Mechanism of Inhibition by Co^{2+} of the Growth of *Thiobacillus ferrooxidans* on Sulphur-Salts Medium

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When *Thiobacillus ferrooxidans* AP19-3 was incubated in sulphur-salts medium with 1 mM- Co^{2+} , the sulphur:ferric-ion oxidoreductase (SFORase) of washed intact cells completely disappeared and a concomitant cessation of cell growth was observed. However, when reduced glutathione (GSH), which is absolutely required for SFORase activity, was added to the cells seemingly lacking SFORase activity, the activity was completely restored. The total GSH content of the cells incubated with or without Co^{2+} was 0.10 and 0.16 $\mu\text{mol (mg protein)}^{-1}$, respectively. The SFORase activity of cell-free extracts in the presence of added GSH was 74% of the whole cell activity without Co^{2+} , indicating that an active SFORase was still present but that the GSH required for SFORase activity was in short supply after incubating the cells in sulphur-salts medium with Co^{2+} . Incubating SFORase with 1 mM- Co^{2+} did not decrease its activity, whereas incubating with Co^{2+} plus GSH markedly decreased activity. Sulphite (1 mM), one of the products of sulphur oxidation by SFORase, partially restored this loss of SFORase activity. A new type of mechanism for the inhibition by Co^{2+} of the sulphur metabolism of *T. ferrooxidans* is proposed: Co^{2+} stops cell growth on sulphur by decreasing the intracellular GSH concentration to a level at which SFORase is no longer active, and the cells then cannot obtain energy by oxidizing elemental sulphur.

INTRODUCTION

Thiobacillus ferrooxidans inhabits acidic environments containing mineral sulphides. Bacteria that have a metal-tolerant sulphur-oxidizing system are more valuable for enhanced leaching of sulphide ores. However, the sulphur-oxidizing system of *T. ferrooxidans* is more sensitive to heavy metals than its iron-oxidizing system. Since cobaltous ion (Co^{2+}) is a potent inhibitor of the growth of *T. ferrooxidans* on sulphur-salts medium (Razzell & Trussel, 1963; Tuovinen *et al.*, 1971; Sugio *et al.*, 1984), it is important to study the mechanism of this inhibition in order to obtain a valuable strain that has a metal-tolerant sulphur-oxidizing system.

The mechanism of sulphur oxidation by *T. ferrooxidans* has been considered to be similar to that of other thiobacilli (Silver & Lundgren, 1968*a, b*; Vestal & Lundgren, 1971; Eccleston & Kelly, 1978). We recently reported the presence of a ferric-ion-reducing system (FIR system) that catalyses the reduction of Fe^{3+} with elemental sulphur in the pure strain of *T. ferrooxidans* AP19-3, and proposed an alternative sulphur-oxidation route that comprises both the FIR and iron-oxidizing system (Sugio *et al.*, 1985). It was found that *de novo* synthesis not only of the FIR system but also of the iron-oxidizing system was an absolute requirement for growth of *Thiobacillus ferrooxidans* AP19-3 on sulphur-salts medium (Sugio *et al.*, 1988). The FIR system was purified to an electrophoretically homogeneous state and characterized (Sugio *et al.*, 1987*b*).

Abbreviations: SFORase, sulphur:ferric-ion oxidoreductase; FIR system, ferric-ion-reducing system; GSH, reduced glutathione; GSSG, glutathione.

We named this enzyme sulphur:ferric-ion oxidoreductase (SFORase). It is localized in the periplasmic space of this strain and has an absolute requirement for GSH (Sugio *et al.*, 1987*b*). Results supporting the involvement of this enzyme in aerobic sulphur oxidation have been presented elsewhere (Sugio *et al.*, 1986*a, b*; 1987*a*).

Though *T. ferrooxidans* is one of the most important bacteria for bacterial leaching, little has been published on the mechanism of inhibition by heavy metals. Cupric ion (Cu^{2+}) competes at the active site of SFORase (FIR system) with Fe^{3+} , which is the obligate acceptor of electrons from elemental sulphur; this competitive inhibition was suggested to be the main cause of the inhibition of sulphur oxidation by Cu^{2+} (Sugio *et al.*, 1986*b*). In this work the mechanism of inhibition by Co^{2+} of the sulphur metabolism of *T. ferrooxidans* AP19-3 was investigated.

METHODS

Micro-organism. *T. ferrooxidans* AP19-3 (Sugio *et al.*, 1984) was used throughout this study.

Media and conditions of cultivation. The compositions of sulphur-salts medium and iron-salts medium were described previously (Sugio *et al.*, 1984, 1985). Iron-grown cells (20 mg protein) were inoculated into 200 ml sulphur-salts medium with or without 1 mM-cobaltous sulphate in a 500 ml flask and cultured by shaking at 30 °C. The pH of the culture medium was adjusted to 2.5 with NaOH every 12 h. To obtain washed intact cells, a portion of culture was withdrawn and centrifuged at 12000 *g* for 10 min; the cells were then resuspended in 0.1 M-sodium phosphate buffer, pH 6.5. The amount of cell protein and the activity of the FIR system were determined as described below.

Activities of the FIR system and SFORase. The activity of the FIR system was determined by measuring either Fe^{2+} or sulphite produced in the reaction mixture using the methods described previously (Sugio *et al.*, 1985, 1987*b*). The reaction mixture used for determining activity by measuring Fe^{2+} production contained 4 ml 0.1 M- β -alanine sulphate buffer, pH 3.0, washed intact cells (5.0 mg protein), Fe^{3+} (25 μmol), sodium cyanide (25 μmol) and elemental sulphur (100 mg) in a total volume of 5.0 ml. The reaction mixture used for determining activity by measuring sulphite production contained 0.1 M-sodium phosphate buffer, pH 6.5 (4 ml), washed intact cells (5 mg protein) and elemental sulphur (100 mg) in a total volume of 5 ml.

SFORase activity was determined by measuring the rate of sulphite production during aerobic oxidation of elemental sulphur at pH 6.5 using the methods described previously (Sugio *et al.*, 1987*b*). After *T. ferrooxidans* AP19-3 had been incubated for 8 d on sulphur-salts medium $\pm$ 1 mM- Co^{2+} , cells were collected by centrifugation at 12000 *g* for 10 min, washed with 0.1 M-sodium phosphate buffer, pH 7.5, and disrupted by passage through a French pressure cell at 1500 kg cm^{-2} . Cell-free extracts obtained were centrifuged at 105000 *g* for 60 min and the resulting supernatant solution was used in the determination of SFORase activity. The composition of the reaction mixture was 0.1 M-sodium phosphate buffer, pH 6.5 (4 ml), 105000 *g* supernatant solution (0.2 mg protein), bovine serum albumin (0.2 mg), elemental sulphur (100 mg) and GSH (20 μmol) in a total volume of 5.0 ml. Sulphite was determined spectrophotometrically by the pararosaniline method (West & Gaeke, 1956).

Determination of GSH. This was done spectrophotometrically by measuring the production of S-lactoylglutathione, which is a condensation product of GSH and methylglyoxal, a reaction catalysed by glyoxalase I. The reaction mixture contained 0.1 M-potassium phosphate buffer, pH 6.5 (2 ml), GSH (0–0.5 μmol), methylglyoxal (10 μmol ; Sigma) and glyoxalase I (4 U; Sigma), in a total volume of 3.0 ml. Washed intact cells (20 mg protein) suspended in 1 ml deionized water were boiled for 10 min in a test-tube; the boiled cell suspension was then dialysed against 20 ml deionized water for 24 h. The concentration of GSH dialysed into 20 ml deionized water was assayed enzymically by the method described above.

Protein content. This was determined by the biuret method (Layne, 1957) or the Lowry method, with crystalline bovine serum albumin as the reference protein.

RESULTS AND DISCUSSION

Activity of the FIR system of cells incubated in sulphur-salts medium containing Co^{2+}

Cells (20 mg protein), grown in iron-salts medium, were inoculated into 200 ml sulphur-salts medium with or without Co^{2+} , to check the activity of the FIR system during incubation. No growth was observed in sulphur-salts medium lacking elemental sulphur. When Co^{2+} was added to sulphur-salts medium at the start of the incubation, the growth rate was markedly inhibited and the specific activity of the FIR system rapidly decreased (Fig. 1). After 8 d incubation, the FIR system activity of cells incubated in sulphur-salts medium with Co^{2+} was completely lost. The same result was obtained when Co^{2+} was added to the medium after 3 d incubation. In a control culture without Co^{2+} , cell protein and the specific activity of the FIR system increased during incubation, suggesting that *de novo* synthesis of the FIR system

Fig. 1

Fig. 1. Effects of Co²⁺ on the growth rate of *T. ferrooxidans* on sulphur-salts medium and on the FIR system activity of the cells. Washed intact cells of iron-grown *T. ferrooxidans* AP19-3 were added to 200 ml sulphur-salts medium with or without 1 mM-Co²⁺. O, Growth without Co²⁺; Δ, growth with Co²⁺ (added at the start of incubation); □, growth with Co²⁺ (added after 3 d incubation); ●, FIR system activity after growth without Co²⁺; ▲, FIR system activity after growth with Co²⁺ (added at the start of incubation); ■, FIR system activity after growth with Co²⁺ (added after 3 d incubation).

Fig. 2

Fig. 2. Activity of the SFORase of cells incubated in sulphur-salts medium with (O) or without (●) 1 mM-Co²⁺.

occurred when the strain was grown on sulphur-salts medium. An increase in FIR system activity was also observed when *T. ferrooxidans* AP19-3 subcultured on sulphur-salts medium 53 times was incubated in fresh sulphur-salts medium (Sugio *et al.*, 1988). These results indicate that the inhibition of growth by Co²⁺ correlates well with the decrease in specific activity of the FIR system.

There are two possible explanations for the lack of FIR system activity in the cells incubated in sulphur-salts medium supplemented with Co²⁺. One is that the FIR system of these cells was damaged by Co²⁺; the other is that GSH, which is absolutely required for SFORase (FIR system) activity, was lost during incubation on sulphur-salts medium with Co²⁺. To distinguish between these two possibilities, SFORase activity was measured in cell-free extracts, prepared from cells incubated in sulphur-salts medium with or without Co²⁺, in the presence of added GSH. Cells lacking FIR system activity were found to possess 74% of the SFORase activity of cells incubated without Co²⁺, indicating that an active SFORase was present in cells after incubation with Co²⁺ (Fig. 2).

The second possibility was tested as follows. GSH was added to washed cells lacking FIR system activity and FIR system activity was then determined. The activity observed was similar to that of cells incubated in sulphur-salts medium without Co²⁺ (Fig. 3*b*). When FIR system activity was determined in cells grown in sulphur-salts medium without Co²⁺, there was no need to add GSH to the reaction mixture, probably because the amount of GSH in the periplasmic space of this strain is sufficient to enable the reaction to proceed there (Fig. 3*a*). In the case of cells grown on sulphur-salts medium without Co²⁺, addition of GSH caused inhibition. These results strongly suggest that when grown on sulphur-salts medium with Co²⁺, the GSH content of cells decreases to such a low level that the SFORase in the periplasmic space cannot operate normally.

Fig. 3. Effect of GSH on the FIR system activity of cells incubated in sulphur-salts medium with (b) or without (a) Co^{2+} . ●, FIR system activity determined without GSH; ■, FIR system activity with 20 μmol GSH.

Table 1. GSH content of cells grown on sulphur-salts medium with or without Co^{2+}

T. ferrooxidans AP19-3 was grown on sulphur-salts medium with or without Co^{2+} . A sample of the culture was centrifuged at 12000 g for 10 min, and the cells were then washed three times with water. The GSH content of the washed cells was determined as described in Methods. Values shown are means of duplicate experiments. ND, Not determined.

Addition to growth medium	Incubation time (d) ...	GSH content [$\mu\text{mol (mg cell protein)}^{-1}$]			
		0	2	3	8
None		0.140	ND	0.160	ND
Co^{2+} (1 mM)		0.140	0.101	0.100	0.100

This hypothesis was further supported by measuring the GSH content of the cells and studying the effect of diamide on FIR system activity. The total GSH content of cells grown on sulphur-salts medium without Co^{2+} increased from 0.140 to 0.160 $\mu\text{mol (mg protein)}^{-1}$ after 3 d incubation (Table 1), whereas the total GSH content of cells incubated in sulphur-salts medium with 1 mM- Co^{2+} decreased from 0.140 to 0.101 $\mu\text{mol mg protein}^{-1}$ after 2 d incubation.

Diamide [diazine dicarboxylic acid bis-(*N,N*-dimethylamide)], oxidizes GSH stoichiometrically in the cell to GSSG (Kosower *et al.*, 1965, 1969; Kosower & Kosower, 1969; Apontowiel & Berends, 1975). FIR system activity was completely inhibited by 0.5 mM-diamide (data not shown), suggesting that GSH in the periplasmic space was oxidized by the diamide to give GSSG, which does not stimulate SFORase activity.

Effect of GSH and Co^{2+} on SFORase activity

SFORase purified to the $(\text{NH}_4)_2\text{SO}_4$ fractionation stage (Sugio *et al.*, 1987b) was incubated with GSH, Co^{2+} or sulphite (1 mM) for 10 h, and then dialysed three times against 0.1 M-sodium phosphate buffer, pH 7.5, to remove GSH, Co^{2+} or sulphite completely. Incubation of SFORase with GSH increased its activity about 2.5-fold (Fig. 4). Incubation of SFORase with Co^{2+} did not affect its activity. However, incubation of SFORase with Co^{2+} plus GSH markedly decreased its activity. This inhibition was partially overcome by reincubating the inhibited enzyme with 1 mM-sulphite (but not with 5 mM-sulphite) for 10 h. The decrease in SFORase activity was not observed when SFORase was incubated with Co^{2+} , GSH and sulphite (1 mM) for 10 h, indicating that sulphite, at a low concentration, has a protective effect on the inactivation of SFORase by Co^{2+} plus GSH. Incubation of SFORase with 1 mM-sulphite increased its activity about 1.5-fold.

Fig. 4. Effect of incubating SFORase with GSH, Co²⁺ or sulphite. SFORase, purified to the (NH₄)₂SO₄ fractionation stage (Sugio *et al.*, 1987*b*), was incubated with GSH, Co²⁺ or sulphite at 1 mM for 10 h and then dialysed three times against 0.1 M-sodium phosphate buffer, pH 7.5. SFORase activity was then determined. ▲, Incubated with GSH; ▽, incubated with sulphite; ●, incubated with Co²⁺; ■, incubated with Co²⁺ plus GSH; ▼, incubated with Co²⁺ plus sulphite; △, incubated with Co²⁺, GSH and sulphite; ○, incubated without Co²⁺, GSH and sulphite; □, SFORase incubated with Co²⁺ plus GSH was further incubated with 1 mM-sulphite for 10 h.

We propose the following mechanism for inhibition by Co²⁺ of the growth of *T. ferrooxidans* AP19-3 on sulphur-salts medium. Co²⁺ added to the medium penetrates into the periplasmic space and, together with GSH, partially inhibits SFORase activity. [GSH is assumed to be synthesized in the cytoplasm and then transported to the periplasmic space. We have previously shown that [³⁵S]GSH incorporated into *T. ferrooxidans* leaks out of the cells during washing with buffer (Sugio *et al.*, 1982).] SFORase may be partially inhibited by Co²⁺ plus GSH because sulphite produced by the SFORase has a protective effect. A partially inhibited SFORase produces less Fe²⁺ than cells grown on sulphur-salts medium lacking Co²⁺. As a result, the amount of GSH synthesized in the cells decreases, which further decreases SFORase activity. Eventually, both sulphur oxidation and cell growth stop completely. The mechanism by which GSH and Co²⁺ act in concert to inhibit SFORase is now under investigation.

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