any sulfur source for 20 min at 85 °C and then subjected to mass analysis of nondigested proteins. MALDI-TOF analyses were performed on a Microflex II mass spectrometer (Bruker, Germany). A saturated solution of sinapinic acid in 50:50 acetonitrile/water, 0.1% trifluoroacetic acid was used as matrix. A 0.7 mg·ml⁻¹ protein solution was used for the deposit according to the thin layer method; mixtures were dried at room temperature. Data were acquired in a positive linear mode, range was set from 2 to 20 kDa, and pulsed ion extraction was fixed to 200 ns. External mass calibration was done just before the acquisition of the sample using protein calibration standard I (Bruker Daltonics). Mass spectra were treated in Flex Analysis software by a Gaussian smoothing (width = 2 cycles = 2).

Microscopy

**Spheroplasts Immunolabeling**—For this experiment, spheroplast formation was optimized as described; fresh cells grown on S⁰ were resuspended in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.75 M sucrose, 1.5 mM EDTA. The cell suspension was frozen and thawed and centrifuged at 10,000 g for 20 min to weaken the cells. The pellet was resuspended in buffer C supplemented with lysozyme from chicken egg (0.4 mg·ml⁻¹) and then incubated at 37 °C for 24 h. The efficiency of cell wall degradation was monitored by microscopy. The spheroplast suspension was centrifuged at 1,500 g for 20 min at 4 °C, and the pellet containing the spheroplasts was washed in buffer C. To be able to visualize the proteins of interest directly on the spheroplast preparation, purified immunoglobulins raised against Sqr, hydrogenase, or SbdP were labeled using the Alexa Fluor 488 protein labeling kit (Invitrogen). To determine the degree of labeling, the ratio of absorbance of Alexa Fluor 488 at 494 nm was compared with absorbance at 280 nm for proteins. To determine the degree of labeling, the ratio of absorbance of Alexa Fluor 488 at 494 nm was compared with absorbance at 280 nm for proteins. We obtained a ratio between 5 and 7 mol of Alexa Fluor 488 dye per mol of antibody. The spheroplasts were then adsorbed on a 1% uranyl acetate. Several washes were performed for 4 h until the temperature reached −40 °C. Then the samples were embedded in Lowicryl HM20. Polymerization was done during 48 h at −40 °C using UV light. Thin sections of 80 nm were cut with an ultramicrotome RMC MTX and collected onto nickel grids. They were incubated into 0.5 m ammonium chloride in TBS for 5 min. After washing in TBS, sections were incubated into TBS-saturating solutions containing 0.5% BSA for 10 min followed by a wash containing 0.1% BSA. Sections were then incubated for 3 h using primary antibody solution (anti-SbdP antibodies) diluted 1:100 in TBS containing 0.1% BSA. After washing in the same buffer, samples were labeled with 15 nm colloidal gold anti-rabbit IgG diluted 1:30 in the same buffer for 1 h as done previously. After several washes, fixation was performed with 2% glutaraldehyde for 5 min and washed in distilled water several times. Grids were examined using an electron microscope Zeiss EM 912, and digital images were acquired with CCD camera Gatan Bioscan model 792.

RESULTS

**SbdP, a Soluble Rhodanese That Interacts with Membrane**—SbdP has been previously characterized as a rhodanese protein with sulfurtransferase activity in vitro (26). It has been purified from the cytoplasmic compartment of *A. aeolicus* spheroplasts, but its exact subcellular localization had never been investigated. For this purpose and as a first approach, cells grown in the presence of S⁰ have been analyzed by immunogold labeling using specific SbdP antibodies in thin sections of intact cells (Fig. 1A). We confirmed a cytoplasmic localization of SbdP, and its presence near the membrane was less expected. However, it has been reported that a purely cytoplasmic protein is likely to be partially stained in a manner indistinguishable from a membrane-associated protein (34). To further confirm a potential localization of SbdP at the membrane, periplasmic extraction has been performed, and the resulting cells have been fractionated into membrane and cytoplasmic fractions. Western blot analysis with SbdP-specific antibodies was performed on these fractions after migration on denaturing gel. SbdP is mainly found in the cytoplasmic fraction with a main band corresponding to the monomeric form (Fig. 1B, 4th lane). Even in denaturing and reducing conditions, some tetrameric forms can be observed due to the high stability of the oligomeric state that we have previously shown to be thermostable (26). Once again, a non-negligible amount of SbdP is found in the solubilized membrane fraction (Fig. 1B, 1st lane) even though no evidence from the primary sequence of SbdP could explain this result. The absence of the tetrameric form in the membrane could either have a physiological signification, yet unknown, or could be due to experimental procedures (use of different buffers and SbdP concentrations). To better understand the potential interaction of SbdP with the membrane, the samples were treated with NaBr, a chaotropic reactant, that allowed the strength of the protein/membrane association to be estimated. Proteins interacting partially with the membrane are then usually separated from membrane-anchored proteins. Under these conditions, the majority of SbdP is released from the membrane (Fig. 1B, 2nd lane) and is found in the soluble washed fraction called the NaBr supernatant (Fig. 1B, 3rd lane). This result was confirmed by a quantitative measurement of the rhodanese.
activity determined in vitro in each fraction (Fig. 1C). This correlates perfectly with SbdP activity because no other active cytoplasmic rhodanese is present in this organism under these growth conditions (26).

Overall, these results highlight the existence of a fraction of SbdP in contact with the membrane, which, to our knowledge, is the first experimental characterization of a membrane localization for a soluble rhodanese. This raises the question of the role of such interaction and the identity of the potential membrane protein partners of SbdP.

Identification of Potential Membrane Protein Partners of SbdP—To determine the physiological membrane protein partners interacting with SbdP, we incubated cells with a cross-linking agent (dithiobis(succinimidyl propionate)) that has been shown to diffuse across the membrane. After treatment of the cells with dithiobis(succinimidyl propionate), the membrane fraction was isolated, solubilized, and separated on a sucrose gradient. Most of the membrane-bound SbdP proteins were found in the heavy fractions (high percentage of sucrose that contains heavy proteins, data not shown). To identify SbdP partners, the fraction containing SbdP activity was loaded on a native gel to allow in gel enzymatic activity detection. Hydrogenase activity could then be observed in the same band that was revealed by specific SbdP antibodies (Fig. 2A). To precisely define the composition of this potential complex, the protein band was cut out, digested with trypsin, and analyzed by ion trap MS. In this band, the presence of SbdP was confirmed, and several membrane proteins, either cross-linked to SbdP or just co-migrating with SbdP due to their intrinsic properties, were clearly identified (supplemental Fig. S1). By using this approach, we noticed that several identified proteins belong to a similar pathway as follows: SR (with its two subunits SreA and SreC), hydrogenase (which belongs to the SR supercomplex), and Sqr. These proteins are involved in sulfur energy metabolism (Fig. 2B) that makes them likely candidates as rhodanese partners. This first screen using intact cells for doing the cross-linking and trying to snapshot potential transient complexes allowed us to direct our research toward membrane proteins involved in sulfur energy metabolism as potential partners for the rhodanese SbdP.

SbdP and Sulfur Reductase, Two Proteins Involved in the Same Pathway?—Its membrane localization and the identification of the SR in the in vivo cross-linked complex led us to test in vitro whether SbdP interacts with partially purified SR complex. Both proteins were preincubated at 85 °C for 30 min and subsequently loaded on a native gel (gel shift assay). The migration pattern of SbdP was then visualized with specific antibodies (Fig. 3A). Strikingly, a completely different migration pattern of SbdP was observed when SbdP was incubated with the SR complex. A gel shift of SbdP due to interaction with a protein present in the enriched fraction of SR was observed. The corresponding band had been cut out from the native gel, and MS allowed the identification of SbdP as well as of the subunit SreA of SR. As these two proteins co-migrate in native conditions, a direct interaction between the rhodanese SbdP and the sulfur reductase SR was strongly supported. To confirm this result, we decided to detect SbdP by following rhodanese activity on gel, a method
previously described for other rhodaneses that we have adapted to our system (32). The presence of SbdP in the additional band is consistent with the previous result obtained by Western blot and MS (Fig. 3B). This also indicates that SbdP retains rhodanese activity when joined to SR.

A complementary approach, using SPR, was carried out to observe potential binding between these two partners in real time. We covalently immobilized SbdP on a chip, and we sequentially injected SR complex at different concentrations, in a continuous flow that passed over the chip and monitored binding events. The data show an unambiguous interaction between the two partners (Fig. 3C). Nevertheless, because of the presence of an equilibrium between multimeric forms of SbdP and the heterogeneity of SR samples, the sensorgrams obtained could not be fitted with the simplest 1–1 reaction model, preventing us from estimating kinetic constants.

The physical interaction between the SbdP and SR complex explains the finding of part of SbdP at the membrane. It also raises the question of the role of such an interaction.

SbdP, a Rhodanese Able to Bind Long Sulfur Chain—Sulfur reductase is known to reduce sulfur chains (such as S0 or poly-sulfide) to H2S. We hypothesized that soluble SbdP might bring the sulfur substrate to membrane SR. To fulfill such a function, SbdP should be able to load sulfur chains. In previous studies, SbdP has been shown to transfer sulfur atoms in vitro from S2O32−/H2O2 to the sulfur acceptor KCN. To better understand which SbdP form is involved in the presence of S2O32−, the molecular masses of both SbdP and SbdP incubated with this substrate were determined by MALDI-TOF MS. Additional sulfur atoms bound to SbdP catalytic cysteine was expected, as observed for other rhodaneses (35). As shown in Fig. 4, purified SbdP without any treatment (“as prepared”) already shows three distinct masses in approximately equivalent amounts. (i) An ion had been measured at m/z 12805.64 and could be assigned to unmodified SbdP (theoretical average m/z 12805). (ii) A population with an m/z of 12837.98 could correspond to one sulfur (mass of a sulfur atom, 32 Da) bound to SbdP (called SbdP-SH). (iii) The third population appeared at m/z 12867.77 and matched SbdP binding two sulfur atoms (SbdP-S-SH). After S2O32− incubation, all three initial species (unmodified SbdP-SH, SbdP-S-SH, and SbdP-S-S-SH) might react similarly with S2O32− because no drastic change in the proportions of these initial species could be observed. Additional masses were detected (Fig. 4A), which probably corre-
spond to addition of oxygen and sulfur atoms (from the $S_2O_3^{2-}/H_2O_2$) to the various initial SbdP species (Fig. 4B). As we were able to show that in vitro this rhodanese could catalyze the transfer of sulfur atom to KCN with $S_0$ as sulfur donor (data not shown), an identical approach was performed using $S_0$ as sulfur donor for SbdP. The MS results also show that this rhodanese can bind up to five sulfur atoms ($m/z$ 12963.06) under our experimental conditions (Fig. 4). Additional peaks (with higher $m/z$) are clearly visible on the mass spectrum; however, it is difficult to know exactly which forms of SbdP they match.

In summary, this experiment shows that SbdP can covalently bind several sulfur atoms on its unique catalytic cysteine. We confirmed it by using an alkylating agent that reacts specifically with cysteine residues. No sulfur fixation on SbdP could then be observed after $S_0$ addition (supplemental Fig. S2). These overall data support our hypothesis of a potential role as sulfur donor to enzyme using long sulfur chains as substrate.

**Positive Effect of SbdP to Key Enzyme of Sulfur Energy Metabolism**—SPR was previously used to visualize the interaction in real time between the SbdP and SR complex (Fig. 3C). We postulate that if SbdP plays a critical role in transferring sulfur to SR, the presence of sulfur bound to SbdP may modify the kinetics or the level of interaction between these two proteins. To test it, we carried out an experiment that consisted of immobilizing the SR complex on the chip and injecting SbdP in the presence or not of a sulfur compound. As we could not use insoluble $S_0$ with the BiACore apparatus, $S_2O_3^{2-}/H_2O_2$ was chosen for the experiment. As observed in Fig. 5A, the interaction between SR complex and SbdP “loaded” with sulfur is drastically modified, with a faster complex formation and higher stability of the resulting complex. As SR cannot catalyze the reduction of $S_2O_3^{2-}/H_2O_2$ to $H_2S$ (9), we assume that the effect is mainly due to SbdP loaded with sulfur atoms that might stabilize the interaction of SbdP to SR.

On the basis of this experiment, we proposed that SbdP could indeed act as a sulfur donor for SR once it is loaded with sulfur atoms. To test this potential functional role of SbdP on SR, we measured sulfur reductase activity in the presence of its usual substrate (S0) as well as in the presence of different amounts of SbdP. A difference observed in $H_2S$ production of SR due to SbdP loaded with sulfur atoms that might stabilize the interaction of SbdP to SR.

![FIGURE 4. Loading of SbdP with sulfur atoms. A, analysis of covalent modification of SbdP by MS: SbdP as prepared (top) or incubated with either $S_2O_3^{2-}$ (middle) or $S_0$ (bottom)].

**TABLE 1. Potential modifications of SbdP to some peaks observed on mass spectra.**

| Name          | $m/z$   | Potential modifications                      |
|---------------|---------|----------------------------------------------|
| As prep       | 12805.64| SbdP-SH                                      |
|               | 12837.98| SbdP-S-SH                                    |
|               | 12867.77| SbdP-S-S-S-SH                                |
| $+S_2O_3^{2-}$|         |                                              |
|               | 12803.56| SbdP-SH                                      |
|               | 12835.46| SbdP-S-SH                                    |
|               | 12867.40| SbdP-S-S-SH                                  |
|               | 12915.80| SbdP-S-S-S-O$_3$                             |
|               | 12948.67| SbdP-S-S-S-S-O$_3$                           |
|               | 12983.63| SbdP-S-S-S-S-S-O$_3$                         |
| $+S_0$        | 12802.14| SbdP-SH                                      |
|               | 12834.08| SbdP-S-SH                                    |
|               | 12866.68| SbdP-S-S-SH                                  |
|               | 12899.39| SbdP-S-S-S-SH                                |
|               | 12932.64| SbdP-S-S-S-S-SH                              |
|               | 12963.06| SbdP-S-S-S-S-S-SH                            |

![As prepared](attachment:attachment.png)
likely not in an excess of one over the other. Results presented correspond to yet unknown. A clear concentration dependence illustrates that we are most translated because the oligomerization state of the active form of both proteins is incubation. Ratio between the different proteins, however, cannot be calculated because the oligomerization state of the active form of both proteins is dilution at 4710 RU on a CM5 sensor chip, and SbdP (0.6 μg/ml) was injected. Thiosulfate alone was also injected as a control, without modification of the signal. B and C, sulfur reductase activity measurements of SR complex (50 μg/ml, final concentration) B or solubilized NaBr-washed membranes (50 μg/ml, final concentration) C performed with the insoluble Su in presence (white circles) or absence (black circles) of exogenous SbdP (5 μg/ml, final concentration) as well as in presence of increasing concentrations of SbdP (1, 5, 10, 15, and 20 μg/ml, final concentration) (inset, B and C) of incubation. Ratio between the different proteins, however, cannot be calculated because the oligomerization state of the active form of both proteins is diluted. A control experiment was made using the alkylated form of SbdP that is inactive as a rhodanese in vitro and is not able to increase SR activity (supplemental Fig. S3), and this further demonstrates a role of SbdP in substrate presentation and is not a consequence of SbdP interaction to SR.

These in vitro experiments support the hypothesis of a role of SbdP as a sulfur donor to SR. In vivo, SbdP might play a role of a cytoplasmic shuttle that would not only convert the sulfur compound into a more “usable” substrate but also bring it to its membrane partner.

Link between SbdP and Another Membrane Protein Involved in Sulfur Metabolism?—Another protein, Sqr, involved in sulfur energy metabolism, has been identified in the in vivo cross-linking experiment (Fig. 2). It catalyzes the oxidation of H₂S to S⁰ (11), and we hypothesized that SbdP could take care of the product of the reaction and per se would allow sulfur recycling. Interaction between Sqr and SbdP was therefore investigated using gel shift assay, as described previously. There was no significant difference in the migration pattern of SbdP or of Sqr when they were incubated alone or together (Fig. 6, A and B). The absence of interaction between the two proteins was further confirmed with SPR experiments (supplemental Fig. S4). In addition, A. aeolicus Sqr has been classified as an integral monotopic membrane protein, meaning that this protein is only exposed to one side of the membrane, for which the periplasmic orientation has been suggested (36). In parallel to our in vitro interaction studies, we investigated the orientation of Sqr; we consequently designed an optimized method adapted for this extremophile organism to create spheroplasts. As observed in Fig. 6, C, E, and G, the cells show a characteristic round shape after treatment due to the depletion of their external membrane and of their periplasmic compartment. We then incubated these cells with fluorescent antibodies directed against Sqr (Fig. 6D), hydrogenase (Fig. 6F), or SbdP (Fig. 6H). A fluorescent staining using confocal microscopy should be visualized only if the protein of interest is oriented toward the periplasm. Hydrogenase, known to be oriented toward the periplasm in A. aeolicus, was used as a positive control. In con-
contrast to SbdP, fluorescence could be clearly detected, revealing the periplasmic orientation of Sqr (Fig. 6D). This experiment confirms that SbdP cannot bind Sqr in vivo and is consistent with the absence of interaction observed in vitro. This indicates that Sqr functions independently of SbdP.

**SOR, Another Partner of SbdP**—We propose that SbdP plays a role of a shuttle transferring long sulfur chains to enzymes that require this substrate for fulfilling their enzymatic reactions. Another enzyme (SOR) uses $S^0$ as substrate and has been proposed to be involved in the energy pathway in various Acidithiobacillus species (13, 14, 37). As SQR is present and active in *A. aeolicus* cytoplasm under our growth conditions, we first decided to test if SQR from *A. aeolicus* was also able to interact partially with the membrane to determine whether it could be part of the sulfur energy metabolism. As shown in Fig. 7A, a portion of SQR can be found in the membrane fraction and is released after treatment with a chaotropic agent.

As SR and SQR use the same substrate ($S^0$), we considered that SbdP might play a central role in sulfur energy metabolism...
and could supply sulfur substrate to the SOR as well. Unfortunately, gel shift assay does not give clear results (supplemental Fig. S5A). The formation of a very large complex (SOR being at least a 16-mer and SbdP oligomerizing), might prevent the incorporation into the gel under the conditions tested. We thus modified the physicochemical conditions (pH and temperature), and we used a cross-linking agent (EDC) to lock this complex and stabilize the interaction. As observed in Fig. 7B (5th lane), a band corresponding to the SbdP-SOR complex is clearly detected. The presence of SbdP and SOR in the cross-linked complex was not only observed using antibodies (supplemental Fig. S5, B and C) but also when rhodanese activity assay was performed in gel and showed a similar pattern (supplemental Fig. S5D). This result also shows that in these conditions, even in the presence of a cross-linking agent, SbdP in the complex is still active as a rhodanese. We decided to confirm the interaction between both purified proteins by SPR. Despite the fact that they present different oligomerization states making it difficult to obtain kinetic constants, the result obtained supports the conclusion of an interaction observed for the first time between SOR and a rhodanese (Fig. 7C). This interaction suggested a potential role of this rhodanese, as for SR, to bring SOR substrates and optimize their presentation to the active site of SOR.

**DISCUSSION**

In this study, we have described the use of in vitro approaches to determine the function of SbdP, a cytoplasmic rhodanese present in *A. aeolicus*. This enzyme has unique features as it can load, on its catalytic cysteine, long chains of sulfur atoms after incubation with S0. Another unusual feature among rhodaneses is that SbdP has two different partners, SR and SOR. Both enzymes use long sulfur chains as substrates, and both are key players in sulfur energy metabolism. The presence of SbdP would optimize substrate presentation to its partner enzymes. On the basis of these data, we propose that SbdP might play a central role in sulfur energy pathways as a shuttle in the transfer of sulfur substrate to catalytically active enzymes (SR or SOR). We present alternative approaches to describe rhodanese function. Indeed, characterizing rhodanese physiological function in an organism is a challenge due to the abundance of potential rhodanese/rhodanese-like proteins within the same genome. As an example, *E. coli* and *Pseudomonas aeruginosa* have, respectively, 9 and 10 annotated rhodaneses or rhodanese-like proteins in their genome (18, 35). This redundancy supports the notion that rhodaneses are involved in distinct cellular processes. However, this makes it difficult to attribute a defined in vitro function just by analyzing the phenotype of the corresponding mutant. Indeed, a different rhodanese can compensate for the absence of an other one (22, 35).

**SbdP, a Rhodanese That Binds Long Sulfur Chains**—Our attempt to determine the number of sulfurs bound to SbdP shows that, after incubation of SbdP in the presence of S0 and S2O52−, several species of the protein can be identified by MS. Some of them bind as many as five sulfur atoms. To our knowledge, this result has never been observed by MS for other rhodaneses, although binding of sulfur compounds has already been studied (35, 38). In most cases the presence of only one or two sulfur atoms bound to the catalytic cysteine has been observed. It is however noticeable that the rhodanese-like protein Sud from *W. succinogenes* has been suggested to be able to bind up to 10 sulfur atoms per monomer, based on biochemical studies (17). However, only two sulfur atoms bound to Sud were detected by MS (39) and five were observed after determination of the NMR structure (40). The authors explained this difference by suggesting that some sulfur atoms bind more tightly due to their protection by the active site, whereas a long chain of sulfur exposed might easily dissociate upon MS procedures (39). This explanation may also apply to SbdP, for which we did not see a homogeneous population binding five sulfur atoms. The docking of a pentasulfide-sulfur molecule on the modeled structure of SbdP (supplemental Fig. S6) shows that the sulfur chain is exposed, meaning that it could be used as a substrate for SR and SOR enzymes.

**SbdP, a Shuttle for Carrying Reactive Sulfur Compounds to Enzymes of Sulfur Metabolism**—The fact that SbdP has the potentiality to interact with at least two partners (SR and SOR) is another unusual feature for rhodaneses. The complex SR-SbdP is reminiscent of the Psr-Sud system of *W. succinogenes*, whereas the complex formed between SbdP and SOR has not yet been observed. SOR and SR have some common characteristics. One of them is the ability to use a long chain of sulfur atoms as substrates. In *A. aeolicus*, both enzymes are present simultaneously in the cell and have a similar location; SR is a three-subunit membrane enzyme oriented toward the cytoplasm, and SOR is a soluble cytoplasmic enzyme that we show to be partially located at the membrane. Moreover, both enzymes possess buried active sites. As an example, the structure of Psr, an SR homologous protein from *Thermus thermophilus*, has been resolved (41), and it has been shown that its active site is located in the subunit A buried at the end of a narrow funnel, as for other molybdoenzymes of this family. How the substrate can get access to this active site is still unclear (42). Likewise, the structure of *A. ambivalens* SOR shows that the SOR active site is also not very accessible and a chimney-like protrusion has been suggested to be the likely entry routes for a linear sulfur chain (43). We proposed that SbdP might act as a sulfur supplier by providing long sulfur chains to these buried active site enzymes. Once loaded with sulfur chain, SbdP could bind the catalytic subunits of its partners and the long chain could then be optimally presented to the cavity leading to the active site. The stabilization of the SbdP-SR complex observed in presence of the substrate supports this hypothesis (Fig. 5A). Overall, we proposed that SbdP might increase the efficiency of sulfur energy metabolism in this bacterium by optimizing the presentation of the substrate to its partner enzymes. We were able to strengthen this theory by demonstrating that SbdP presence accelerates H2S production of its partner enzyme SR (Fig. 5, B and C). This impressive effect due to an active role of SbdP might be explained by the following facts: (i) SbdP binds a chain of sulfur and by an appropriate interaction with SR, the substrate is correctly and efficiently presented to the active site of the SR; (ii) SbdP increases both the local concentration of substrate and the time of substrate presentation to the active site of the enzyme by forming a
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After growth with $S^0$ as sulfur source, membrane-bound hydrogenase/SR (Ref. 9) and Sqr/bc$_1$ complex/cytochrome c oxidase supercomplexes (11) are present and active, as well as cytoplasmic and membrane-associated SOR enzyme (Ref. 12 and this paper). $H_2S$ gas, produced by SR and SOR, might be used by Sqr as a substrate and converted into polysulfide or elemental sulfur that has been proposed to be incorporated into cytoplasmic sulfur globules (36). These sulfur globules (9), which probably serve as sulfur storage for the bacteria, might be in turn used by SbdP as an alternative sulfur source. Finally, sulfite and thiosulfate produced by SOR might be used by still unknown systems participating in sulfur energy metabolism as proposed previously (13, 14). By interacting with both SOR and SR, the rhodanese SbdP is at a key position in this scheme, delivering substrate as a long chain of sulfur atoms, a very suitable form of substrate for these enzymes and, finally, might increase the efficiency of the sulfur energy metabolism of A. aeolicus. Continuous lines represent the pathways demonstrated in this paper; dotted lines represent proposed sulfur traffic. Black points represent quinone molecules; $S_8$ is for sulfur (the physiological substrate and the number of sulfur atoms in the molecule in vivo are not known); cyt c oxidase is for cytochrome c oxidase, and c is for monoheme cytochrome c$_{555}$.

FIGURE 8. Central role of SbdP in the model of energy sulfur recycling in A. aeolicus. After growth with $S^0$ as sulfur source, membrane-bound hydrogenase/SR (Ref. 9) and Sqr/bc$_1$ complex/cytochrome c oxidase supercomplexes (11) are present and active, as well as cytoplasmic and membrane-associated SOR enzyme (Ref. 12 and this paper). $H_2S$ gas, produced by SR and SOR, might be used by Sqr as a substrate and converted into polysulfide or elemental sulfur that has been proposed to be incorporated into cytoplasmic sulfur globules (36). These sulfur globules (9), which probably serve as sulfur storage for the bacteria, might be in turn used by SbdP as an alternative sulfur source. Finally, sulfite and thiosulfate produced by SOR might be used by still unknown systems participating in sulfur energy metabolism as proposed previously (13, 14). By interacting with both SOR and SR, the rhodanese SbdP is at a key position in this scheme, delivering substrate as a long chain of sulfur atoms, a very suitable form of substrate for these enzymes and, finally, might increase the efficiency of the sulfur energy metabolism of A. aeolicus. Continuous lines represent the pathways demonstrated in this paper; dotted lines represent proposed sulfur traffic. Black points represent quinone molecules; $S_8$ is for sulfur (the physiological substrate and the number of sulfur atoms in the molecule in vivo are not known); cyt c oxidase is for cytochrome c oxidase, and c is for monoheme cytochrome c$_{555}$.

Stable complex; and (iii) the substrate (sulfur chain) supplied by SbdP is more efficiently used by the enzyme than the initial cyclic sulfur $S_8$ substrate, for example.

We propose that SbdP plays a similar function of substrate-donating protein for the SOR enzyme. However the impact of SbdP on the kinetics of SOR product formation is difficult to detect on account of the simultaneous reduction and oxidation of $S^0$ to $SO_2^-$, $S_2O_3^{2-}$, and $H_2S$. Moreover, some products of the reaction ($SO_2^-$ and $H_2S$) are unstable, and others ($S_2O_3^{2-}$) can be used by SbdP as a substrate.

Sulfur globules have been observed in A. aeolicus cytoplasm (9). These kind of sulfur structures are usually present either in the periplasmic compartment or in the extracellular environment (44). In any case, the chemical nature of sulfur in the globules is still a debated question, and it seems that three different forms could be found, depending on the approach used: cyclo-octasulfur ($S_8$), polyanthionates ([(S(SO$_3$)$_2$)$_2$]$_2^{-}$), and sulfur chains (45). These three forms can all be used as substrates by SbdP. In A. aeolicus, a role as sulfur storage has been suggested based on previous studies showing that in the absence of exogenous $S^0$, these inclusions disappeared over time. The fact that A. aeolicus possesses two enzymes that use long sulfur chains in the cytoplasm allows us to speculate that sulfur globules might be one of their sources of substrate, with SbdP acting as a shuttle.

Sulfur Energy Metabolism of A. aeolicus, an Overview—As illustrated in Fig. 8, a broad picture of A. aeolicus sulfur energy metabolism is becoming visible. Pieces of this intricate puzzle of sulfur metabolism are now better defined by the association of complementary approaches (proteomics and enzymatic studies) with the main players, SR, Sqr, SOR, well characterized (as described in Fig. 8 legend) (9, 11, 12). A role of SOR in the sulfur energy pathway is strongly supported by its membrane location also observed in Acidithiobacillus tengchongensis (37), which would allow transfer of its products to membrane energy enzymes (13, 14). Here, we define the function of an additional protein involved in this complex sulfur energy metabolism of A. aeolicus. We propose a model in which SbdP would be at the central position of this sulfur energy network, as represented in Fig. 8, playing the role of a shuttle to carry a sulfur chain from different sources (such as the sulfur globules) to the cytoplasmic or membrane-located enzymes and allowing a better availability of the substrate to optimize the sulfur traffic. This substrate channeling toward its targets would also allow us to prevent any interference with other pathways, a reasonable possibility on account of the reactivity of sulfur compounds, in a similar way as metallochaperones for highly reactive metals (46). SbdP may represent the prototype of a new subfamily of rhodanese involved in sulfur traffic in cells. The periplasmic rhodanese-like protein Sud from W. succinogenes, which presents 32% of identity with SbdP, might as well belong to this family especially because biochemical data and metabolic context suggest that it might supply sulfur substrate to the Psr enzyme (17). In other organisms such as Acidithiobacillus ferrooxidans (47), A. ambivalens and some green sulfur bacteria (48), with similar sulfur metabolism, rhodanese or rhodanese-like proteins, might play an identical function.

Finally, our findings not only allow a better understanding of the functional role of the rhodanese enzymes but also open the way to a deeper investigation of the sulfur compounds traffic and recycling between the actors of an intricate sulfur energy metabolism network.
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