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Kazuo Kamimura · Emi Higashino · Tadayoshi Kanao
Tsuyoshi Sugio

Effects of inhibitors and NaCl on the oxidation of reduced inorganic sulfur compounds by a marine acidophilic, sulfur-oxidizing bacterium, *Acidithiobacillus thiooxidans* strain SH

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Abstract The effect of NaCl and the pathways of the oxidation of reduced inorganic sulfur compounds were studied using resting cells and cell-free extracts of *Acidithiobacillus thiooxidans* strain SH. This isolate specifically requires NaCl for growth. The oxidation of sulfur and sulfite by resting cells was strongly inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide. Carbonylcyanide *m*-chlorophenyl-hydrazone and monensin were also relatively strong inhibitors. Thiosulfate-oxidizing activity was not inhibited by these uncouplers. Valinomycin did not inhibit the oxidation of sulfur compounds. NaCl stimulated the sulfur- and sulfite-oxidizing activities in resting cells but not in cell-free extracts. The tetrathionate-oxidizing activity in resting cells was slightly stimulated by NaCl, whereas it did not influence the thiosulfate-oxidizing activity. Sulfide oxidation was biphasic, suggesting the formation of intermediate sulfur. The initial phase of sulfide oxidation was not affected by NaCl, whereas the subsequent oxidation of sulfur in the second phase was Na^+-dependent. A model is proposed for the role of NaCl in the metabolism of reduced sulfur compounds in *A. thiooxidans* strain SH.

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K. Kamimura (✉)
Department of Biological Function,
Faculty of Agriculture, Okayama University,
1-1-1 Tsushima-Naka, Okayama 700-8530, Japan
E-mail: kamimura@cc.okayama-u.ac.jp
Tel.: +81-86-251-8305
Fax: +81-86-251-8388

E. Higashino · T. Sugio
Division of Science and Technology for Energy Conversion,
Graduate School of Natural Science and Technology,
Okayama University, 3-1-1 Tsushima-Naka,
Okayama 700-8530, Japan

T. Kanao
Department of Instrumental Analysis,
Advanced Science Research Center,
Okayama University, 3-1-1 Tsushima-Naka,
Okayama 700-8530, Japan

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Introduction

Acidophilic chemolithotrophic sulfur-oxidizers are generally soil, freshwater, or acid mine water bacteria, and their ability to live in saline environments is limited. Some exceptional acidophilic isolates that require or are tolerant to NaCl include the sulfur-oxidizer *Thiobacillus prosperus* (Huber and Stetter 1989), the iron-oxidizing bacterium KU2-11 (Kamimura et al. 2001a), and the moderately halophilic *Acidithiobacillus thiooxidans* strain SH (Kamimura et al. 2003). *A. thiooxidans* strain SH is a marine isolate and grows optimally in sulfur medium in the presence of 2% NaCl. NaCl stimulates the sulfur- and sulfite-oxidizing activities in resting cells. KCl and LiCl also stimulate, but they do not replace the NaCl requirement for growth.

Some marine heterotrophic bacteria require NaCl for optimum growth and use a Na^+ gradient across the membrane for the uptake of substrates and the rotation of flagella. For example, a Na^+ gradient is generated by the NADH:quinone oxidoreductase functioning as an efflux pump of Na^+ in *Vibrio alginolyticus* (Unemoto and Hayashi 1993; Ventosa et al. 1998). Thus, in bacteria that require NaCl for growth, Na^+ gradients are generated across the membrane during active metabolism of substrates. As *A. thiooxidans* SH specifically requires NaCl for growth, it is hypothesized that a Na^+ gradient is generated and used for physiological functions of the cell. Therefore, a Na^+ gradient across the membrane may be generated during the oxidation of reduced sulfur compounds. The purpose of this study was to examine the oxidation of reduced inorganic sulfur compounds in *A. thiooxidans* SH, with emphasis on enzymes that specifically require NaCl for their activity. Several inhibitors were also tested in order to charac-

terize the respiration and electron transport in *A. thiooxidans* SH.

Materials and methods

Bacterium and growth conditions

Acidithiobacillus thiooxidans strain SH was grown under static culture conditions at 30°C in sulfur medium containing (w/v) 1% sulfur, 0.3% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.01% KCl, 0.001% $Ca(NO_3)_2$, and 2% NaCl. The pH of medium was adjusted to 4.0 with 1 N sulfuric acid.

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

For harvest, cultures were filtered (cellulose paper) to remove sulfur particles. Cells were harvested by centrifugation, washed three times with 0.1 M β-alanine-SO_4 buffer (pH 3.0), and resuspended in the buffer for resting cell experiments. For cell-free extracts, cells were resuspended 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 cell three times at 1,500 kg/cm^2. The homogenate was centrifuged at 12,000 *g* for 15 min and the supernatant (S_{12}) was used as a cell-free extract. The membrane fraction (P_{105}) was obtained from S_{12} by further centrifugation at 105,000 *g* for 60 min and resuspended in 0.1 M Tris-HCl buffer (pH 7.5).

Oxidation of reduced sulfur compounds

The oxidation of elemental sulfur, sulfite, thiosulfate, tetrathionate, and sulfide was measured as oxygen uptake with a Biological Oxygen Monitor (Yellow Spring Instrument, Yellow Springs, Ohio, USA). The reaction mixture contained 0.1 M buffer, resting cells (0.5–1 mg of protein) or S_{12} (0.2–1 mg of protein), and 32 mg of elemental sulfur, 0.3 mM sulfite, 5 mM thiosulfate, 200 μM tetrathionate, or 200 μM sulfide in a total volume of 3 ml. The optimum pH values for oxygen uptake activity by resting cells or S_{12} were determined in separate experiments with each sulfur compound. For sulfur-, sulfite-, and sulfide-oxidizing activities, cells were resuspended in 0.1 M β-alanine-SO_4 buffer (pH 4.0). For thiosulfate- and tetrathionate-oxidizing activities in resting cells, β-alanine-SO_4 buffers at pH 2.5 and pH 2.0 were used, respectively. For sulfur- and sulfite-oxidizing activities in S_{12}, MES buffer (pH 7.0) was used. The sulfur-oxidizing activity in S_{12} was tested with colloidal sulfur (Sigma-Aldrich Japan, Osaka, Japan). The thiosulfate-oxidizing activity in S_{12} was tested in β-alanine-SO_4 buffer (pH 3.0). Cells or S_{12} were pre-incubated at 30°C for 10 min, and the reaction was started by adding the test sulfur compound to the reaction mixture. Cells were pre-incubated for 3 min with inhibitors [cyanide, antimycin A, and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO)] or uncouplers [carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP), monensin and valinomycin] before starting the reaction and measuring the substrate-dependent oxygen uptake rates. Reference experiments with boiled cells and S_{12} were carried out to account for purely chemical reactions. The biological oxidation activities were obtained by subtracting the control value.

Enzyme assays

The activity of cytochrome *c*:acceptor oxidoreductase was measured in 10 mM sodium phosphate buffer (pH 5.5) at 30°C as the decrease in absorbance at 550 nm due to the oxidation of reduced mammalian cytochrome *c* (from horse heart, Sigma Chemical, St. Louis, Mo., USA) in a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan) as previously described (Iwahori et al. 1998). The *N,N,N′*,N′-tetramethyl-*p*-phenylendiamine (TMPD) dehydrogenase activity was measured at 30°C as the increase in absorbance at 560 nm. The reaction mixture contained 50 mM MES buffer (pH 5.5), S_{12} (0.5 mg protein), and 10 mM TMPD in a total volume of 1 ml. One unit of TMPD dehydrogenase activity was defined as the amount of enzyme that oxidized 1 μmol TMPD in 1 min at 30°C. The activity of ubiquinol:acceptor oxidoreductase was measured in 0.1 M MES buffer (pH 5.5) at 30°C as the increase in absorbance at 275 nm due to the oxidation of ubiquinol (Q_2H_2) as previously described (Kamimura et al. 2001b). One unit of ubiquinol:acceptor oxidoreductase activity was defined as the amount of enzyme that oxidized 1 μmol Q_2H_2 in 1 min at 30°C. Sulfite dehydrogenase and thiosulfate dehydrogenase activities were measured spectrophotometrically at 30°C as the reduction of ferricyanide, according to the method previously described (Nakamura et al. 1995, 2001). For sulfite dehydrogenase and thiosulfate dehydrogenase activities in S_{12}, 0.1 M Bis-Tris buffer (pH 6.0) and β-alanine-SO_4 buffer (pH 4.0) were used, respectively. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μmol ferricyanide in 1 min at 30°C. The adenylsulfate [= adenosine-5′-phosphosulfate (APS)] reductase activity was followed as the sulfite-dependent ferricyanide reduction in the presence of 1 mM AMP in 0.1 M Bis-Tris buffer (pH 6.0), according to the method previously described (Zimmermann et al. 1999).

Preparation of pyridine ferrohemochromes from P_{105}

Hemes were extracted according to the method of Rieske (1967). P_{105} (3 mg protein) was homogenized with 1 ml cold acetone and centrifuged at 12,000 *g* for 10 min. The precipitate thus obtained was mixed with 1 ml cold chloroform:methanol (2:1). The solution was centrifuged

at 12,000 *g* for 10 min, and the precipitate was mixed with 1 ml cold acetone. The hemes of cytochromes *a* and *b* were extracted from the protein by three successive homogenizations with the solution of 0.5 ml cold acetone in 0.024 M HCl. The acetone extract was pooled and evaporated to dryness *in vacuo*, and then immediately dissolved in 60 µl pyridine and 60 µl 0.2 M KOH. The protein residue after the extraction with HCl-acetone retained cytochrome *c*. The residue was homogenized in 30 µl pyridine and 30 µl 0.2 M KOH. The absorption spectra for pyridine ferrohemochromes were measured with a Shimadzu UV-1200 spectrophotometer.

Cytochrome studies

Cytochromes that were reduced upon addition of substrates were studied by measuring spectra of cells at 22 ± 2°C with a model MPC-3100 Shimadzu spectrophotometer with a 1-cm light path. Spectra of cells with substrates were obtained by adding 15 mM colloidal sulfur, 200 µM sulfite or 5 mM thiosulfate to the reaction mixture containing 5 mg intact cells in 0.1 M β-alanine-SO_4 buffer (pH 4.0) in a total volume of 1 ml. The complete reduction of cytochromes was achieved by the addition of dithionite (sodium hydrosulfide).

Other analytical methods

Sulfite was analyzed spectrophotometrically with *p*-rosaniline as the indicator, according to the method of West and Gaeke (1956). Protein was measured by the method of Lowry et al. (1951), with crystalline bovine serum albumin as the reference.

Results

Cytochromes

The dithionite-reduced spectrum showed a number of absorption peaks (Fig. 1) at 420 (c_γ), 530 (b_β), 573 (b_α), and 620 nm (d_α). A heme *a* was detected by a pyridine ferrohemochrome analysis, although the amount was lower than that of a heme *b* (data not shown). Cytochrome *c*, *b*, *a*, and *d* types were also reported in *A. thiooxidans* grown on sulfur (Tano et al. 1982) and thiosulfate (Masau et al. 2001). The initial reduction of a *b*-type cytochrome was detected within 1 min upon the addition of colloidal sulfur or sulfite, while the initial reduction of a *c*-type cytochrome was detected within 1 min upon the addition of thiosulfate (Fig. 2).

Effect of NaCl on the oxidation of reduced inorganic sulfur compounds by resting cells

The characteristics of the oxidation of sulfur or sulfite by resting cells of strain SH have been previously reported

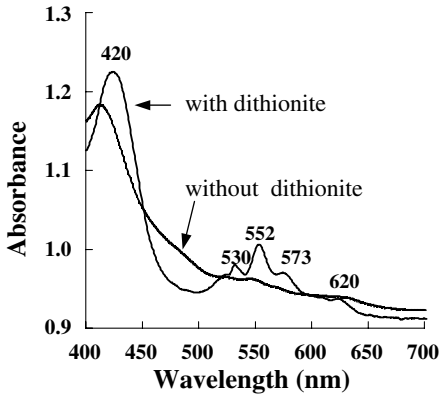

Fig. 1 Absorption spectra of *Acidithiobacillus thiooxidans* strain SH cells. Cytochromes were completely reduced by the addition of dithionite

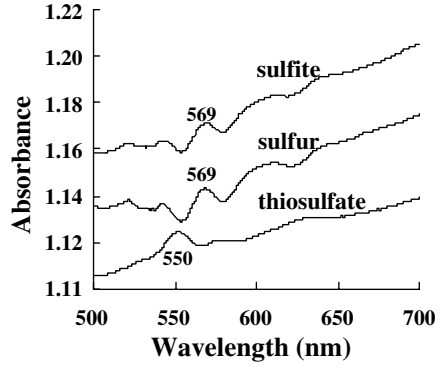

Fig. 2 Cytochrome reduction upon the addition of colloidal sulfur, sulfite, or thiosulfate to strain *A. thiooxidans* SH cells. Absorption spectra of cells (5 mg/ml) were measured after a 1-min incubation with each substrate

(Kamimura et al. 2003). The sulfur-oxidizing activity by resting cells was detected in a broad pH range from 2–6 without a clear optimum (Fig. 3). Sulfite (0.3 mM/30 min/mg) accumulated during the oxidation of sulfur by resting cells in the presence of 1 µM HQNO. HQNO inhibited the sulfur- and sulfite-oxidizing activities by resting cells by approximately 40 and 90%, respectively. The sulfite-, thiosulfate-, tetrathionate-, and sulfide-oxidizing activities by resting cells had the optimum pH of 4–5, 2–3, 2, and 6, respectively (Fig. 3). The time course of the sulfide-dependent oxygen uptake by resting cells exhibited a biphasic pattern, indicating transient accumulation of an intermediate. The reaction during the initial phase had the optimum pH of 6.0 (Fig. 3). The optimum pH of the second phase could not be determined. A ratio of 2 nmol sulfide oxidized and 1 nmol O_2 consumed was observed in the initial phase.

The sulfur-, sulfite-, and tetrathionate-oxidizing activities in resting cells were stimulated about 10-, 4.5-, and 2-fold by 1–3% NaCl (Fig. 4). The thiosulfate-oxidizing activity and the initial phase of sulfide oxidation were not affected by NaCl, whereas the second phase of

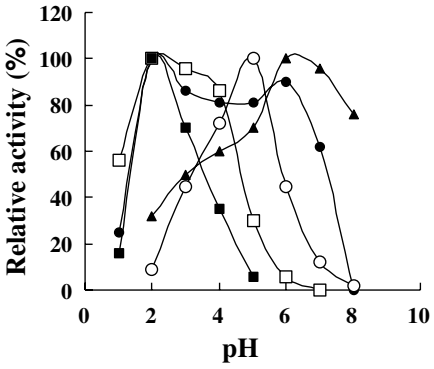

Fig. 3 Effect of pH on the oxidation of sulfur (*filled circle*), sulfite (*open circle*), tetrathionate (*filled square*), thiosulfate (*open square*), and sulfide (*filled triangle*) in resting cells of *A. thiooxidans* strain SH

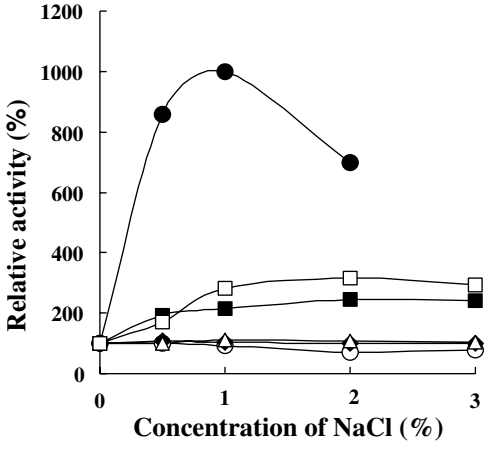

Fig. 4 Effect of NaCl on the oxidation of sulfur (*filled circle*, *open circle*), thiosulfate (*filled triangle*, *open triangle*), and tetrathionate (*filled square*, *open square*) by resting cells of strain *A. thiooxidans* SH. Activities were measured in the presence (*open*) or the absence (*filled*) of 1 μM 2-heptyl-4-hydroxyquinoline-*N*-oxide

sulfide oxidation was stimulated about fourfold with 1–3% NaCl (data not shown). The stimulation of sulfur-oxidizing activity by NaCl was not observed in the presence of 1 μM HQNO. The tetrathionate-oxidizing activity in resting cells was stimulated about twofold with 1–3% NaCl both in the presence and in the absence of 1 μM HQNO (Fig. 4).

Effect of NaCl on enzymes involved in the metabolism of reduced sulfur compounds

The colloidal sulfur-oxidizing activity in S_{12} had the optimum pH of 7 (13 nmol O_2/min/mg) and was not affected by NaCl at up to 4% concentration. Sulfur oxidation in S_{12} of *A. thiooxidans* requires reduced glutathione (Suzuki 1994). However, reduced glutathione did not affect the activity in S_{12} of strain SH. The sulfite-oxidizing activity in S_{12} had the optimum pH of 7 (8 nmol O_2/min/mg) and was not stimulated by NaCl. Approximately 30% of the activity was inhibited in the presence of 2% NaCl. The sulfite dehydrogenase activity was not detected in S_{12} of strain SH, whereas Nakamura et al. (1995) detected it in S_{12} of *A. thiooxidans* JCM7814. Furthermore, the sulfite dehydrogenase activity in the presence of 1 mM AMP (APS reductase activity) was not detected in S_{12} of strain SH.

Cytochrome *a*, which is a well-known component of cytochrome *c* oxidase, was not clearly detected in strain SH. The cytochrome *c*:acceptor oxidoreductase activity, measured with reduced mammalian cytochrome *c* as an electron donor, was not detected in P_{105}. The TMPD dehydrogenase activity was present in P_{105} (0.012 U/mg) and was unaffected by NaCl at up to 4% concentration. The ubiquinol:acceptor oxidoreductase activity was detected in P_{105}, had the optimum pH of 5.5 (0.49 U/mg), and was stimulated 1.3-fold with 1% NaCl. Similarly, the activity was stimulated in the presence of 1% KCl or LiCl. The thiosulfate-oxidizing activity with S_{12} had the optimum pH at 3 (9.2 nmol O_2/min/mg), similar to that observed with resting cells, and was not stimulated by NaCl. A thiosulfate dehydrogenase that oxidizes thiosulfate to tetrathionate has been isolated from *A. thiooxidans* JCM7814 (Nakamura 2001). This activity was detected in S_{12} of strain SH with an optimum pH at 4.0 and was not stimulated by NaCl.

Effects of inhibitors and uncouplers on the oxidation of reduced inorganic sulfur compounds

The effects of inhibitors and uncouplers on the oxidation of reduced inorganic sulfur compounds are summarized in Table 1. The sulfur-oxidizing activity was inhibited by KCN, HQNO, CCCP, and monensin. The sulfite-oxidizing activity was strongly inhibited by KCN, HQNO, and CCCP. The activity was not affected by antimycin A at up to 50 μM concentration. The thiosulfate-oxidizing activity was inhibited by KCN and HQNO, but not by CCCP and monensin. The tetrathionate-oxidizing activity was inhibited by KCN, HQNO, CCCP, and monensin. The inhibition pattern for tetrathionate oxidation by inhibitors and uncouplers was similar to that seen for sulfur oxidation. The initial phase of sulfide oxidation was inhibited by KCN, but relatively resistant to HQNO, CCCP, and monensin. The second phase of sulfide oxidation was inhibited by KCN, HQNO, CCCP, and monensin. The inhibition of the second phase of sulfide oxidation exhibited a mixed pattern; the inhibition levels shown by CCCP, monensin, and valinomycin were similar to that seen for sulfite oxidation, whereas the inhibition levels shown by KCN and HQNO were similar to that seen for sulfur oxidation. Valinomycin slightly inhibited the sulfur- and tetrathionate-oxidizing activities, whereas sulfite-, thiosulfate-, and sulfide-oxidizing activities were not affected by this uncoupler.

Table 1 Effect of NaCl, inhibitors, and uncouplers on the oxidation of reduced sulfur compounds by resting cells

Oxidative substrate[a]	Optimum pH	NaCl	Oxidation rate (%)					
			None	KCN (100 μM)	HQNO (10 μM)	CCCP (10 μM)	Monensin (10 μM)	Valinomycin (10 μM)
S^0	2–7	+[d]	100	40	34	36	36	83
SO_3^{2-}	4–5	+	100	19	3	8	52	93
$S_2O_3^{2-}$	2–3	−[e]	100	40	51	89	95	92
$S_4O_6^{2-}$	2	+	100	42	45	55	48	72
$S^{2-,b}$	6	−	100	53	78	78	106	101
$S^{2-,c}$	−	+	100	62	31	7	51	93

[a]The oxidation rates of reduced sulfur compounds measured in the absence of inhibitors and uncouplers were as follows: S^0, 107 nmol O_2/min/mg; SO_3^{2-}, 74 nmol O_2/min/mg; $S_2O_3^{2-}$, 170 nmol O_2/min/mg; $S_4O_6^{2-}$, 78 nmol O_2/min/mg; $S^{2-,b}$, 180 nmol O_2/min/mg; $S^{2-,c}$, 58 nmol O_2/min/mg

[b]Initial phase of sulfide oxidation
[c]Second phase of sulfide oxidation
[d]The activity was stimulated by NaCl
[e]The activity was not stimulated by NaCl

Discussion

Little information is available on how the sodium motive force is generated and used in chemoautotrophic bacteria. As *Acidithiobacillus thiooxidans* strain SH specifically requires NaCl for growth (Kamimura et al. 2003), it is to be expected that a Na^+ gradient across the membrane is coupled with physiological functions. As a prelude to characterizing the sodium motive force in strain SH, the effect of NaCl on activities of enzymes of reduced sulfur metabolism was examined.

Thiobacilli and other sulfur-oxidizing bacteria oxidize sulfur via intermediate sulfite to sulfate (Pronk et al. 1990; Kelly et al. 1997; Friedrich 1998; Kelly 1999, 2003; Friedrich et al. 2001; Kappler and Dahl 2001). Based on the results obtained in this study, Fig. 5 presents the sequence of the oxidation reactions and their cellular localization together with the specific sites for the inhibitors. Cytochrome components in SH cells were similar to those characterized in *A. thiooxidans* (Tano et al. 1982; Masau et al. 2001). At least two pathways were involved in the reduced sulfur metabolism. One pathway was through cytochrome *c*, and the other involved cytochromes *b* and *d*. Although a cytochrome *c*:acceptor oxidoreductase activity was not detected with mammalian cytochrome *c* as the electron donor, a TMPD dehydrogenase activity was detected. Thus, strain SH has a cytochrome *c* oxidase that uses endogenous cytochrome *c* as an electron donor. Ubiquinol:acceptor oxidoreductase activity involved cytochromes *b* and *d*, suggesting a *bd*-type ubiquinol oxidase. Cytochromes *b* and *d* were reduced upon the addition of sulfite while HQNO strongly inhibited the sulfite oxidation. These data suggest that the ubiquinone-cytochrome *b* complex is involved in the transfer of electrons from sulfite to oxygen. Antimycin A, a specific inhibitor of the bc_1 complex, did not inhibit sulfite oxidation, suggesting that the electrons from sulfite oxidation bypass the bc_1 complex. The involvement of a ubiquinone-cytochrome *b* complex has already been suggested to account for the transfer of electrons from sulfite to oxygen in *A. thiooxidans* (Tano et al. 1982; Masau et al. 2001) as well as *A. caldus* strain KU (Hallberg et al. 1996).

The oxidation of sulfite was strongly inhibited by CCCP and monensin but not by valinomycin, evidence that the oxidation occurred in the cytoplasm. Although the oxidation of sulfite by resting cells was stimulated by

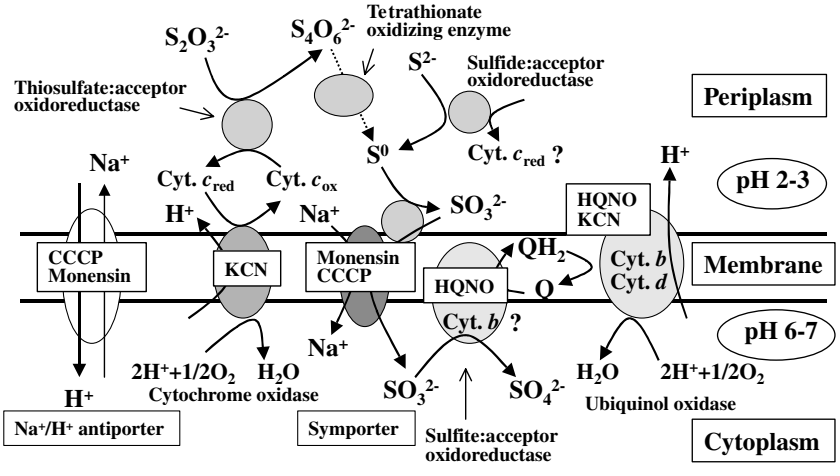

Fig. 5 Tentative scheme of the oxidation pathways for inorganic reduced sulfur compounds in *A. thiooxidans* strain SH

NaCl, the activity in S_{12} (optimum pH 7.0) did not respond to NaCl. These results suggest that the H^+ and Na^+ gradients across the inner membrane were involved in the translocation of sulfite to the cytoplasm in strain SH. As the oxidation of sulfur to sulfate involves sulfite as the intermediate, intermediate sulfite needs to be translocated into cells for further oxidation. In resting cells, the inhibitory effect of CCCP and monensin on sulfur oxidation was lower as compared to their effects on sulfite oxidation. These data suggested that the oxidation of sulfur to sulfite did not require NaCl for the activity, and the translocation of intermediate sulfite was inhibited by the uncouplers. The stimulation of the sulfur-oxidizing activity in resting cells by NaCl was not observed in the presence of HQNO. As the subsequent oxidation of sulfite was inhibited by HQNO, the results supported the hypothesis that NaCl is not necessary for the oxidation of sulfur to sulfite. The sulfur-oxidizing activity in the presence of HQNO was inhibited by CCCP, suggesting that, in intact cells, membrane energization is required for sulfur metabolism involving reductive reactions, as previously shown for the oxidation elemental sulfur by *A. ferrooxidans* (Bacon and Ingledew 1989).

The periplasmic and cytoplasmic pH range in acidophilic bacteria is thought to be 2.0–3.0 and 6.0–7.0, respectively. The optimum pH for the oxidation of thiosulfate and tetrathionate in thiosulfate-grown *A. thiooxidans* was 5.0 (Masau et al. 2001). The pH optima for the oxidation of these compounds in strain SH were in the range 2.0–3.0, suggesting that they were oxidized in the periplasm. Thiosulfate is partially oxidized to tetrathionate in *A. thiooxidans* and *A. caldus* (Hallberg et al. 1996; Masau et al. 2001). Periplasmic thiosulfate dehydrogenases with an optimum pH of 3.0–5.5 have been reported for *A. thiooxidans* (Nakamura et al. 2001), *A. acidophilum* (Meulenberg et al. 1993), and *Thiobacillus* sp. W5 (Visser et al. 1997). The pH 2.5 optimum of thiosulfate-oxidizing activity in resting cells, the pH 4.0 optimum of the thiosulfate dehydrogenase in S_{12}, and the lack of inhibition of thiosulfate oxidation by CCCP and monensin in resting cells (Table 1) strongly suggested that the thiosulfate was converted in the periplasm.

The tetrathionate-oxidizing activity in resting cells in the presence and absence of HQNO was stimulated by NaCl (Fig. 4), suggesting that enzymes involved in the tetrathionate oxidation require NaCl or salts for the activity. Tetrathionate hydrolase localized in the periplasm of *A. caldus* catalyzes the formation of thiosulfate, pentathionate, and sulfate with an optimum pH of 3.0 (Bugaytsova and Lindström 2004). In strain SH, the optimum pH of tetrathionate oxidation was 2.0, suggesting the periplasmic oxidation. The inhibitory effects of inhibitors and uncouplers indicated that sulfur was produced during tetrathionate oxidation.

The oxidation of sulfide by strain SH exhibited a biphasic pattern of oxygen consumption, indicating a transient accumulation of an intermediate. Meulenberg et al. (1992) reported that the oxidation of reduced sulfur compounds followed a biphasic pattern because of the accumulation of elemental sulfur. In the present study, the association between the concentration of sulfide used and the amount of oxygen consumed in the initial phase of sulfide oxidation strongly suggested the accumulation of sulfur. The results obtained with ionophores and other inhibitors suggested that, in the initial phase, sulfide was converted to sulfur in the periplasmic side of the cytoplasmic membrane, followed by sulfur oxidation by a NaCl-dependent pathway. The stimulation of the second phase by NaCl is consistent with this hypothesis.

Inhibition of sulfite oxidation by uncouplers has been reported in *A. caldus* and *A. thiooxidans* (Hallberg et al. 1996; Masau et al 2001), but sulfite oxidation was not stimulated by NaCl. In the case of resting cells of *A. caldus*, the effect of CCCP was attributed to the inhibition of the translocation of sulfite into the cytoplasm (Hallberg et al. 1996). For *A. thiooxidans*, Masau et al. (2001) elaborated on the electrochemical potential essential for electron flow from sulfite through cytochrome *c* into the *b* region of the electron transport system. The sulfite-binding site is influenced by proton motive force in intact cells, whereas in S_{12} the sulfite oxidation system is exposed without constraints of closed cell membranes, and sulfite oxidization is not affected by uncouplers (Masau et al. 2001). In strain SH, intact cells required both H^+ and Na^+ gradients for the oxidation of sulfite. The activity in S_{12} was not stimulated by NaCl. The results suggest that a Na^+ gradient is used to translocate sulfite into the cytoplasm.

NaCl did not specifically affect the enzyme activities involved in the metabolism of reduced sulfur compounds, except for the tetrathionate-oxidizing activity. The conversion of tetrathionate to other reduced sulfur compounds occurred in the periplasm rather than in the membrane and, therefore, the tetrathionate-decomposing enzyme does not function as an efflux pump of Na^+. Thus, the Na^+ gradient was not directly generated during the metabolism of reduced sulfur compounds by enzymes involved in the efflux of Na^+. A Na^+ gradient played a role in sulfite oxidation in strain SH, and it is possible the gradient was generated by a Na^+/H^+ antiporter. Although some acidophiles use a K^+/H^+ antiporter to regulate internal pH (Matin 1999; Konings et al. 2002), a Na^+/H^+ antiporter may play an important role in marine acidophiles such as *A. thiooxidans* strain SH.

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