The ability of *Thiobacillus ferrooxidans* to oxidize not only ferrous ion but also reduced sulfur compounds confers on this bacterium an important position among the bacteria used for leaching of sulfide ores. To breed a more valuable strain for bacterial leaching, it is important to study the mechanism of sulfur oxidation by *T. ferrooxidans*. The mechanism of sulfur oxidation by *T. ferrooxidans* has been considered to be similar to that of other thiobacilli.1~4) We purified a sulfur : ferric ion oxidoreductase (SFORase) to an electrophoretically homogeneous state from iron-grown *T. ferrooxidans* AP19-3.5) In the presence of glutathione (GSH) the enzyme catalyzes the oxidation of hydrogen sulfide, FeS, and elemental sulfur with Fe$^{3+}$ as an electron acceptor to give Fe$^{2+}$ and sulfite ion. An alternative sulfur oxidation route with both SFORase and iron-oxidase was proposed for this strain.5~10) The problem of how elemental sulfur or other reduced sulfur compounds insoluble in water are oxidized by industrially important bacteria of the genus *Thiobacillus* has attracted many investigators from both scientific and practical points of view. However, there have been few reports about the mechanism of the initial attack of this bacterium on insoluble sulfur particles.11~13) We have found that the actual substrate for SFORase in the oxidation of elemental sulfur was not solid elemental sulfur, but hydrogen sulfide, which was produced chemically by incubating elemental sulfur with GSH. Thus, we gave the enzyme an alternative name, hydrogen sulfide : ferric ion oxidoreductase (SFORase).14) From the observation that SFORase was completely inhibited by hydrogen sulfide at 0.5mM, and that the inhibition was specifically restored by Fe$^{2+}$...
added to the reaction mixture, we proposed the possibility that FeS was formed in the periplasmic space as an intermediate during the sulfur oxidation by this strain.\(^{14}\) FeS produced in the periplasmic space of this bacterium could continuously supply a harmless level of hydrogen sulfide to SFORase.

Here, we show that (i) a heat stable, reduced sulfur compound, which supplies hydrogen sulfide to a purified SFORase, is formed in the plasma membrane of \textit{T. ferrooxidans} API9-3 when treated with hydrogen sulfide solution; (ii) a novel hydrogen sulfide-binding protein (SBP), which can bind hydrogen sulfide reversibly, was purified from the plasma membrane of this strain to an electrophoretically homogeneous state; and (iii) hydrogen sulfide is stabilized in the presence of SBP, and the hydrogen sulfide-bound SBP supplies hydrogen sulfide to SFORase to give sulfite ion. These results strongly suggest that the hydrogen sulfide-binding protein (SBP) purified in this work is involved in the oxidation of elemental sulfur by \textit{T. ferrooxidans} API9-3.

**Materials and Methods**

\textbf{Microorganism, medium, and conditions of cultivation.} \textit{T. ferrooxidans} API9-3 was used throughout this study.\(^{15}\) The composition of iron-salts medium used for the large-scale production of cells and the method for cultivation were described previously.\(^{7}\)

\textbf{Activity of hydrogen sulfide:ferric ion oxidoreductase.} The activity of hydrogen sulfide:ferric ion oxidoreductase (SFORase)\(^{5,14,16,17}\) was measured under aerobic conditions by measuring sulfite ion by the method described previously. The method for purification of SFORase from iron-grown \textit{T. ferrooxidans} API9-3 was described previously\(^{5}\) and an enzyme solution at the stage of the Mono Q column chromatography\(^{5}\) was used throughout this study. The reaction mixture contained 4 ml of 0.1 M sodium phosphate buffer (pH 6.5), enzyme, 0.2 mg of bovine serum albumin, 100 mg of elemental sulfur, and 20 \(\mu\)mol of GSH (adjusted to pH 6.5 with dilute NaOH). The total volume was 5.0 ml.

The activity of hydrogen sulfide:ferric ion oxidoreductase was also measured with intact cells by measuring the concentration of Fe\(^{2+}\) in the reaction mixture.\(^{6}\) The presence of ferrous and sulfite ions was measured colorimetrically by a modification of the o-phenanthroline method\(^{18}\) and the pararosaniline method,\(^{19}\) respectively.

\textbf{Purification of hydrogen sulfide-binding protein.} A plasma membrane fraction (360 mg of protein) of \textit{T. ferrooxidans} API9-3 was solubilized with 0.1 M sodium phosphate buffer (pH 7.5) supplemented with 1% sodium dodecyl sulfate (SDS) for 1 hr at 25°C and centrifuged at 105,000 \(\times\) g for 30 min. A brown supernatant solution thus obtained was put on a DEAE-cellulose column (1 by 15 cm) equilibrated with 0.02 M sodium phosphate buffer (pH 7.5) supplemented with 1% SDS. A red brown fraction (10 ml), which did not absorb to DEAE-cellulose, was collected. After hydrogen sulfide solution was added to 1 mM and the mixture heated for 5 min in a boiling water bath, the fraction was put on a column (3 by 60 cm) of Sephadex G-100 equilibrated with 0.02 M sodium phosphate buffer (pH 7.5) supplemented with 1% SDS. A deep green fraction from the column of Sephadex G-100 (8.1 mg of protein) showed a single band on SDS-PAGE and was reserved as a purified sulfide-binding protein (SBP).

\textbf{Analysis.} Hydrogen sulfide was measured by the methylene blue method.\(^{20}\) \(N,N\)-dimethyl-p-phenylenediamine sulfate (400 mg) and ferric chloride (600 mg) were dissolved separately in 100 ml of 6 M HCl. Diamine-ferric chloride solution was prepared by mixing equal volumes of the two above-described solutions just before analysis. A sample of an enzyme reaction mixture was withdrawn and centrifuged at 12,000 \(\times\) g for 2 min to discard solid elemental sulfur. Diamine-ferric chloride solution (0.2 ml) was added to the supernatant solution (0.5 ml). Total volume was adjusted with H\(_2\)O to 2.5 ml. The resulting blue color due to the production of methylene blue was measured by a Shimadzu UV-140 spectrophotometer at 670 nm. A good linearity was obtained between hydrogen sulfide concentration (0~ approx. 0.01 \(\mu\)mol hydrogen sulfide per ml) and the absorbance at 670 nm.

The iron in the purified hydrogen sulfide-binding protein was measured by atomic absorption spectroscopy with a Shimadzu AA-625-01 spectrophotometer using an air-acetylene flame. The spectral line chosen was at 2482 Å. Purified SBP (0.86 mg of protein) was ashed in a crucible at about 450°C for 10 min. The ash was dissolved in 2 N HCl and the iron concentration of this solution was measured. The standard solution of iron was prepared by diluting 1000 \(\mu\)g/ml standard iron solution for atomic absorption spectroscopy (Ishizu Pharmaceutical Co., Ltd.) with 0.1 N HCl.

The molecular weight of hydrogen sulfide-binding protein was measured by sodium dodecyl sulfate-PAGE by the method of Weber and Osborn.\(^{21}\) Protein was measured by the method of Lowry et al.\(^{22}\) with crystalline bovine serum albumin as a reference protein.
Results and Discussion

Formation of a heat stable, reduced sulfur compound after the treatment of intact cells with hydrogen sulfide

Purified hydrogen sulfide : ferric ion oxidoreductase (SFORase) absolutely required glutathione (GSH) for its activity. When it oxidizes elemental sulfur, the actual substrate of this enzyme was not solid elemental sulfur but hydrogen sulfide, which is easily produced chemically by incubating solid elemental sulfur with GSH at pH 6.5, the pH optimum of this enzyme. However, since a high concentration of hydrogen sulfide rather strongly inhibited the enzyme activity and only ferrous iron (Fe$^{2+}$) specifically protected SFORase from this inhibition, it was supposed that something like FeS is transiently formed in the periplasmic space of *T. ferrooxidans* AP19-3 during the sulfur oxidation of this strain.

To establish more clearly whether FeS or any other form of sulfur than FeS is formed in the cells in the presence of high concentrations of hydrogen sulfide, we artificially treated the cells with externally added hydrogen sulfide and then analyzed the substance formed after this treatment. When sodium sulfide is dissolved in the buffer solution at pH 6.5, almost all the sulfide ion are present as hydrogen sulfide (H$_2$S) at this pH, but not as sulfide ions. So, washed intact cells (5 mg of protein) were incubated with 100mM of hydrogen sulfide solution in 0.1 M sodium phosphate buffer (pH 6.5) (the pH of the sodium sulfide solution added to the cell suspension had been adjusted to 6.5 with sulfuric acid) for 1 hr at 30°C. Hydrogen sulfide-treated cells were then washed three times with 0.1 M sodium phosphate buffer (pH 6.5) to discard hydrogen sulfide which was not fixed to the cells. To identify any reduced sulfur compound in hydrogen sulfide-treated cells, SFORase activity of hydrogen sulfide-treated cells were measured with the reaction mixture with or without elemental sulfur. Elemental sulfur-dependent reduction of Fe$^{3+}$ to Fe$^{2+}$ was observed in control cells without hydrogen sulfide treatment, indicating that SFORase in control cells used externally added elemental sulfur as an electron donor to reduce Fe$^{3+}$ (Fig. 1A). In contrast, a similar level of Fe$^{2+}$ observed in control cells was produced when hydrogen sulfide-treated cells were incubated in the reaction mixture lacking elemental sulfur (Fig. 1B), suggesting that hydrogen sulfide itself or a reduced sulfur compound produced from the hydrogen sulfide was present in the treated cells. The addition of elemental sulfur to sulfide-treated cells did not affect the amount of Fe$^{2+}$ produced, suggesting that the reduced sulfur compound formed after the treatment is preferable as a substrate for SFORase that externally added elemental sulfur.

The substrate specificity of purified SFORase is narrow. That is, among reduced sulfur compounds tested, only FeS, hydrogen sulfide, and elemental sulfur could be used as a substrate for this enzyme. Thus, by using the purified SFORase, we tried to identify the reduced sulfur compound formed in the cells after the hydrogen sulfide treatment. The hydrogen sulfide-treated cells (25 mg of protein) were dried for 1 hr at 150°C and powdered. The cells thus powdered were incubated with purified SFORase to test
Fig. 2. Formation of a Heat Stable Reduced Sulfur Compound in the Cells after Treatment of Intact Cells with Hydrogen Sulfide.

Purified hydrogen sulfide:ferric ion oxidoreductase (SFORase) at the stage of Mono Q column chromatography (12) was used throughout this study. The method for analysis of sulfite ions produced by the oxidation of reduced sulfur compound by purified SFORase was described in Materials and Methods. The hydrogen sulfide-treated cells or untreated cells (20 mg of protein preparation described in the text) were dried for 1 hr at 150°C and powdered. The hydrogen sulfide-treated cells thus produced were incubated with (A) purified SFORase or (A) 10 min-boiled SFORase. The hydrogen sulfide-untreated cells thus produced were incubated with (O) purified SFORase or (O) 10 min-boiled SFORase.

whether the powder could supply sulfide ions to the enzyme and produce sulfite ion in the reaction mixture. Sulfite ions were produced when hydrogen sulfide-treated dried cells were used as a substrate for purified SFORase (Fig. 2). In contrast, no sulfite ions were produced in control dried cells without hydrogen sulfide treatment. Hydrogen sulfide is completely decomposed after incubation for 1 hr at 150°C. So, the reduced sulfur compound formed in hydrogen sulfide-treated cells may not be hydrogen sulfide itself, but FeS or a FeS-like compound because FeS is stable after 1 hr of heat treatment at 150°C and can be used as a substrate for the purified SFORase.

Localization of the heat stable reduced sulfur compound formed in the cells of T. ferrooxidans AP19-3 after hydrogen sulfide treatment

Plasma membrane (50 mg of protein) and cytosol (50 mg of protein) fractions prepared from iron-grown T. ferrooxidans AP19-3 were dialyzed with 100 ml of 10 mM hydrogen sulfide solution (pH 6.5) for 12 hr, and then dialyzed three times with one liter of 0.1 M sodium phosphate buffer (pH 6.5) to discard hydrogen sulfide. The hydrogen sulfide-treated plasma membrane and cytosol fractions were dried for 1 hr at 150°C and powdered. The hydrogen sulfide-treated plasma membrane and cytosol fractions were incubated with purified SFORase. The amount of sulfite ions produced chemically was always checked throughout this study by using 10 min-boiled SFORase instead of native SFORase. (B) The same hydrogen sulfide-treatment described above was carried out with pronase-treated plasma membrane (O) and cytosol fraction (A) (50 mg of protein).

Fig. 3. Localization of the Heat Stable Reduced Sulfur Compound Formed in the Cells of T. ferrooxidans AP19-3.

Symbols: (A) Plasma membrane (O) and cytosol fractions (A) (50 mg of protein) prepared from iron-grown T. ferrooxidans AP19-3 were dialyzed with 100 ml of 10 mM hydrogen sulfide solution (pH 6.5) for 12 hr, and then dialyzed three times with one liter of 0.1 M sodium phosphate buffer (pH 6.5) to discard hydrogen sulfide. The hydrogen sulfide-treated plasma membrane and cytosol fractions were dried for 1 hr at 150°C and powdered. The hydrogen sulfide-treated plasma membrane and cytosol fractions were incubated with purified SFORase. The amount of sulfite ions produced chemically was always checked throughout this study by using 10 min-boiled SFORase instead of native SFORase. (B) The same hydrogen sulfide-treatment described above was carried out with pronase-treated plasma membrane (O) and cytosol fraction (A) (50 mg of protein).
hydrogen sulfide-binding protein in plasma membrane or cytosol fraction, the hydrogen sulfide treatment was done after both fractions were treated with 10 units of pronase E (Sigma type XIV) in 0.1 M sodium phosphate buffer (pH 7.5) for 2 hr at 30°C. Hydrogen sulfide solution (pH 6.5) was added to pronase-treated plasma membrane (50 mg of protein) and cytosol (50 mg of protein) fractions at 10 mM, and then dialyzed three times with one liter of 0.01 M sodium phosphate buffer (pH 6.5). The pronase- and sulfide-double treated plasma membrane and cytosol fractions were dried for 1 hr at 150°C, powdered as described above, and incubated with purified SFORase. The amount of sulfite ions produced in the reaction mixture was markedly decreased when pronase- and hydrogen sulfide-double treated plasma membrane was used as a substrate for purified SFORase (Fig. 3). In contrast, no noticeable effect of pronase treatment on sulfite production was observed in the pronase and hydrogen sulfide-double treated cytosol fraction, suggesting that hydrogen sulfide can bind to a specific protein of plasma membrane. We tentatively called the plasma membrane protein, which binds hydrogen sulfide, hydrogen sulfide-binding protein (SBP).

**Purification and some properties of hydrogen sulfide-binding protein**

Hydrogen sulfide-binding protein (SBP) was purified to an electrophoretically homogeneous state by the method described in Materials and Methods. The procedure gave 11-fold purification over the 1% SDS-solubilized fraction of plasma membrane. The apparent molecular weight of hydrogen sulfide-binding protein was 16,000 by SDS polyacrylamide disc gel electrophoresis. Iron content of a purified SBP was measured by atomic absorption spectroscopy and found that one molecule of SBP contains 4.5 atoms of iron. The SBP dialyzed sufficiently with 0.1 M sodium phosphate buffer (pH 7.5) did not show any absorption peak in a visible region. However, when hydrogen sulfide solution was added to the dialyzed SBP at 50 μM, characteristic absorption peaks appeared at 435, 533, and 637 nm (Fig. 4). These three absorption peaks disappeared upon dialyzing the protein with 0.1 M sodium phosphate buffer (pH 7.5), suggesting that hydrogen sulfide reversibly bound to the protein. The SBP dialyzed with 500 ml of 3 mM α,α'-dipyridyl for 12 hr and dialyzed again sufficiently with 0.1 M sodium phosphate buffer (pH 7.5) did not show any peaks in a visible region though hydrogen sulfide solution was added at 50 μM, suggesting that iron in SBP is crucial in trapping hydrogen sulfide.

We showed that hydrogen sulfide was chemically produced when elemental sulfur was incubated with GSH in 0.1 M sodium phosphate buffer (pH 6.5). The amount of hydrogen sulfide produced chemically was markedly increased in proportion to the amount of SBP added to the reaction mixture (Fig. 5), suggesting that SBP can protect hydrogen sulfide from chemical oxidation by molecular oxygen. SBP could stabilize hydrogen sulfide between pH 5.5 and 8.0 (data not shown). The SBP dialyzed with 500 ml of 3 mM α,α'-dipyridyl completely lost the ability to increase the production of hydrogen sulfide, suggesting an possible role of iron in the SBP to increase hydrogen sulfide in the reaction.
Fig. 5. Effects of Hydrogen Sulfide-binding Protein on the Amount of Hydrogen Sulfide Produced Chemically with Elemental Sulfur and GSH.

The amount of hydrogen sulfide produced chemically with elemental sulfur (100 mg) and GSH (20 μmol) was measured in 5 ml of 0.1 M sodium phosphate buffer (pH 6.5) containing bovine serum albumin (10 mg) (○), ferric ion (18 μg) (×), hydrogen sulfide-binding protein (●, 18 μg; △, 36 μg; ■, 61 μg) or without ferric ion and hydrogen sulfide-binding protein (○ and ×). After 30 min, purified SFORase was added to some of the reaction mixture (△, △, ●, and △), but not to the other reaction mixtures (○, ○, ×).

mixture. Bovine serum albumin and Fe³⁺ did not have a similar enhancing effect on hydrogen sulfide production as that of SBP. After incubation of elemental sulfur with GSH for 30 min, purified SFORase was added to the reaction mixture. The amount of hydrogen sulfide in the reaction mixture decreased, indicating that SFORase oxidized hydrogen sulfide produced chemically in the presence of SBP (Fig. 5).

We tried to measure the hydrogen sulfide bound to SBP. To prepare the SBP whose binding sites for hydrogen sulfide are fully saturated (S²⁻-SBP), purified SBP was treated with 1 mM of hydrogen sulfide for 30 min. The S²⁻-SBP thus produced was precipitated by adding ethanol to the reaction mixture at 50%, and washed sufficiently with 0.1 M sodium phosphate buffer to remove hydrogen sulfide not bound to the SBP. When purified SFORase was added to the S²⁻-SBP, sulfite ion was produced in the reaction mixture. The amount of hydrogen sulfide bound to SBP was calculated from the amount of sulfite ion produced in the reaction mixture (Fig. 6). The calculated value, 2.3 molecules of sulfide per molecule of SBP, suggests that one molecule of SBP binds 2 or 3 molecules of hydrogen sulfide.

It seems very reasonable for *T. ferrooxidans* AP19-3 to have a hydrogen sulfide-binding protein, because when the bacterium oxidizes elemental sulfur by SFORase, hydrogen sulfide produced chemically by reducing elemental sulfur with GSH is an actual substrate for this enzyme.¹⁴

References

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