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Actual substrate for elemental sulfur oxidation by sulfur:ferric ion oxidoreductase purified from *Thiobacillus ferrooxidans*

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Initial step of elemental sulfur (S^0) oxidation by a purified sulfur:ferric ion oxidoreductase from *Thiobacillus ferrooxidans* was investigated. When S^0 and reduced glutathione (GSH), which was absolutely required for S^0 oxidation by sulfur:ferric ion oxidoreductase, were incubated in a buffer solution (pH 6.5), hydrogen sulfide (H_2S) and GSSG were chemically produced at the rate of 0.021 and 0.082 μmol/ml per h, respectively. If sulfur:ferric ion oxidoreductase was added to the incubation mixture, H_2S production immediately stopped and sulfite production opened, suggesting that H_2S is an actual substrate of sulfur:ferric ion oxidoreductase. Among the reduced sulfur compounds tested, S^0, H_2S and FeS were utilized as an electron donor of sulfur:ferric ion oxidoreductase and a mechanism of initial steps of S^0 oxidation was proposed. It was also found that when S^0 was oxidized by sulfur:ferric ion oxidoreductase in the presence of GSH, contact of sulfur:ferric ion oxidoreductase with solid element sulfur was unnecessary.

Introduction

Thiobacillus ferrooxidans has an ability to oxidize not only ferrous ions but also reduced sulfur compounds under acidic conditions, and the ability to oxidize two types of reduced inorganic compounds makes the bacterium most valuable microorganism for bacterial leaching. The problem how elemental sulfur or insoluble substrate in water is oxidized by the industrially important bacteria genus *Thiobacillus* has attracted many investigators from scientific point of view. The necessity for *Thiobacillus thiooxidans* to contact directly with elemental sulfur before sulfur oxidation could occur was proposed [1,2], and some properties of energy-dependent cell–sulfur adhesion in *T. thiooxidans* were investigated by Takakuwa [3]. However, there has been no report about mechanism of initial attack on insoluble sulfur particles by *T. ferrooxidans*.

Sulfur-oxidizing enzyme has been partially purified in a number of thiobacilli [4–9]. Suzuki studied a mechanism of elemental sulfur oxidation with sulfur dioxygenase partially purified from *T. thiooxidans*, which catalizes sulfur oxidation in the presence of catalytic quantities of GSH, and proposed sulfur oxidation involving glutathione polysulfide as intermediate [5]. The mechanism of sulfur oxidation of *T. ferrooxidans* has been considered similar to that of other thiobacilli [8,10–12]. We recently reported the presence of a ferric ion-reducing system (FIR system) that catalyzes the reduction of Fe^{3+} with elemental sulfur in a pure strain of *T. ferrooxidans* AP19-3, and proposed an alternative sulfur oxidation route that is composed of both the FIR and iron-oxidizing systems [13]. A sulfur:ferric ion oxidoreductase, which corresponds to the FIR system and catalyzes the oxidation of elemental sulfur with Fe^{3+} as an electron acceptor, was purified to an electrophoretically homogeneous state from iron-grown *T. ferrooxidans* AP19-3 [14]. Results strongly suggesting a physiological importance of sulfur:ferric ion oxidoreductase in an aerobic sulfur oxidation of this strain have been presented elsewhere [15–20].

The properties of sulfur:ferric ion oxidoreductase were distinct from those of sulfur dioxygenase purified from *T. thiooxidans* by Suzuki [5] except for GSH as being absolutely required for enzymatic activity. Since GSH is commonly required for a solid sulfur oxidation, studying the role of GSH in sulfur oxidation by sulfur:ferric ion oxidoreductase may add valuable information to clarify the mechanism of oxidation of insolu-

Abbreviations: GSH, glutathione; GSSG, oxidized glutathione; FIR, ferric ion-reducing; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

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ble inorganic sulfur compounds. In this report, we clarified the role of GSH in elemental sulfur oxidation by sulfur:ferric ion oxidoreductase and determined an actual substrate of sulfur:ferric ion oxidoreductase. Furthermore, evidence is presented that a physical contact of sulfur:ferric ion oxidoreductase with solid elemental sulfur is not necessary to oxidize sulfur.

Materials and Methods

Microorganism, medium, and conditions of cultivation. *T. ferrooxidans* AP19-3 was used throughout this study [21]. The composition of iron-salts medium used for the large-scale production of cells and the method for cultivation were described previously [22].

Purification of sulfur:ferric ion oxidoreductase from iron-grown T. ferrooxidans AP19-3. Sulfur:ferric ion oxidoreductase, which catalyzes the oxidation of elemental sulfur with Fe^{3+} to give sulfite and Fe^{2+}, was purified from iron-grown *T. ferrooxidans* AP19-3 by the method described previously [14]. Enzyme solution at the stage of mono Q column chromatography [14] was used throughout this study. The enzyme solution showed one main and three faint bands on polyacrylamide gel, and the sulfur:ferric ion oxidoreductase activity (specific activity: 965.3 units per mg protein) was observed only in the main band.

The activity of sulfur:ferric ion oxidoreductase was determined under aerobic conditions by measuring sulfite [14] instead of measuring Fe^{2+} produced under anaerobic conditions in the presence of Fe^{3+}. Under aerobic conditions, there is no necessity to add Fe^{3+} in the reaction mixture probably because a trace amount of Fe^{3+} in sulfur:ferric ion oxidoreductase, which is reduced by S^0, is reoxidized rapidly by molecular oxygen at pH 6.5 [14]. The reaction mixture contains 4 ml 0.1 M sodium phosphate buffer (pH 6.5); enzyme; 0.2 mg bovine serum albumin; 100 mg elemental sulfur; and 40 μmol GSH (adjusted to pH 6.5 with dilute NaOH), Total volume was 5.0 ml. The sulfite produced was determined colorimetrically by the pararosaniline method [23]. The amount of sulfite produced chemically was always checked throughout this study by using a 10 min-boiled sulfur:ferric ion oxidoreductase instead of native sulfur:ferric ion oxidoreductase.

Analysis. Sulfide sulfur was determined by the Methylene blue method [24]. *N,N*-dimethyl-*p*-phenylenediamine sulfate (400 mg) and ferric chloride (600 mg) were dissolved into 100 ml 6 M HCl. Diamine-ferric chloride solution was prepared by mixing equal volumes of two above-described solutions just before analysis. A reaction mixture was withdrawn and centrifuged at $12\,000 \times g$ for 2 min to discard a 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_2O to 2.5 ml. A 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 μmol hydrogen sulfide per ml) and absorbance at 670 nm.

Oxidized glutathione (GSSG) was determined spectrophotometrically by measuring oxidation of NADPH in the presence of glutathione reductase. The composition of reaction mixture for GSSG assay was as follows: 0.5 ml 0.2 M potassium phosphate buffer (pH 7.0); 2 mM NADPH, 50 μl 0.5 U glutathione reductase (Sigma); and 50 μl supernatant solution obtained by centrifuging each of reaction mixtures at $12\,000 \times g$ for 2 min. Total volume was 1.0 ml.

Results

Substrate specificity of sulfur:ferric ion oxidoreductase and absolute requirement of GSH for sulfur:ferric ion oxidoreductase activity

Sulfur:ferric ion oxidoreductase purified from *T. ferrooxidans* AP19-3 to an electrophoretically homogeneous state catalyzes the oxidation of elemental sulfur by Fe^{3+} in the presence of GSH to give Fe^{2+} and sulfite [14]. We first tried to search for another compound that has an enhanced effect on a sulfur:ferric ion oxidoreductase activity as GSH does. The GSH requirement was not replaced by GSSG or D-panthethine, and by *s*-methylglutathione and *s*-hexylglutathione, in which a hydrogen atom of sulfhydryl group of GSH is replaced by a methyl- or hexyl-group, indicating that hydrogen atom of a sulfhydryl-group of GSH plays an important role in sulfur oxidation by sulfur:ferric ion oxidoreductase (data not shown). Another reagent possessing a sulfhydryl group, such as cysteine, cysteine plus glutamic acid, cysteine plus glycine, cysteine plus glutamic acid plus glycine, cystein plus *r*-L-glutamylglycine, and 2-mercaptoethanol, did not have an enhanced effect on sulfur:ferric ion oxidoreductase activity. Reducing reagents such as dithiothreitol and ascorbic acid also did not have any enhanced effect for the activity.

Whether sulfur:ferric ion oxidoreductase can utilize reduced sulfur compounds other than elemental sulfur as an electron donor, was studied. Inorganic sulfur compounds such as thiosulfate, dithionate, tetrathionate, and metal sulfides except FeS, and organic sulfur compounds such as 2-mercaptoethanol, dithiothreitol, cysteine, cystine, methionine, thiophene, dibenzothiophene, trithioformaldehyde, vitamine B_1, biothin, and D-panthethine, did not serve as electron donor for sulfur:ferric ion oxidoreductase (Table I). When FeS was utilized as an electron donor for sulfur:ferric ion oxidoreductase, 40% of the activity observed when elemental sulfur was utilized as a substrate was obtained, suggesting that elemental sulfur was a superior substrate than FeS. GSH was also required when FeS was oxidized

TABLE I

Substrate specificity for sulfur:ferric ion oxidoreductase purified from T. ferrooxidans AP19-3

The composition of the reaction mixture and the methods used for analysis are described in Materials and Methods. Sulfur:ferric ion oxidoreductase (3.5 μg of protein) was used.

Additions	Concn.	Sulfite produced (nmol/min per ml)	Additions	Concn. (mM)	Sulfite produced (nmol/min per ml)
Elemental sulfur	20 mg/ml	0.68	dithiothreitol	40	0.00
Sodium sulfide	0.02 mM	0.69	2-mercaptoethanol	40	0.00
	0.06 mM	0.57	cysteine	40	0.00
	0.50 mM	0.00	methionine	40	0.00
Thiosulfate	1 mM	0.00	vitamine B_1	40	0.00
	40 mM	0.00	biotin	40	0.00
Dithionate	1 mM	0.00	D-panthethine	40	0.00
	40 mM	0.00	thiourea	40	0.00
Tetrathionate	40 mM	0.00	thiophene	40	0.00
FeS	20 mg/ml	0.28	dibenzothiophene	40	0.00
CuS	20 mg/ml	0.00	trithioformaldehyde	40	0.00
CoS	20 mg/ml	0.00			
NiS	20 mg/ml	0.00			
ZnS	20 mg/ml	0.00			
PbS	20 mg/ml	0.00			

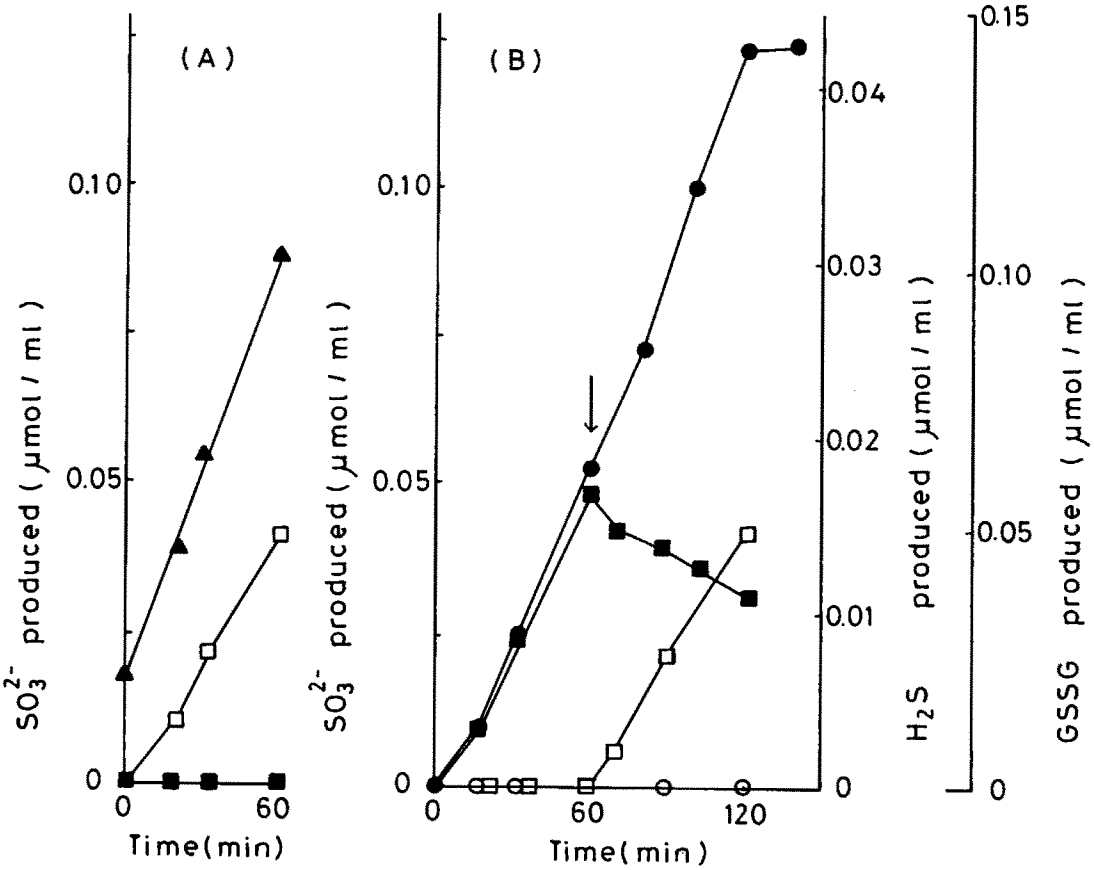

Fig. 1. Production of hydrogen sulfide, sulfite, and GSSG in elemental sulfur oxidation by sulfur:ferric ion oxidoreductase purified from *T. ferrooxidans* AP19-3. The composition of the standard assay mixture for sulfur:ferric ion oxidoreductase is described in materials and Methods. Sulfur:ferric ion oxidoreductase (3.5 μg of protein) was used. Symbols: (A) A sulfur:ferric ion oxidoreductase was added to the standard assay mixture at start of reaction, and the concentration of hydrogen sulfide (■) and sulfite (□) were determined. (B) Two flasks containing both GSH and elemental sulfur were incubated forl h. One of the flasks was further incubated without the addition of sulfur:ferric ion oxidoreductase and concentration of hydrogen sulfide (●) and sulfite (○) in the reaction mixture were determined. Sulfur:ferric ion oxidoreductase (3.5 μg of protein) was added to another flask at the point of arrow, and the concentration of hydrogen sulfide (■) and sulfite (□) were determined. A chemical production of GSSG in the standard reaction mixture without sulfur:ferric ion oxidoreductase was also ploted (▲).

by sulfur:ferric ion oxidoreductase to give sulfite. This may be due to a protective effect of GSH on the decomposition of sulfite produced by sulfur:ferric ion oxidoreductase reaction (data not shown). A low concentration fo sodium sulfide (0.06 mM) was utilized as a substrate for sulfur:ferric ion oxidoreductase and gave sulfite as a product, but a high concentration of sodium sulfide (0.5 mM) could not be utilized.

The role of GSH on elemental sulfur oxidation by sulfur:ferric ion oxidoreductase

To study a specific role of GSH in sulfur oxidation by sulfur:ferric ion oxidoreductase, we tried to identify the products appeared in the incubation mixture when elemental sulfur and GSH were incubated in 0.1 M sodium phosphate buffer (pH 6.5) in the absence of sulfur:ferric ion oxidoreductase. Hydrogen sulfide (H_2S) and GSSG were chemically produced at the rate of 0.021 and 0.082 μmol/ml per h, respectively (Fig. 1A and B). When sulfur:ferric ion oxidoreductase was added to the reaction mixture 1 h later, the H_2S production immediately stopped and sulfite production opened. No H_2S production was observed when sulfur:ferric ion oxidoreductase was added to the reaction mixture containing elemental sulfur and GSH at the start of reaction (Fig. 1A). When the incubation mixture obtained by incubating S^0 with GSH applied to a column (1.5 × 36 cm) of Sephadex G25 equilibrated with 0.1 M sodium phosphate buffer, the same elution pattern as that of authentic sodium sulfide solution was obtained. Also the peak corresponding hydrogen sulfide disappeared when the incubation mixture was treated with sulfur:ferric ion oxidoreductase for 2 h (data not shown), suggesting that the active compound in the incubation mixture, which was utilized as a substrate of this enzyme, was H_2S, but not glutathione hydrosulfide. From the results obtained in Table I and Fig. 1, it was concluded that H_2S is an actual substrate for sulfur:ferric ion oxidoreductase in oxidation of insoluble solid sulfur by *T. ferrooxidans* AP19-3.

In Table I we showed that 0.5 mM of sodium sulfide did not serve as a substrate for sulfur:ferric ion oxidoreductase at pH 6.5, at which any sulfide in solution exist mainly as H_2S. To solve why a high concentration of H_2S could not be utilized as a substrate, the effect of H_2S on the oxidation of elemental sulfur by sulfur:ferric ion oxidoreductase was studied. Oxidation of elemental sulfur by sulfur:ferric ion oxidoreductase was strongly inhibited by H_2S at 0.5 mM (Fig. 2). However, when 4 mM of Fe^{2+} was added to the reaction mixture containing 0.5 mM of H_2S, the H_2S was oxidized by sulfur:ferric ion oxidoreductase and gave sulfite as a product (Fig. 3). The Fe^{2+} requirement was not replaced by other metal ions, such as Fe^{3+}, Sn^{2+}, Ni^{2+}, and MoO_4^{2-} at 4 mM, suggesting that by adding Fe^{2+} in the reaction mixtures, FeS was produced and as a result, H_2S concentration decreased to harmless level for sulfur:ferric ion oxidoreductase. FeS, which can continuously supply a low concentration of H_2S to

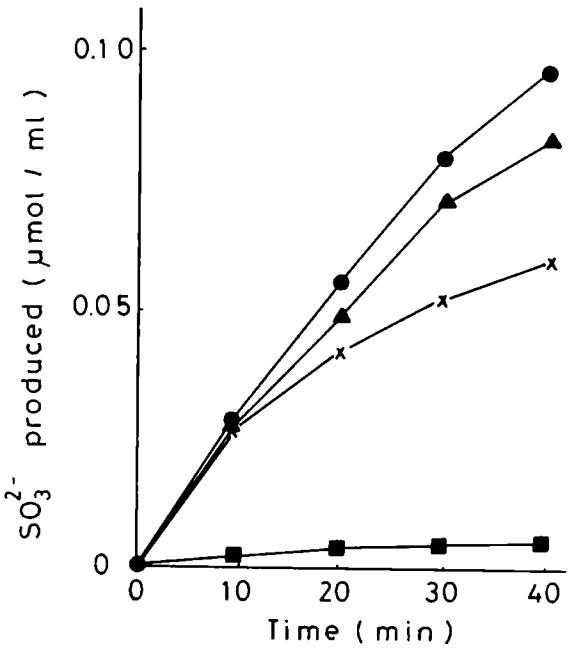

Fig. 2. Effect of sodium sulfide on the oxidation of elemental sulfur by sulfur:ferric ion oxidoreductase purified from *T. ferrooxidans* AP19-3. The composition of the standard assay mixture for sulfur:ferric ion oxidoreductase is described in materials and Methods. Sulfur:ferric ion oxidoreductase (12.5 μg of protein) was used. Concentration of sodium sulfide was checked by the Methylene blue method as described in materials and Methods. Symbols: sodium sulfide was added to standard assay mixture for sulfur:ferric ion oxidoreductase at 0.02 mM (▲), 0.10 mM (×), and 0.50 mM (■). No sodium sulfide was added to the standard assay mixture (●).

TABLE II

Effect of metal ions on the activity of sulfur:ferric ion oxidoreductase purified from T. ferrooxidans

The composition of the reaction mixture and the methods used for analysis are described in Materials and Methods. Enzyme purified at the stage of mono Q column chromatography (12.5 μg of protein) was added to the reaction mixture.

Metal ions	Concn. (mM)	Sulfite produced (nmol/min per ml)
none	–	1.60
$FeSO_4 \cdot 7\,H_2O$	1.0	1.38
$Fe_2(SO_4)_3$	1.0	1.42
$CuSO_4 \cdot 5\,H_2O$	0.001	0.95
	0.01	0.00
$CoSO_4 \cdot 7\,H_2O$	0.1	0.31
	0.5	0.00
$NiSO_4$	0.1	0.30
$ZnSO_4 \cdot 7\,H_2O$	1.0	1.10
$Pb(NO_3)_2$	1.0	1.15
$Cr_2(SO_4)_3$	4.0	1.60
$UO_2SO_4 \cdot 3\,H_2O$	1.0	1.40
$Na_2MoO_4 \cdot 2\,H_2O$	2.0	1.60
$MnSO_4$	1.0	1.60
$MgSO_4 \cdot 7\,H_2O$	1.0	1.60

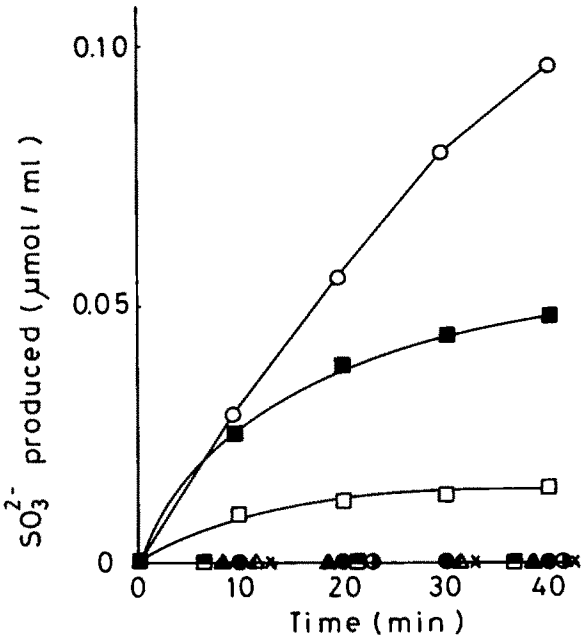

Fig. 3. Effect of metal ions on the oxidation of sodium sulfide by sulfur:ferric ion oxidoreductase purified from *T. ferrooxidans* AP19-3. The composition of the standard reaction mixture for sulfur:ferric ion oxidoreductase is described in materials and Methods. Sulfur:ferric ion oxidoreductase (12.5 μg of protein) was used. Sodium sulfide instead of elemental sulfur was added to standard assay mixture at 0.5 mM as a substrate of sulfur:ferric ion oxidoreductase. Symbols: metal ions described below were added at the following concentration; □, Fe^{2+} (1 mM); ■, Fe^{2+} (4 mM); ●, Fe^{3+} (1 mM); ▲, Sn^{2+} (1 mM); ⊟, Ni^{2+} (1 mM); ▲, Cr^{3+} (4 mM); ◑, MoO_4^{2-} (4 mM). No metal ion was added (×). Sulfite production with standard assay mixture, in which both metal ions and sulfide were omitted (○).

sulfur:ferric ion oxidoreductase, could be utilized as a substrate for sulfur:ferric ion oxidoreductase (Table I). The amount of H_2S produced chemically by incubating elemental sulfur and GSH (approx. 0.021 mM H_2S was produced after 1 h incubation) was low, and it may not give any harmful effect on sulfur:ferric ion oxidoreductase activity. From the results, it can be said that a low concentration of H_2S, which does not inhibit sulfur:ferric ion oxidoreductase activity, can serve as a substrate for sulfur:ferric ion oxidoreductase.

It is strange that various metal sulfide except FeS could not be utilized as an electron donor of sulfur:ferric ion oxidoreductase (Table I). This may be explained by the inhibitory effect of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+} on sulfur:ferric ion oxidoreductase activity because these metal ions inhibited sulfur:ferric ion oxidoreductase activity at 1 mM (Table II). In contract, the inhibitory effect of Fe^{2+} on sulfur:ferric ion oxidoreductase activity was rather low.

Oxidation of elemental sulfur without a contact of SFORase with a solid elemental sulfur

If H_2S is produced chemically by elemental sulfur, and GSH is an actual substrate of sulfur:ferric ion oxidoreductase, it is supposed that in the presence of GSH a contact of sulfur:ferric ion oxidoreductase with a solid elemental sulfur is not necessary for the enzyme to oxidize elemental sulfur. To check this supposition, elemental sulfur (200 mg) suspended in 2.0 ml of 0.1 M sodium phosphate buffer (pH 6.5) supplemented with or without GSH (40 μmol) was put into dialyzing bags, and incubated against 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) for 8 h at 30 °C. After 8 h incubation, the dialyzing bag containing a solid elemental sulfur was discarded and the dialyzate thus obtained was tested whether sulfite is produced by incubating with sulfur:ferric ion oxidoreductase in the absence of elemental sulfur. Sulfite production was observed in the dialyzate obtained by incubating both elemental sulfur and GSH in dialyzing bag (Fig. 4). By contrast, no sulfite was produced in the dialyzate obtained by incubating elemental sulfur with buffer solution lacking GSH, indicating that there was no necessity for sulfur:ferric ion oxidoreductase to contact with a solid elemental sulfur if GSH is present.

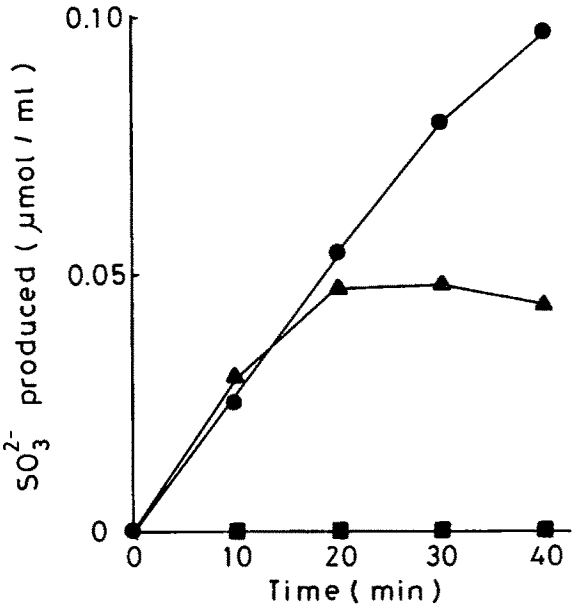

Fig. 4. Oxidation of elemental sulfur without a contact of sulfur:ferric ion oxidoreductase with a solid elemental sulfur. Elemental sulfur (200 mg) suspended with 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) supplemented with or without GSH (40 μmol) was put into dialyzing bag, and incubated for 8 h at 30 °C. The dialyzates obtained 8 h incubation were tested for the production of sulfite by incubating them with sulfur:ferric ion oxidoreductase in the absence of elemental sulfur. Symbols: ▲, the dialyzate obtained by incubating both element sulfur and GSH in dialyzing bag was used as a substrate for sulfur:ferric ion oxidoreductase: ■, the dialyzate obtained by incubating elemental sulfur with buffer solution lacking GSH was used as a substrate for sulfur:ferric ion oxidoreductase; ●, sulfite production with standard assay mixture. Sulfur:ferric ion oxidoreductase (12.5 μg of protein) was used.

Discussion

To clarify the oxidation mechanism of a solid elemental sulfur by *T. ferrooxidans,* the role of GSH in sulfur oxidation by a sulfur:ferric ion oxidoreductase purified from *T. ferrooxidans* AP19-3 [14] was investigated and it was clarified that elemental sulfur is chemically reduced by GSH to give hydrogen sulfide before it was oxidized by sulfur:ferric ion oxidoreductase. It has been known that sulfide is produced chemically by incubating elemental sulfur with GSH [4,25]. Hydrogen sulfide thus produced was concluded to be an actual substrate for sulfur:ferric ion oxidoreductase because it was rapidly oxidized by the enzyme to give sulfite. This was supported from the results that a low concentration of H_2S and FeS were utilized as a substrate of sulfur:ferric ion oxidoreductase and gave sulfite as a product.

Suzuki showed that sulfur dioxygenase partially purified from *T. thiooxidans* oxidizes elemental sulfur in the presence of catalytic amount of GSH, and proposed sulfur oxidation mechanism involving glutathione polysulfide as intermediate [5]. Unlike sulfur dioxygenase, sulfur:ferric ion oxidoreductase requires a large amount of GSH for the appearance of maximum activity [14]. Since GSH added to the reaction mixture is utilized to reduce elemental sulfur and give H_2S, it seems rather preferable to say that GSH is one of substrates, but not a cofactor in sulfur oxidation by *T. ferrooxidans* AP19-3. Since hydrogen sulfide was identified as an actual substrate of sulfur:ferric ion oxidoreductase in this text, we should like to change the enzyme name from sulfur:ferric ion oxidoreductase to an alternative name hydrogen sulfide : ferric ion oxidoreductase. The enzyme, which catalyzes the oxidation of sulfide directly to sulfite, has been purified from *Thiobacillus denitrificans* [26].

A de novo synthesis of not only sulfur:ferric ion oxidoreductase but also iron oxidase was observed when *T. ferrooxidans* AP19-3 grows on sulfur-salts medium [19]. Another physiological significance of sulfur:ferric ion oxidoreductase in an aerobic sulfur oxidation of this strain has been published elsewhere [15–18]. An involvement of cytochrome bc_1 segment of respiratory chains in sulfur:ferric ion oxidoreductase reaction was ruled out from the following results. (i) 2-Heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) did not inhibit sulfur:ferric ion oxidoreductase activity at 40 μM [14]. (ii) A sulfur:ferric ion oxidoreductase purified to an electrophoretically homogeneous state did not show any absorption peaks of cytochromes (data not shown). (iii) Though the production of Fe^{2+} was observed in the culture medium, *T. ferrooxidans* AP19-3 could not grow on sulfur-salts medium with Fe^{3+} as a terminal electron acceptor under anaerobic conditions [19].

According to our proposition, a solid elemental sulfur is not oxidized on the surface of outer membrane of *T. ferrooxidans* AP19-3 because a sulfur:ferric ion oxidoreductase was found to be present in periplasmic space of this strain [14], but it may be oxidized by sulfur:ferric ion oxidoreductase in periplasmic space after passing through the outer membrane by a mechanism that remains to be established [14]. Since no sulfur:ferric ion oxidoreductase activity was observed at pH 4.5, but 36.1 and 57.7% of the activity was observed at pH 5.5, and 6.0, respectively, the periplasmic pH of this strain seems to be above pH 4.5 [14].

A reduced glutathione (GSH) was absolutely required for elemental sulfur oxidation by sulfur:ferric ion oxidoreductase. The importance of GSH in sulfur oxidation by sulfur:ferric ion oxidoreductase was also suggested from the study on mechanism for the inhibition by Co^{2+} of sulfur metabolism of *T. ferrooxidans* AP19-3, in which Co^{2+} stops cell growth on sulfur by decreasing the intracellular GSH concentration to a level at which sulfur:ferric ion oxidoreductase is no longer active, and the cells then cannot obtain energy by oxidizing elemental sulfur [20]. GSH synthesized in cytosol of *T. ferrooxidans* AP19-3 seems to be transported to periplasmic space because sulfur:ferric ion oxidoreductase is localized in periplasmic space [14]. We have previously shown that ^{35}S-GSH incorporated into *T. ferrooxidans* leaks out of the cells during washing with buffer [27], and recently presented properties of ^{35}S-GSH uptake and release by *T. ferrooxidans* AP19-3 [28], suggesting that GSH can transport from cytosol to periplasmic space, and vice versa. From the information now available we propose the following mechanism for the recycling of GSSG to 2 GSH: the GSSG transported back to cytoplasm seems to be reduced with NADH by glutathione reductase. The existence of glutathione reductase was reported in *T. thiooxidans* [29] and *T. ferrooxidans* [30]. The NADH required for carbon dioxide fixation and GSSG reduction may be produced through ATP-dependent reduction of NAD^+ [31].

Elemental sulfur, H_2S (at low concentration), and FeS could be utilized as a substrate of sulfur:ferric ion oxidoreductase (Table I). A sulfur:ferric ion oxidoreductase was strongly inhibited by H_2S at 0.5 mM, but this inhibition was specifically restored by adding Fe^{2+} to the reaction mixture (Fig. 3). These results suggest that when H_2S concentration in periplasmic space increases to a harmful level for sulfur:ferric ion oxidoreductase, the H_2S reacts with Fe^{2+} to give a harmless FeS. Another possible role of FeS is that FeS is reserving form of reducing equivalents of elemental sulfur. Namely, unstable reducing equivalents of H_2S may be reserved in a stable form or FeS which can continuously provide a low concentration of H_2S and Fe^{2+} as a substrate for sulfur:ferric ion oxidoreductase and iron oxidase, respectively. From the results presented in this text and previous work [13–20,28], we proposed a mech-

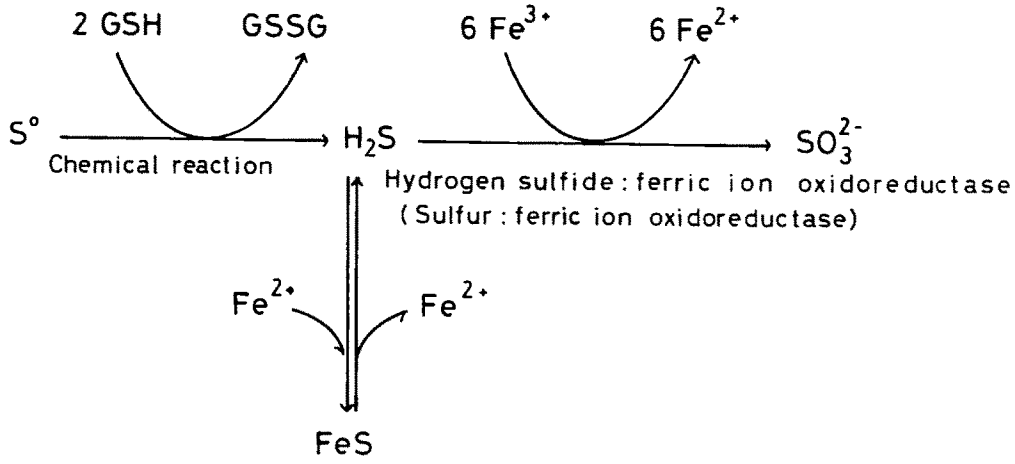

Fig. 5. Initial steps of elemental sulfur oxidation by hydrogen sulfide: ferric ion oxidoreductase (sulfur:ferric ion oxidoreductase) purified from *T. ferrooxidans* AP19-3.

anism of initial steps of sulfur oxidation in *T. ferrooxidans* AP19-3 to be that shown in Fig. 5.

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