Scientific Reports of the Faculty of Agriculture
Okayama University Vol. 91, 23-29 (2002)

Mechanism of Oxidation of Reduced Sulfur Compounds by Sulfur-Grown *Acidithiobacillus caldus* Strain GO-1

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The oxidation of reduced sulfur compounds was studied by using resting cells of sulfur-grown *Acidithiobacillus caldus* strain GO-1. The optimum pHs for the oxidation of thiosulfate, tetrathionate, sulfur, sulfite and sulfide were 2, 3, 3-6, 7 and 7, respectively. The highest oxidation rate was observed with sulfite. The oxidation rates of the reduced sulfur compounds were measured in the absence or presence of inhibitors and uncouplers. 2, 4-dinitrophenol (DNP) and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) strongly inhibited the oxidations of sulfur and sulfite. *N*-Ethylmaleimide (NEM) strongly inhibited the oxidation of tetrathionate and sulfur. 2-heptyl-4-hydroxy-quinoline-*N*-oxide (HQNO) inhibited the oxidation of sulfur and sulfite. The results suggested that tetrathionate was oxidized in the periplasmic space, and sulfur and sulfite were oxidized in the cytoplasm. Pyridine ferrohemochromes prepared from the membrane of strain GO-1 cell revealed the involvement of cytochromes *b* and *c*. Ubiquinol oxidase activity was detected in strain GO-1 cell, but cytochrome *c* oxidase measured by using mammalian cytochrome *c* as an electron donor was not detected in the cell. On the basis of the results a model for the metabolism of the reduced sulfur compounds by *At. caldus* strain GO-1 was proposed.

Key words : *Acidithiobacillus caldus*, acidophile, moderately thermophilic bacterium, sulfur-oxidizing bacterium, sulfite oxidation

Introduction

Acidithiobacillus caldus is a moderately thermophilic acidophile which was first found in environments such as coal spoil heaps[1]. This bacterium is capable of oxidizing reduced sulfur compounds, but incapable of oxidizing ferrous iron. The bacterium obtains its carbon by reductive fixation of atmospheric CO_2. The optimum growth pH and temperature was 2-2.5 and 45°C, respectively. *At. caldus* strain GO-1 was isolated from the water of Goshiki hot spring in Hokkaido, Japan. It is an obligately chemolithotrophic sulfur-oxidizing bacterium having a pH optimum for growth of 2.0 and an optimum growth temperature of 45°C[2].

At. caldus was frequently found in bioleaching processes operating at temperatures above 40°C and thought to be the primary sulfur oxidizer in the process[3]. We found that the growth of *At. caldus* strain GO-1 was activated in medium containing 1 mM cadmium ion[4]. Cadmium ion is known to be toxic to most microorganisms. In the bioleaching process, many heavy metals including cadmium are leached from sulfidic ores. Therefore, it is important and interesting not only from a scientific point of view but also from the applicatory point of view to clarify the

Received October 1, 2001

mechanism of the growth-activation of strain GO-1 by cadmium ion. The activation of growth by cadmium ion seems to be related to the oxidation of reduced sulfur compounds.

Hallberg *et al.* reported the mechanism of the oxidation of reduced sulfur compounds by *At. caldus* strain KU cell by studying the effect of inhibitors on the oxidizing activities and stoichiometries of metabolite accumulation[5]. Based on the data, the following metabolism was proposed: thiosulfate was oxidized to tetrathionate, elemental sulfur was formed during the oxidation of tetrathionate and sulfide, and sulfite was found as an intermediate of tetrathionate and sulfur metabolism.

The characteristics of *At. caldus* strain GO-1 are slightly different from strain KU. Although strain KU can grow mixotrophically with sulfur and yeast extract or glucose, strain GO-1 is an obligately chemolithotrophic sulfur-oxidizing bacterium. Therefore, the mechanism of the oxidation of reduced sulfur compounds in strain GO-1 cell may be different from that in strain KU cell. In this paper, the oxidation of reduced sulfur compounds by resting cells of strain GO-1 grown in sulfur medium was studied in the presence or absence of uncouplers and inhibitors, and the components of cytochromes and terminal oxidases were examined to clarify the mechanism of the oxidation of reduced sulfur compounds in *At. caldus* strain GO-1 cell.

Materials and Methods

Bacterium and growth condition

At. caldus strain GO-1 was used throughout this study. The strain was cultivated in sulfur salt medium (pH 2.5), as described previously[2].

Preparation of resting cell, cell-free extract and membrane fraction

Cells were grown in sulfur salt medium (pH 2.5) in a 500 ml Erlenmeyer flask containing 200 ml of the medium under aerobic condition at 45°C. The culture was filtrated through filter paper (No. 2 filter paper, Advantec Co., Ltd.) to remove sulfur particles. Cells were harvested by centrifugation, washed with 0.1 M β-alanine-SO_4 buffer (pH 3.0), suspended in the buffer, and used as resting cells. Cells washed with 0.1 M β-alanine-SO_4 buffer (pH 3.0) were suspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM $MgSO_4$ and 1% glycerol and broken by passing through a French press at 1,500 kg/cm^2 three times. The solution was centrifuged at 12,000 × g for 15 min. The supernatant was used as a cell-free extract. The cell-free extract was further centrifuged at 105,000 × g for 60 min. The precipitate was suspended in 0.1 M Tris-HCl buffer (pH 7.5) and used as a membrane fraction.

Assay for activity of reduced sulfur compound oxidation

The oxidizing activities of reduced sulfur compounds by using resting cells were measured polarographically by the oxygen uptake caused by the oxidation of reduced sulfur compounds with oxygen electrode (YSI Model 5300 Biological Oxygen Monitor, Yellow Spring Instrument Co., Inc., Ohio, USA). The reaction mixture contained 0.1 M buffer, 1 mg of cell protein, and substrates in a 3-ml reaction volume. The reaction was started by adding substrate to the reaction mixture and measured at 45°C. Substrate-dependent oxygen uptake rates in the presence of inhibitors and uncouplers were assayed after a pre-incubation of the cell suspension for 3 min with the inhibitors or uncouplers.

Preparation of pyridine ferrohemochromes from membrane fraction

A membrane fraction (3 mg protein) was homogenized with 1 ml of cold acetone and centrifuged at 12,000 × g for 10 min, and the precipitate thus obtained was mixed with 1 ml of cold chloroform and methanol (2:1). The solution was centrifuged at 12,000 × g for 10 min, and the precipitate was mixed with 1 ml of cold acetone. The hemes of cytochromes *a* and *b* were then extracted from the protein by three successive

homogenizations with the solution of 0.5 ml of cold acetone in 0.024 N HCl. The acetone extract was pooled and evaporated to near dryness *in vacuo*, and then immediately dissolved in 60 μl of pyridine and 60 μl of 0.2 N KOH. The protein residue after the extraction with HCl-acetone retained cytochrome *c*. The residue was homogenized in 30 μl of pyridine and 30 μl of 0.2 N KOH. Absorption spectrum for each pyridine ferrohemochrome was measured with a Beckman DU-65 spectrophotometer.

Measurement of cytochrome c oxidase

The activity of cytochrome *c* oxidase was measured at 45°C by the decrease in absorbance at 550 nm due to the oxidation of reduced mammalian cytochrome *c* (Sigma, from horse heart) in a Shimadzu UV-1200 spectrophotometer. The reaction mixture was composed of 0.1 M sodium phosphate buffer (pH 5.5), 0.15 mg of membrane protein and 0.4 mg of reduced mammalian cytochrome *c* in a total volume of 1.5 ml.

Measurement of ubiquinol oxidase

The activity of ubiquinol oxidase was measured spectrophotometrically at 45°C by the increase in absorbance at 275 nm with a Shimadzu UV-1200 spectrophotometer. The reaction mixture contained 50 mM MES-NaOH (pH 5.5), 30 μM Q_2H_2, 0.02% Tween 20, 0.15 mg of membrane protein in a total volume of 1 ml. The reaction was started by adding Q_2H_2 to the reaction mixture.

Protein measurement

Protein was measured by the method of Lowry *et al.* with crystalline bovine serum albumin as the reference protein[6].

Results and Discussion

Oxidation activities of reduced sulfur compounds

The resting cells of *At. caldus* strain GO-1 grown in sulfur medium had thiosulfate-, tetrathionate-, sulfur-, sulfide- and sulfite-oxidizing activities. The optimum pHs and activities for the oxidation of reduced sulfur compounds are summarized in Table 1. pHs of periplasmic space and cytoplasmic space in an acidophilic bacterium having the optimum growth pH at 2-3 are thought to be 2-3 and 6-7, respectively. Judging from the data of optimum pHs for the oxidation of reduced sulfur compounds, thiosulfate and tetrathionate were oxidized in the periplasm and sulfur, sulfite and sulfide were oxidized in the cytoplasm. The oxygen consumption rate with sulfite was the highest among them. Although the optimum pH for sulfite oxidation with tetrathionate-grown resting cells of *At. caldus* strain KU cell was reported to be 3[5], the optimum pH for sulfite oxidation with sulfur-grown *At. caldus* strain GO-1 cell was 7 as shown in Table 1. Although a different growth substrate was used in each study, the different optimum pH for the oxidation of sulfite in each strain suggested that the location of the enzyme for the oxidation of sulfite in strain GO-1 cell was different from strain KU cell. Enzyme purification studies will clarify the location of the enzyme. In the case of strain KU, the highest activity for reduced sulfur compounds oxidation with tetrathionate-grown resting cells of strain KU was observed for the oxidation of thiosulfate at pH 3, and the oxygen consumption rates for sulfite and sulfide were in the same range as tetrathionate and sulfur at pH 3[5]. The tetrathionate-oxidizing activity of sulfur-grown cells of strain GO-1 examined in this study was lowest among the activities of reduced sulfur compounds oxidation. The results suggest that the enzyme for the oxidation of tetrathionate is inducible, as reported

Table 1 Optimum pHs and activities of oxidation of reduced sulfur compounds by resting cells of *At. caldus* GO-1

Oxidative substrate	Optimum pH	Activity (μlO_2/min/mg)
$S_2O_3^{2-}$	2	0.9
$S_4O_6^{2-}$	3	0.1
S^0	3-6	0.9-1.1
SO_3^{2-}	7	2.7
S^{2-}	7	0.2

previously[5]. Tetrathionate decomposing enzyme was purified from tetrathionate-grown *Thiobacillus thiooxidans* and *T. ferrooxidans*[7,8]. The enzymes were reported to be located in the periplasm for *T. thiooxidans* and in the membrane for *T. ferrooxidans*, and catalyzed the decomposition of tetrathionate to thiosulfate. The optimum pHs of the enzymes were reported to be both 3. In the case of strain KU cell, tetrathionate was proposed to be transported into the cytoplasm and then oxidized to sulfur. Since the optimum pH for the oxidation of tetrathionate in strain GO -1 cell was 3, tetrathionate seemed to be oxidized in the periplasm in the cell. Although thiosulfate was converted to tetrathionate and the enzyme was inducible in strain KU cell, a relative high thiosulfate-oxidizing activity was observed in strain GO-1 cell.

Effect of uncouplers and inhibitors on oxidation of reduced sulfur compounds

Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) and 2, 4-dinitrophenol (DNP) are known as uncouplers or protonophores. These agents affected the membrane potential of *Thiobacillus acidophilum* grown with glucose as growth substrate[9]. As shown in Table 2, DNP and CCCP strongly inhibited the oxidations of sulfur and sulfite in resting cells of strain GO-1. Since the optimum pH for the oxidation of these compounds was around 7, the results suggested that these compounds must be transported across the membrane before oxidation. DNP and CCCP inhibited tetrathionate oxidizing activity slightly, but did not inhibit thiosulfate-oxidizing activity at all. Twenty μM CCCP reduced the rate of thiosulfate- and tetrathionate-oxidation to 60% and 55%, respectively. *N* - Ethylmaleimide (NEM), known as a thiol-binding agent and inhibitor of sulfur oxidation, strongly inhibited the oxidations of tetrathionate and sulfur. The same results were reported in many other sulfur-oxidizing bacteria[5,10,11]. The rate of thiosulfate oxidation was not inhibited by 0.5 mM NEM, and was reduced to 50% at 2 mM NEM. The oxidation of tetrathionate in strain GO-1 cell was not completely inhibited by CCCP and had optimum pH at 3. Although tetrathionate was oxidized in the cytoplasm of strain KU cell because CCCP strongly inhibited the oxidation of tetrathionate, the results obtained with strain GO-1 cell suggested that the oxidation of tetrathionate seemed to occur in the periplasmic space of strain GO-1 cell. Slight inhibitions of tetrathionate oxidation in the presence of DNP and CCCP seemed to indicate that tetrathionate could be oxidized to sulfur in the periplasm, but sulfur formed could not be oxidized in the cytoplasm in the presence of uncouplers because of the inhibition of sulfur-uptake. Since NEM strongly inhibited the oxidation of tetrathionate, tetrathionate may be decomposed in the periplasm with the following reaction involving thiol groups as a catalytic region; $S_4O_6^{2-} + 1/2O_2 + H_2O \rightarrow 2S + 2SO_4^{2-} + 2H^+$, as described by Masau *et al.*[11]. The oxidation of thiosulfate with strain GO-1 cell was not completely inhibited by either uncouplers or inhibitors and had optimum pH 3. Since strain GO -1 cannot grow in thiosulfate medium at pH 2.0[4], the consumption of oxygen may be due to the increased reactivity of thiosulfate at acidic pH.

Table 2 Effects of uncouplers and inhibitors on the oxidation of reduced sulfur compounds by *At. caldus* GO-1 cell

Substrate	Activity (%)[a]			
	DNP	CCCP	NEM	HQNO
$S_2O_3^{2-}$	100	100	100	100
$S_4O_6^{2-}$	73	65	0	57
S^0	10	20	0	18
SO_3^{2-}	10	18	ND[b]	0
S^{2-}	27	100	93	ND

[a] Activities were measured by oxygen uptake with oxygen electrode, and expressed % activity of the control without inhibitors as 100.

[b] ND; not determined. Following concentrations of inhibitors were used: 2, 4-DNP; 20 μM, CCCP; 5 μM, MEM; 0.5 mM, HQNO; 10 μM.

Strain GO-1 cell grown in sulfur medium had sulfide-oxidizing activity (0.2 μl O_2/min/mg) and optimum pH at 7 as shown in Table 1. For this reason, sulfide was thought to be oxidized in the cytoplasm. DNP reduced the rate of oxidation of sulfide to 27%, but the rate of oxidation was not inhibited by CCCP and NEM as shown in Table 2. Although it was reported that sulfide was incorporated into the cytoplasm and converted to sulfur in strain KU cell, the mechanism of the oxidation of sulfide in strain GO-1 cell was not clear in this study. 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), known as an inhibitor of cytochrome bc_1 and ubiquinol oxidase, strongly inhibited the oxidation of sulfur and sulfite. The results suggested the involvement of cytochrome *b* in the oxidation pathway of these compounds.

Cytochrome composition in strain GO-1 cell

Cytochromes from the plasma membrane of strain GO-1 cell were extracted. Pyridine ferrohemochromes prepared from the extracts showed absorption peaks at 556 nm and 552 nm, as shown in Fig. 1A and Fig. 1B, respectively, indicating the involvement of cytochrome *b* (Fig. 1A) and cytochrome *c* (Fig. 1B) in strain GO-1 cell. The peak for hemes *a* was not clearly observed in the absorption spectra of pyridine ferrohemochromes prepared from the membrane of strain GO-1 cell.

Terminal oxidase in strain GO-1 cell

In various Thiobacilli and other sulfur-oxidizing bacteria, sulfur is oxidized via sulfite as intermediate to sulfate[12–15]. One of the pathways of sulfite oxidation in sulfur-oxidizing bacteria involve the molybdenum-containing sulfite: cytochrome *c* oxidoreductase. The reduced cytochrome *c* is oxidized by using cytochrome *c* oxidase as a terminal oxidase. Alternatively, sulfite can be oxidized via an oxidative adenylsulfate (adenosine-5'-phosphosulfate; APS) pathway in which the substrate is oxidized by an APS reductase with AMP and an electron acceptor. The product APS is used for substrate-level phosphorylation. Recently, sulfite: acceptor oxidoreductase activity was detected in the extremely thermophilic and acidophilic archaeon *Acidianus ambivalens* which did not involve detectable cytochrome *c*, and suggested the involvement of sulfite: quinone oxidoreductase in sulfite oxidation[16]. The reduced quinone can be oxidized by using quinol oxidase as a terminal oxidase. Nogami *et al.* have also suggested the presence of

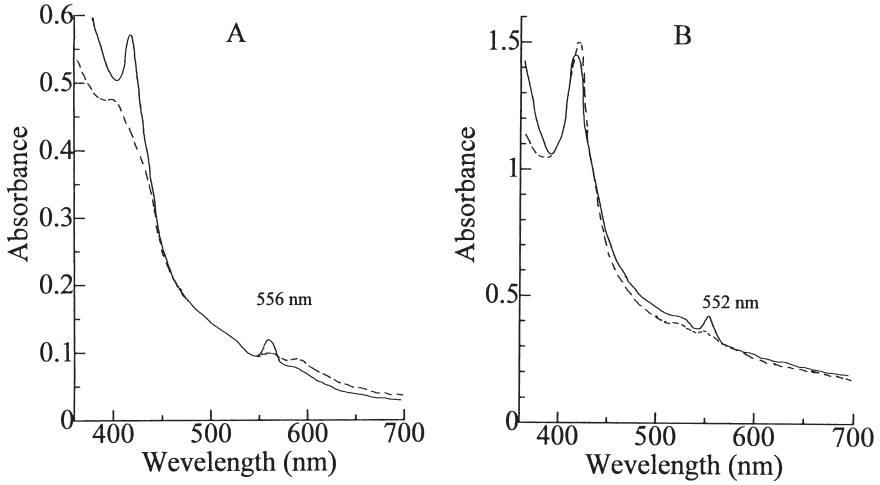

Fig. 1 Absorption spectra of pyridine ferrohemochromes prepared from the membrane fraction of strain GO-1.
(A) Absorption spectra of pyridine ferrohemochrome containing heme *b*, (B) Absorption spectra of pyridine ferrohemochrome containing heme *c*. Solid lines indicate $Na_2S_2O_4$-reduced spectra. Hyphenated lines indicate air-oxidized spectra.

ubiquinol oxidase in *Thiobacillus thiooxidans* strain NB1-3[17]. Kamimura *et al.* have purified ubiquinol oxidase containing cytochrome *b* from *Thiobacillus ferrooxidans* NASF-1 cell grown in iron medium and detected increased activity in the cells grown in sulfur medium[18]. The mechanism of sulfite oxidation has not yet been studied in *At. caldus*. The effect of AMP on the oxidation of sulfite was examined with cell-free extract of strain GO-1 cell. The oxidation of sulfite was not activated by the addition of AMP to the reaction mixture. Thus, APS pathway seemed not to be involved in the oxidation of sulfite in strain GO-1 cell. Since cytochrome *a*, which is a well-known component of cytochrome *c* oxidase, was not clearly detected in strain GO-1 cell, terminal oxidase activities in the cell were measured. Cytochrome *c* oxidase activity measured by using mammalian cytochrome *c* as an electron donor was not detected in the membrane fraction of strain GO-1 cell, while ubiquinol oxidase activity was detected in the membrane fraction of the cell (data not shown). Since strain GO-1 involved cytochrome *c*, endogenous cytochrome *c* may be used as an electron donor for cytochrome *c* oxidase. As described above, cytochrome *a*, a well-known component of cytochrome *c* oxidase was not clearly observed in strain GO-1 cell, cytochrome *b*, known as one of components of ubiquinol oxidase was involved in strain GO-1 cell, and HQNO inhibited the sulfur and sulfite oxidation. These results suggested that ubiquinol oxidase seemed to be involved in sulfite oxidation and function as a terminal oxidase in strain GO-1 cell. The involvement of ubiquinone-cytochrome *b* complex has already been suggested in the transfer of electrons from sulfite to oxygen in *At. caldus* strain KU and *Thiobacillus thiooxidans*[5,11,19].

The following mechanism can be proposed for the oxidation of reduced sulfur compounds in strain GO-1 cell from results obtained in this study; tetrathionate was oxidized in the periplasm and converted to sulfur which was oxidized in the cytoplasm, sulfur was transported into the cytoplasm and oxidized to sulfite, sulfite thus formed was oxidized to sulfate by using sulfite: ubiquinone oxidoreductase, and finally ubiquinol thus formed was oxidized by using ubiquinol oxidase as a terminal oxidase. The detection of intermediates during the oxidation of reduced sulfur compounds and studies of enzyme purification will be needed to clarify the oxidation mechanism proposed in this study.

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硫黄で増殖した *Acidithiobacillus caldus* GO-1株による還元型硫黄化合物の酸化機構

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硫黄で増殖した *Acidithiobacillus caldus* GO-1株の休止菌体を用いて，還元型硫黄化合物の酸化機構を検討した．GO-1株は，チオ硫酸，テトラチオン酸，元素硫黄，亜硫酸及び硫化物の酸化活性を持っており，その最適pHは，それぞれ2，3，3-6，7及び7であった．これらの酸化活性の中で，亜硫酸の酸化速度が最も高かった．阻害剤やアンカップラーを用いて，還元型硫黄化合物の酸化への影響を調べた結果，DNPとCCCPによる元素硫黄及び亜硫酸酸化活性の強い阻害，NEMによるテトラチオン酸及び硫黄酸化活性の強い阻害，およびHQNOによる元素硫黄及び亜硫酸酸化活性の阻害が観察された．これらの結果は，テトラチオン酸がペリプラズマで，硫黄及び亜流酸が細胞質で，それぞれ酸化されることを示唆した．細胞膜からピリジンフェロヘモクロームを調製して，シトクロームの構成成分を検討した結果，ヘム *b* 及び *c* が検出された．また，末端酸化酵素の活性を検討した結果，ユビキノール酸化酵素の活性は検出されたが，哺乳類のシトクロームを用いて測定したシトクローム *c* 酸化酵素の活性は検出されなかった．これらの結果に基づいて，*At. caldus* GO-1株の還元型硫黄化合物の酸化機構を考察した．