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Existence of aa_3-Type Ubiquinol Oxidase as a Terminal Oxidase in Sulfite Oxidation of *Acidithiobacillus thiooxidans*

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It was found that *Acidithiobacillus thiooxidans* has sulfite:ubiquinone oxidoreductase and ubiquinol oxidase activities in the cells. Ubiquinol oxidase was purified from plasma membranes of strain NB1-3 in a nearly homogeneous state. A purified enzyme showed absorption peaks at 419 and 595 nm in the oxidized form and at 442 and 605 nm in the reduced form. Pyridine ferrohaemochrome prepared from the enzyme showed an α-peak characteristic of haem *a* at 587 nm, indicating that the enzyme contains haem *a* as a component. The CO difference spectrum of ubiquinol oxidase showed two peaks at 428 nm and 595 nm, and a trough at 446 nm, suggesting the existence of an aa_3-type cytochrome in the enzyme. Ubiquinol oxidase was composed of three subunits with apparent molecular masses of 57 kDa, 34 kDa, and 23 kDa. The optimum pH and temperature for ubiquinol oxidation were pH 6.0 and 30 °C. The activity was completely inhibited by sodium cyanide at 1.0 mM. In contrast, the activity was inhibited weakly by antimycin A_1 and myxothiazol, which are inhibitors of mitochondrial bc_1 complex. Quinone analog 2-heptyl-4-hydoroxyquinoline *N*-oxide (HOQNO) strongly inhibited ubiquinol oxidase activity. Nickel and tungstate (0.1 mM), which are used as a bacteriostatic agent for *A. thiooxidans*-dependent concrete corrosion, inhibited ubiquinol oxidase activity 100 and 70% respectively.

Key words: *Acidithiobacillus thiooxidans*; ubiquinol oxidase; sulfite oxidation; terminal oxidase; concrete corrosion

The sulfur-oxidizing bacterium *Acidithiobacillus thiooxidans*[1] (formerly *Thiobacillus thiooxidans*) is an acidophilic chemolithotrophic bacterium that derives energy for growth from the oxidation of inorganic reduced sulfur compounds. *A. thiooxidans* and the iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* are known as the most important microorganisms for the bacterial leaching of sulfide ores.[2–5] *A. thiooxidans* is also known as one of the main agents responsible for microbial concrete corrosion.[6–19] The sulfuric acid produced in sewer pipes by *A. thiooxidans*-dependent hydrogen sulfide oxidation reacts with the components of concrete such as calcium oxides and calcium hydroxide to form a sandy calcium sulfate (gypsum). Therefore, it is desirable that sulfur oxidation by *A. thiooxidans* cells should be activated at the practical bacterial leaching sites, but rather inhibited at the surface of concrete structure of sewer pipe. To activate or repress the activity of *A. thiooxidans*, it is important to clarify the enzymes involved in sulfur oxidation of *A. thiooxidans*. The oxidation of elemental sulfur by *A. thiooxidans* is a complex process involving the contact of cells with sulfur particles, the oxidation of sulfur to sulfite, and the oxidation of sulfite to sulfate. The first step of sulfur oxidation from elemental sulfur to sulfite is catalyzed by sulfur dioxygenase.[20–22] Sulfite is considered to be the key intermediate in the oxidation of inorganic sulfur compounds in thiobacilli.[22–27] Sulfur is nearly quantitatively oxidized to sulfite by *A. thiooxidans* cells when the sulfite oxidation is inhibited by 2-*n*-heptyl-4-hydroxyquinoline N-oxide (HOQNO).[28] Many studies have been done on the enzymes involved in the bacterial oxidation of inorganic sulfur compounds since *A. thiooxidans* was first isolated and characterized by Waksman and Joffe.[29] Sulfur dioxygenase that oxidizes elemental sulfur to thiosulfate in the presence of catalytic quantities of reduced glutathione was purified approximately 12 fold from cell extract of *A. thiooxidans*.[20,21] The partially purified enzyme catalyzed the incorporation of ^{18}O into thiosulfate through a

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direct oxygenation reaction,[21] but, it was found later that the initial product of sulfur oxidation by the enzymes from *T. thiooxidans* and *T. thioparus* was sulfite, but not thiosulfate.[22] Recently we purified sulfur dioxygenase 26.4-fold from a $105{,}000 \times g$ soluble fraction of *A. thiooxidans* NB1-3. The enzyme purified from NB1-3 is a monomer enzyme with a molecular mass of 19.5 kDa.[30] The sulfite produced by sulfur dioxygenase is further oxidized to sulfate either through sulfite:cytochrome *c* oxidoreductase,[31] or through adenosine phosphosulfate (APS) reductase.[32] Sulfite dehydrogenase was purified from *A. thiooxidans* JCM 7814.[33,34]

Although a great many studies have been done on the enzymes involved in sulfur oxidation of acidophilic chemolithotrophic sulfur-oxidizing bacteria, no information has been available on the terminal oxidase in sulfur- and/or sulfite-oxidation of *A. thiooxidans*. In this study we found the first evidence that aa_3-type ubiquinol oxidase plays a role as a terminal oxidase in sulfite oxidation of *Acidithiobacillus thiooxidans*.

Materials and Methods

Microorganisms, medium, and condition of cultivation. The sulfur-oxidizing bacterial strains used were *Acidithiobacillus thiooxidans* NB1-3,[14] *A. thiooxidans* strains IFO 13724 and13701, and *A. thiooxidans* ON 106.[35] The sulfur-salt medium (pH 3.0) used for the cultivation of *A. thiooxidans* contained elemental sulfur (1.0%), $(NH_4)_2SO_4$ (3.0%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.01%), and $Ca(NO_3)_2$ (0.001%). The method for the large-scale production of cells was described previously.[25] The culture medium from six 10-liter carboys was filtered with Toyo filter paper (no. 2) to remove the particles of elemental sulfur, and centrifuged at $15{,}000 \times g$ to gather cells. Harvested cells were washed three times with 50 mM MOPS–NaOH buffer (pH 7.0) and suspended in the same buffer. The washed cells obtained was used in the experiment.

Ubiquinol-2. Ubiquinone-2 (Q_2) was kindly supplied by Eizai (Tokyo), and ubiquinol, the reduced form of ubiquinone-2 (Q_2H_2), was prepared as described by Riske.[36]

Ubiquinol oxidase activity. Ubiquinol oxidase activity was measured spectrophotometrically at 30 °C by following the increase in absorbance at 275 nm. The reaction mixture (1.0 ml) contained 50 mM MES–NaOH (pH 6.0), 30 μM Q_2H_2, 0.02% Tween 20, and 10 μg of membranes. The reaction mixture without 30 μM Q_2H_2 was preincubated for 1 min at 30 °C. The reaction was started by adding 10 μl of 3 mM Q_2H_2. A molecular extinction coefficient of 12.25 $mM^{-1}\,cm^{-1}$ at 275 nm was used for the quinone form (Q_2). One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 μmol quinone in one min at 30 °C. Absorption spectrum was measured with a Shimazu UV-1200 spectrophotometer (Kyoto, Japan).

Sulfite-ubiquinone oxidoreductase activity. Sulfite-ubiquinone oxidoreductase activity was assayed spectrophotometrically at 30 °C by monitoring the reduction of Q_2 at 275 nm. An assay mixture (980 μl) containing 50 mM MES–NaOH (pH 8.5), 0.02% Tween 20, 1 mM KCN, 30 μM Q_2H_2, and 50 μg of cytosol or 10 μg membranes was preincubated for 10 min at 30 °C. The reaction was started by adding 10 μl of 3 mM Q_2 and 10 μl of 200 mM $NaHSO_3$.

Preparation of pyridine ferrohemochrome. Cytochrome aa_3 was extracted from a purified ubiquinol oxidase (9 μg) with 1 ml of cold chloroform–methanol (2:1), 1 ml of cold acetone, and then tree times with 0.5 ml cold acetone–0.024 N hydrochloric acid.[37] The acetone was evaporated to near dryness. The residue was suspended in 50 μl of pyridine and 50 μl of 0.2 N potassium hydroxide.

Purification of ubiquinol oxidase. All steps of the purification process were done at 4 °C. Resting cells of *A. thiooxidans* NB1-3 suspended in 50 mM MOPS–NaOH buffer (pH 7.0) were disrupted by passage three times through a French pressure cell at 1,500 kg/cm^2, and centrifuged at $12{,}000 \times g$ for 10 min. The supernatant (cell extract) was further centrifuged at $105{,}000 \times g$ for 60 min to obtain plasma membranes. The plasma membranes (1,022 mg of protein) was suspended in 50 mM MOPS–NaOH buffer (pH 7.0) at concentration of 10 mg protein/ml, and incubated in buffer containing 0.5% *n*-dodecyl-β-D-maltopyranoside (DM) for 1 h to extract ubiquinol oxidase from the membranes. After enzyme extraction, the solution was centrifuged at $105{,}000 \times g$ for 60 min to obtain a soluble fraction (0.5% DM extract). The 0.5% DM extract was equilibrated with 50 mM MOPS–NaOH buffer (pH 7.0) containing 0.1% DM, concentrated, and then applied to a DEAE-Toyopearl 650M column (1 × 10 cm) equilibrated with 50 mM MOPS–NaOH buffer (pH 7.0) containing 0.1% DM. The enzyme, bound to a DEAE-Toyopearl 650M column, was eluted with 50 mM MOPS–NaOH buffer (pH 7.0) containing 50 mM sodium sulfate. The enzyme fraction from DEAE-Toyopearl 650M column chromatography was concentrated and applied to a Phenyl-Toyopearl 650M column (1 × 10 cm) equilibrated with 50 mM MOPS–NaOH buffer (pH 7.0) containing 0.1% DM and 400 mM sodium sulfate. Ubiquinol oxidase activity was eluted with 50 mM MOPS–NaOH buffer (pH 7.0) containing 400 mM sodium sulfate. The enzyme fraction after Phenyl-Toyopearl 650M column chromatography was applied to a Butyl-Toyopearl 650M column equilibrated with a 50 mM MOPS buffer (pH 7.0) containing 0.1% DM and 200 mM sodium sulfate. Ubiquinol oxidase was eluted with a buffer

containing 200 mM sodium sulfate. The enzyme fraction obtained after Butyl-Toyopearl 650M column chromatography were concentrated and applied to a TSKgel G3000SW column (7.5 mm × 60 cm) equilibrated with a 50 mM MOPS–NaOH buffer (pH 7.0) containing 0.05% DM and 50 mM sodium sulfate. Ubiquinol oxidase activity was eluted with the same buffer at a flow rate of 0.5 ml/min at room temperature. The peak having enzyme activity was collected and concentrated and used to determinine subunit structure and enzyme properties.

Protein measurement. Protein concentration was measured by the Lowry method,[38] with crystalline bovine serum albumin as the reference protein.

Results

Existence of sulfite:ubiquinone oxidoreductase and ubiquinol oxidase activities in A. thiooxidans cells

It is known that elemental sulfur is oxidized at first by sulfur dioxygenase to produce sulfite, and that the sulfide thus produced is next oxidized by the sulfite oxidase enzyme system to give sulfate in *A. thiooxidans*, but no information has been available on the terminal oxidase in sulfur- and/or sulfite-oxidation of *A. thiooxidans*. We first investigated whether the cytosol or plasma membranes prepared from *A. thiooxidans* NB1-3 cells has sulfite:ubiquinone oxidoreductase activity. Since ubiquinone (Q_2) has a characteristic absorption peak at 275 nm and ubiquinol does not have a peak at 275 nm, sulfite:ubiquinone oxidoreductase activity was measured by the decrease in absorbance at 275 nm. When plasma membranes was used as an enzyme source, absorbance at 275 nm decreased rapidly with time. In contrast, absorbance at 275 nm did not decrease in the reaction mixture without enzyme or with cytosol. These results indicate that sulfite:ubiquinone oxidoreductase activity (58.5 mU/mg) is present in plasma membranes of *A. thiooxidans* NB1-3 (Fig. 1). Since the reaction product of sulfite oxidation by sulfite:ubiquinone oxidoreductase is ubiquinol (Q_2H_2), the results obtained as above strongly suggest that ubiquinol is next oxidized by ubiquinol oxidase in *A. thiooxidans* cells. Hence ubiquinol oxidase activity was measured spectrophotometrically using cytosol and plasma membranes of strain NB1-3 as an enzyme source. The ubiquinol oxidase activities of the cytoplasm and plasma membranes from strain NB1-3 were 0.1 and 0.83 U/mg respectively (Fig. 2), indicating that ubiquinol oxidase is located in the plasma membranes of *A. thiooxidans* cells. The ubiquinol oxidase activities of plasma membranes from *A. thiooxidans* strains NB1-3, ON 106, IFO 13701, and IFO 13724 were 0.83, 0.07, 0.78, and 0.36 unit/mg protein respectively, indicating that all of the *A. thiooxidans* strains tested have the activity. Strain NB1-3 had the highest ubiquinol oxidase activity among the *A. thiooxidans* strains tested.

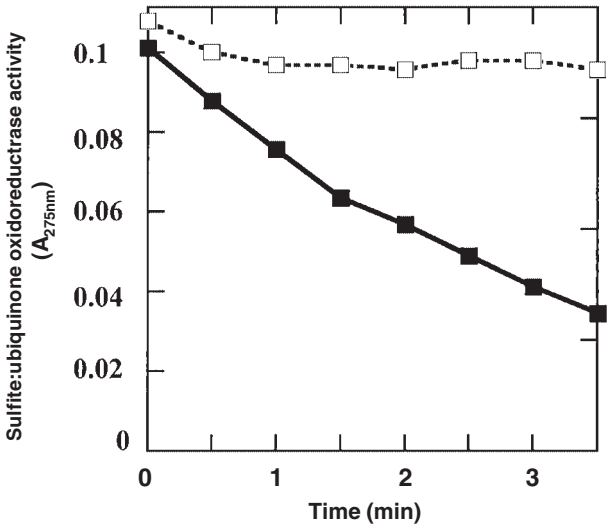

Fig. 1. Sulfite:Ubiquinone Oxidoreductase Activities of Plasma Membranes and the Cytoplasm from *A. thiooxidans* NB1-3.
The enzyme activity was measured as described in "Materials and Methods." Symbols: ■, plasma membranes (50 μg of protein); □, cytoplasm (50 μg of protein).

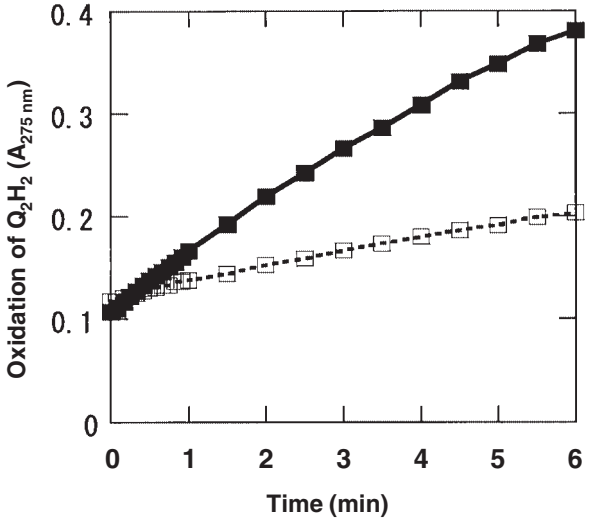

Fig. 2. Ubiquinol-Oxidizing Activities of Plasma Membranes and the Cytoplasm from *A. thiooxidans* NB1-3.
The enzyme activity was measured as described in "Materials and Methods." Symbols: ■, plasma membranes (30 μg of protein); □, cytoplasm (30 μg of protein).

Purification of ubiquinol oxidase

The results of a typical purification are summarized in Table 1. The procedure resulted in 11.5-fold purification over plasma membranes of *A. thiooxidans* NB1-3 with a yield of 0.3%. The enzyme solution obtained by TSKgel G3000W chromatography was nearly homogeneous as estimated by native PAGE (Fig. 3A). Only one faint band was observed under a main protein band on native PAGE gel. The subunit structure of ubiquinol oxidase was analyzed by 12.5% SDS–PAGE (Fig. 3B). Ubiquinol oxidase was composed of three subunits with

Table 1. Summary of Purification Procedure of Ubiquinol Oxidase from the Plasma Membrane of *A. thiooxidans* NB1-3

Step	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Plasma membrane	1,022	848	0.83	1.0	100
Solubilization with 0.5% DM	278	578	2.10	2.5	68.2
DEAE-Toyopearl 650M	108	449	4.18	5.0	53
Phenyl-Toyopearl 650M	65	433	6.78	8.1	51
Butyl-Toyopearl 650M	46	280	6.14	7.4	33
TSKgel G3000SW	0.3	2.7	9.54	11.5	0.3

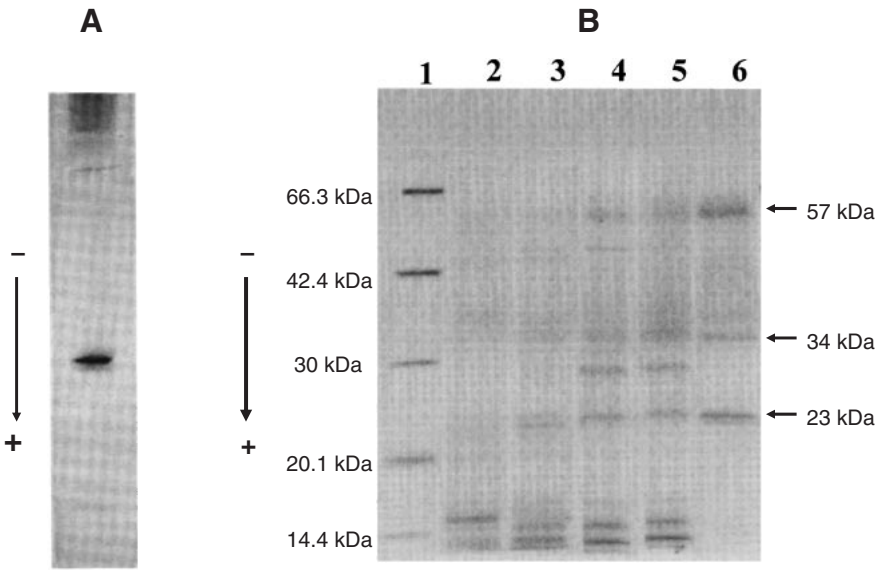

Fig. 3. Native PAGE (A) and SDS–PAGE (B) of a Purified Ubiquinol Oxidase.

A, Disc gel electrophoresis was carried out in 5.0% polyacrylamide gel containing 0.05% DM. A ubiquinol oxidase from TSK gel G3000SW column chromatography (30 µg protein) was applied on the column. The gel was stained with Coomassie Brilliant Blue. B, After SDS–PAGE on a 12.5% polyacrylamide gel, subunits of ubiquinol oxidase were revealed by silver staining. Lane 1, marker proteins of Lysozyme (Mr 14,400), trypsin inhibitor (Mr 20,100), carbonic anhydrase (Mr 30,000), aldolase (Mr 42,400), bovine serum albumin (Mr 66,267), and phosphorylase b (Mr 97,400); lane 2, 0.5% DM extract; lane 3, DEAE-Toyopearl chromatography; lane 4, Phenyl-Toyopearl chromatography; lane 5, Butyl-Toyopearl chromatography; lane 6, TSKgel G3000SW. Each of the enzyme samples applied to a column was 2.5 µg protein.

apparent molecular masses of 57 kDa, 34 kDa, and 23 kDa.

Spectral properties of ubiquinol oxidase

The purified enzyme showed absorption peaks at 419 and 595 nm in the oxidized form and at 442 and 605 nm in the reduced form (Fig. 4). Pyridine ferrohaemochrome prepared from the enzyme showed an α-peak characteristic of haem *a* at 587 nm, indicating that the enzyme contains haem *a* as a component (Fig. 5). The CO difference spectrum of ubiquinol oxidase showed two peaks at 428 nm and 595 nm, and a trough at 446 nm, suggesting that aa_3-type cytochrome is present in the enzyme[39] (Fig. 6).

Enzymatic properties of ubiquinol oxidase

The substrate specificity of ubiquinol oxidase was studied. The purified enzyme used not only ubiquinol (3.13 U/mg) but also 2,3,5,6-tetramethyl-*p*-phenylenediamine (TMPD) (0.45 U/mg) as a substrate, but it did not use reduced mammalian cytochrome *c* as an electron donor (data not shown). The optimum pH for ubiquinol oxidation was pH 6.0 (Fig. 7A). Almost all of the activity was lost when measured at pH below 5.5 and above 8.0. The enzyme was stable between pH 6.0 and 8.0 when incubated in 0.1 M MES–NaOH buffer and 0.1 M sodium phosphate buffer for 60 min, but quite unstable in 0.1 glycine-NaOH buffer with pH above 10 and in 0.1 M sodium citrate buffer with pH below 4.0 (data not shown). The enzyme was stable when incubated in 0.1 M MES–NaOH buffer (pH 6.0) and 0.1 M sodium phosphate buffer (pH 7.0) for 60 min, but quite unstable in 0.1 glycine–NaOH buffer with pH above 10 and in 0.1 M sodium citrate buffer with pH below 4.0 (data not shown). The optimum temperature for ubiquinol oxidation was 30 °C (Fig. 7B). Almost all of the activity was lost when it was measured at temperatures above 40 °C. The enzyme was quite

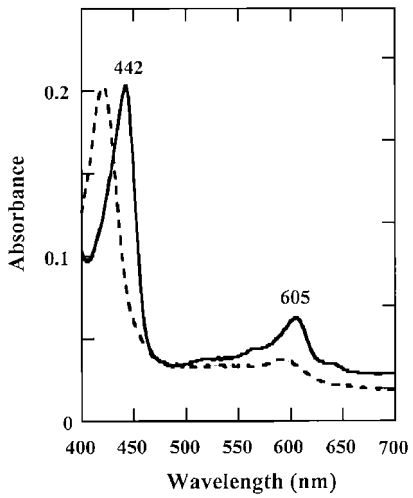

Fig. 4. Absorption Spectra of a Purified Ubiquinol Oxidase from *A. thiooxidans* NB1-3.

The absorption spectra of ubiquinol oxidase were recorded with a Beckman DU65 spectrophotometer. The sample cuvette contained 9.9 μg of a purified enzyme in 100 μl of 50 mM MOPS–NaOH buffer (pH 7.0) containing 0.05% DM and 50 mM sodium sulfate. Symbols: —, enzyme reduced with hydrosulfite; - - -, enzyme in air-oxidized form.

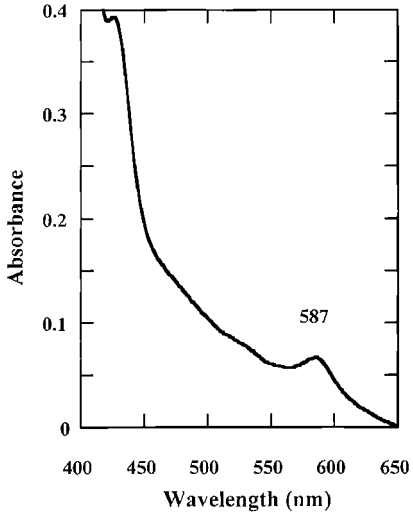

Fig. 5. Pyridine Ferrohemochrome of a Purified Ubiquinol Oxidase from *A. thiooxidans* NB1-3.

Pyridine ferrohemochrome was prepared from 9 μg of a purified enzyme by the method described in "Materials and Methods." The absorption spectrum of pyridine ferrohemochrome was recorded with a Beckman DU65 spectrophotometer.

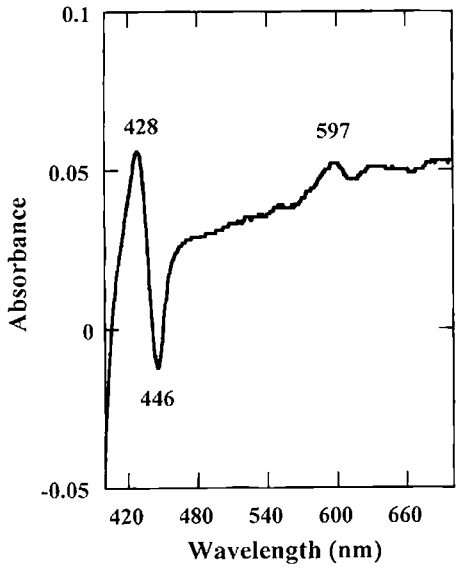

Fig. 6. CO Difference Spectrum of a Purified Ubiquinol Oxidase from *A. thiooxidans* NB1-3.

CO-reduced minus reduced difference spectrum was recorded with a Beckman DU65 spectrophotometer. The sample cuvette contained 9.9 μg of purified enzyme in 100 μl of 50 mM MOPS–NaOH buffer (pH 7.0), containing 0.05% DM and 50 mM sodium sulfate. The enzyme solution was reduced with hydrosulfite, and then CO gas was bubbled in the solution for 1 min.

Table 2. Effects of Metabolic Inhibitors on Ubiquinol Oxidase Activity

Inhibitor	Concentration	Remaining activity (%)
None	—	100
KCN	1.0 μM	76
	10.0 μM	30
	1.0 mM	0
NaN_3	1.0 mM	57
	5.0 mM	32
	10.0 mM	19
Antimycin A_1	1.0 μM	82
	10.0 μM	73
Myxothiazol	1.0 μM	79
	10.0 μM	61
HOQNO	1.0 μM	43
	5.0 μM	39
	10.0 μM	30

unstable when it was incubated in 0.1 M MES–NaOH buffer (pH 6.0) for 30 min at temperatures above 40 °C (data not shown).

The effects of respiratory inhibitors on ubiquinol oxidase activity were studied. KCN, well known inhibitor of cytochrome *c* oxidase activity, completely inhibited ubiquinol oxidase activity at 1.0 mM (Table 2). In contrast, the activity was inhibited weakly by antimycin A_1 and myxothiazol, inhibitors of the mitochondrial bc_1 complex, but a quinone analog, 2-heptyl-4-hydroxy-quinoline *N*-oxide (HOQNO), inhibited ubiquinol oxidase activity strongly. The effects of heavy metals on ubiquinol oxidase activity were also studied. Ag^+, Hg^{2+}, Cd^{2+} and Ni^+, completely inhibited activity at 0.1 mM (Table 3), but manganese and magnesium ions did not affect it. Chromium ions activated the enzyme activity 1.5-fold.

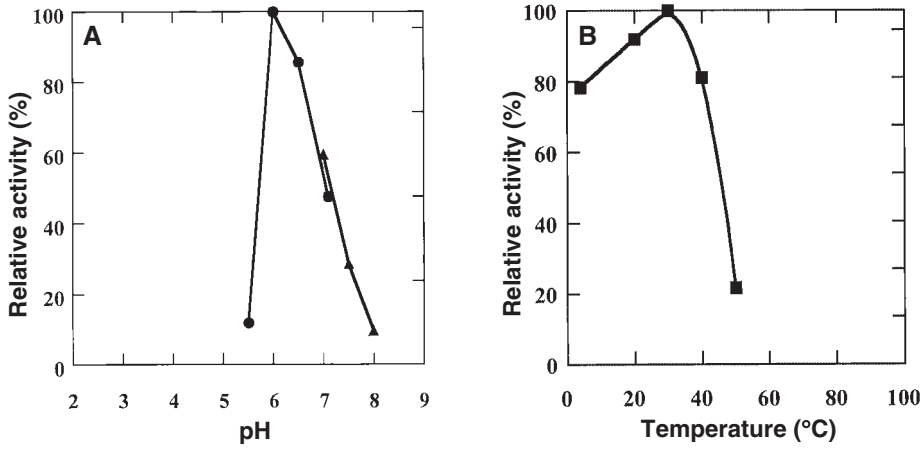

Fig. 7. Effects of pH (A) and Temperature (B) on the Activity of Ubiquinol Oxidase.
A, The activity was measured in 50 mM of MES–NaOH buffer (●) or HEPES–NaOH buffer (▲) at 30 °C. B, The activity was measured in 50 mM of MES–NaOH buffer (pH 6.0). The highest activity was designated 100%.

Table 3. Effects of Heavy Metals on Ubiquinol Oxidase Activity

Heavy metal	Concentration (mM)	Remaining activity (%)
None	—	100
$AgNO_3$	0.1	0
$HgCl_2$	0.1	0
$CdCl_2$	0.1	0
$ZnSO_4$	0.1	29
$CuSO_4$	0.01	42
$NiSO_4$	0.1	0
$CoSO_4$	1.0	76
$MnSO_4$	1.0	100
$MgSO_4$	1.0	117
$CrCl_3$	1.0	151
Na_2WO_4	0.1	30
Na_2MoO_4	0.1	55

Discussion

It is known that there are two pathways for the oxidation of sulfite in thiobacilli. One mechanism, proposed by Peck, involves the formation of adenine 5′-sulphatophosphate (APS) from AMP and sulfite.[40] The APS produced by APS reductase is subsequently converted to ATP with a concomitant regeneration of AMP by the combined action of ADP-sulfurylase and adenylate kinase. The other mechanism, proposed by Charles and Suzuki, does not require AMP and is catalyzed by sulfite oxidase with ferricyanide or cytochrome *c* as an electron accepor.[31,41] Nakamura *et al.* reported that membranes prepared from *Thiobacillus thiooxidans* JCM 7814 contain cytochromes *a*, *b*, and *c* and show sulfite dehydrogenase activity.[33,34] Most of the activities of sulfite oxidation are in the membranes of the bacterium, and the addition of AMP to the reaction mixture neither affects the sulfite oxidation activity nor produces APS.[33] From these results, they concluded that *T. thiooxidans* JCM 7814 has only the AMP-independent pathway. However, although a great many studies have been done on the enzymes involved in the sulfur- and sulfite-oxidation of *A. thiooxidans*, no information is available on an actual terminal oxidase in the sulfur- and sulfite-oxidation of *A. thiooxidans*. Ubiquinol oxidase was first purified in a nearly homogeneous state from plasma membranes of *A. thiooxidans* strain NB1-3 and characterized. The results obtained from pyridine ferrohaemochrome and the CO difference spectrum of a purified ubiquinol oxidase strongly suggest the existence of an aa_3-type cytochrome in the enzyme. We have purified iron oxidase from the moderately thermophilic iron-oxidizing bacterium strain TI-1, which contains an aa_3-type cytochrome.[42] Strong inhibition of ubiquinol oxidase activity by a low concentration of sodium cyanide (1.0 mM), but not by antimycin A_1 or myxothiazol, also support the involvement of an aa_3-type cytochrome in the enzyme activity.[42] The finding that *A. thiooxidans* NB1-3 has sulfite:ubiquinone oxidoreductase activity indicates that the bacterium uses ubiquinol oxidase as a terminal oxidase in the oxidation of elemental sulfur as well as the oxidation of sulfite, an intermediate during elemental sulfur oxidation of this bacterium.

We have shown that concrete supplemented with nickel and/or tungsten is resistant to corrosion,[13,43] and that nickel and tungsten bound to *A. thiooxidans* NB1-3 cells inhibit both sulfur dioxygenase and sulfite oxidase, and as a result, inhibit cell growth.[6,14,15,43] We prepared portland cement bars (40 × 40 × 160 mm) supplemented with various kinds of inorganic and organic compounds, and these bars were hung from the lid of a sludge tank manhole and exposed to a 5–10 ppm hydrogen sulfide atmosphere.[13] After 2 years of exposure, it was found that the surface of the portland cement bar supplemented with nickel was especially smooth, while that without nickel was brittle and rough,[13] suggesting the possibility that nickel can be used as a bacteriostatic agent for the prevention of concrete corrosion. Recently, we showed that when

portland cement bars supplemented with 0.075% metal nickel or with 0.075% metal nickel and 0.075% calcium tungstate were exposed to the atmosphere of a sewage treatment plant containing 28 ppm of H_2S for 2 years, the weight loss of the portland cement bar with metal nickel and calcium tungstate was much lower than that of the cement bar containing 0.075% metal nickel.[43] Both nickel and tungsten are unique as bacteriostatic agents for concrete corrosion, because the former tightly binds to *A. thiooxidans* cells at neutral and slightly alkaline pH, but the latter only at acidic pH below 3.0, and both can inhibit growth of the bacterium markedly at the pH at which these metal ions bind to the cells. However, the precise site of nickel- and tungsten-inhibition on the elemental sulfur oxidation enzyme system of *A. thiooxidans* remains unsolved. From this, the findings that low concentrations of nickel and tungstate (0.1 mM) inhibited ubiquinol oxidase activity 100 and 70% respectively are interesting and important, because these inhibition studies indicate that one of the sites of nickel- and tungstate-inhibition in elemental sulfur oxidation enzyme system is ubiquinol oxidase, a terminal oxidase in sulfite oxidation enzyme system of *A. thiooxidans*.

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