Biogeochemistry 7: 195–201 (1989)

Ferric iron reduction by *Thiobacillus ferrooxidans* at extremely low pH-values

WOLFGANG SAND
Institut für Allgemeine Botanik, Abt. Mikrobiologie, Ohnhorststr. 18, D. 2000 Hamburg 52, FRG

Key words: ferrous iron oxidation, ferric iron reduction, sulfur oxidation, *Thiobacillus ferroxidans*

Abstract. When ferrous iron and sulfur were supplied, cells of *T. ferrooxidans* in a well-aerated medium started growth by oxidizing ferrous iron. After ferrous iron depletion a lagphase followed before sulfur oxidation started. During sulfur oxidation at pH-values below 1.3 ($\pm$0,2) the ferrous iron concentration increased again, although the oxygen saturation of the medium amounted to more than 95%. The number of viable cells did not increase. Thus resting cells of *T. ferrooxidans*, which are oxidizing sulfur to maintain their proton balance, reduce ferric to ferrous iron. The ferrous iron-oxidizing system seemed to be inhibited at pH-values below 1.3. At a pH-value of 1.8 the ferrous iron was reoxidized at once. A scheme for the linkage of iron- and sulfur metabolism is discussed.

Introduction

T. ferrooxidans, an obligate chemolithoauthotroph, grows by ferrous iron oxidation. The energy is preserved as ATP–metabolic energy. Other inorganic compounds, e.g., reduced sulfur compounds can also be utilized as energy substrates (Eccleston & Kelly 1978; Kelly 1982).

According to Brock & Gustafson (1976), *T. ferrooxidans* has a potential to reduce ferric iron while growing on sulfur. The reduction was measurable under anaerobic conditions exclusively. Brock & Gustafson (1976) postulated that under aerobic conditions the cells would readily reoxidize ferrous iron.

As proposed by several authors (Kino & Usami 1982; Lundgren et al. 1983; Sugio et al. 1985) the ferrous iron oxidizing system and the ferric iron reducing system are autonomous. They may feed each other by their end-products.

Other lithoautotrophic pathways like the oxidation of nitrite to nitrate by *Nitrobacter* have been shown to be reversible. Under anaerobic conditions a cell-free system reduced nitrate to nitrite with an artificial electron donator (Sundermeyer-Klinger et al. 1984).

The present work was carried out in order to determine the conditions, by which cultures of *T. ferrooxidans* accumulate ferrous iron. In particular, the pH dependence of the reaction was examined.

Materials and methods

Two pure strains of *T. ferroxidans* were used in this study. They had been kindly supplied by Dr K. Bosecker (BGR, Hannover, FRG). Purity of the strains was achieved by serial dilutions. The highest dilution (10^{-7}) was used for inoculation of a new culture. This procedure was repeated at least 4 times. For the detection of heterotrophic contaminants the cultures were plated on agar containing trypticase soy broth (0.1 g/l) and glucose (1 g/l) according to Harrison (1981). Light microscopy also was used to check for contaminants. Contaminated cultures and assays were discarded. Thus the purity of the cultures and assays was controlled throughout the experiments. Light microscopy was also used for enumeration of bacterial counts (total counts).

The cells (pH-optimum 1.8) were grown in mineral salts culture medium as described by Mackintosh (1978). Stock cultures were kept at 28 °C in the dark for two months. For the experiments the culture medium was supplemented with 50 g/l ferrous sulfate and 10 g/l sulfur (20 μm grain size).

The initial pH-values are adjusted to 1.1/1.3/1.5/1.8/2.0/2.2 with sulfuric acid. The assays (triplicate set) were inoculated with 1×10^8 cells/ml of an actively iron-oxidizing culture of *T. ferrooxidans*. Growth experiments lasted 5 months. The experiments were carried out in shake flasks (300 ml volume, 150 ml medium, 200 rpm). The assays were adjusted weekly for the evaporated water. The pH-value, the ferrous/ferric iron (Anonymous, 1984) and the sulfate content (Dugan & Apel 1978) were regulalry monitored. Oxygen concentration was measured once a month by means of an oxygen electrode (Clarke-type, YSI, USA). For the measurement of the oxygen concentration 5 ml aliquots were taken from the shake flasks. Disturbance of the culture medium was carefully avoided to prevent oxygen addition.

Viability was tested by inoculating aliquots of the cultures into fresh medium with a pH-value of 1.8. Cell burst was not detected.

Results and discussion

During the whole experiment anaerobic conditions favoring ferric iron reduction did not occur in the medium. Oxygen partial pressure was not limiting (5%, Myerson 1981). This could be demonstrated by means of an

oxygen electrode. Values above 95% oxygen saturation were always recorded.

After completion of ferrous iron oxidation a redox potential of + 770 mV was measured. The value decreased to + 710 mV during the formation of ferrous iron.

T. ferrooxidans oxidized consecutively ferrous iron and elemental sulfur, when fed simultaneously. As Fig. 1 clearly shows, at an initial pH of 1.8 the cells first oxidized ferrous iron. After depletion of ferrous iron a lag-phase followed, before the cells started to oxidize sulfur. Sulfuric acid production is indicated by the simultaneous decrease of the pH-value and the increase in the sulfate content. Essentially the same pattern of results was observed for the initial pH-values 1.3, 1.5, and 2.0. For experiments initiated at pH 1.1 and pH 2.2 and for the uninoculated controls a typical example fo the results obtained is given in Fig. 2. The decrease in ferrous iron content in Fig. 2 is probably due to a formation of jarosites and autoxidation reactions with oxygen (Murr & Metha 1982).

Several proton-generating reactions may thus occur during the formation of jarosite (Lawrence & Gunn 1985). Neither ferrous iron nor sulfur was biologically oxidized in these tests. The blanks were checked for contaminants by inoculation of sterile media containing ferrous iron (pH 1.8) or sulfur (pH 3.0) or organic compounds (pH 2.5). Growth could not be detected. Chemical controls with ferric iron and sulfur did also not result in ferrous iron accumulation.

During biological sulfur oxidation the ferrous iron content started increasing again, as is clearly shown by Fig. 1. At the end of the experiment a ferrous iron concentration of up to 2 g/l was measured. It was demonstrated by Brock & Gustafson (1976) that the reduction of ferric to ferrous iron during sulfur oxidation is biologically catalyzed. *T. thiooxidans, T. ferrooxidans* and *S. acidocaldarius* were able to reduce ferric iron. As is evident from Fig. 1, the increase of the ferrous iron concentration coincided in the well-aerated shake flasks with a decrease of the pH-value below 1.3 ($\pm$ 0.2). Below this limit evidence of reoxidation was not observed. In contrast a reoxidation of the ferrous iron could be demonstrated, if the pH-value of the medium was increased above pH 1.6. This is indicated in Fig. 1 at the end of the experiment. By addition of sodium carbonate the pH-value was increased from 0.8 to 1.7. After a short lag of about 20h ferrous iron oxidation started again (in the presence of sulfur).

The cell counts revealed that the cultures were growing while oxidizing ferrous iron. They increased from 1×10^8 at the start to 8×10^8 cells/ml at the depletion of ferrous iron (20 days). During the following time the cell counts decreased slightly. At the end of the experiment they amounted to

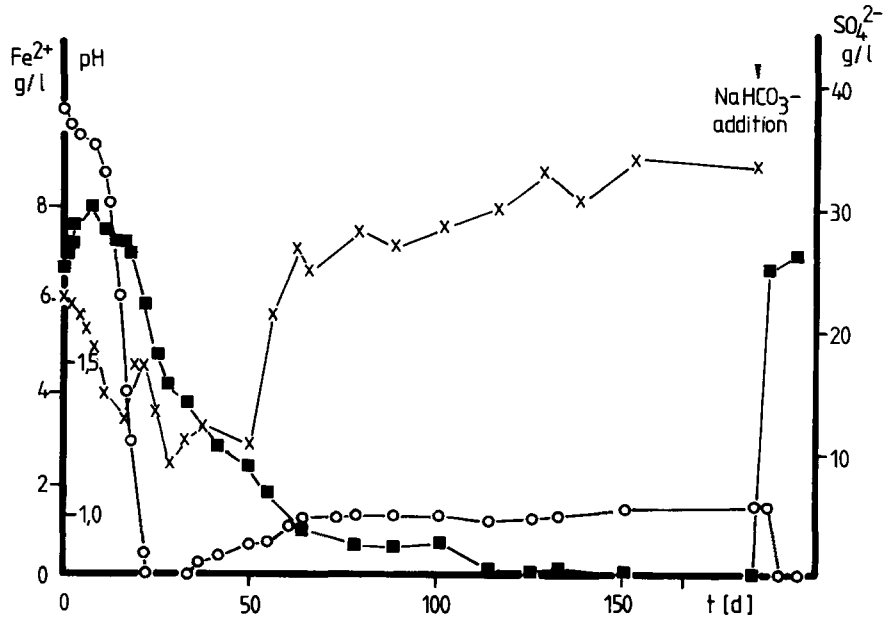

Fig. 1. Typical alterations of the ferrous iron content, the sulfate content, and the pH-value in a culture of *T. ferrooxidans* strain RAM 6 F. The arrow indicates the addition of solid sodiumcarbonate.
○ = ferrous iron content, X = sulfate content, □ = pH-value, standard deviation ± 2%, Shake culture 250 rpm, 30 °C, dark.

6×10^8 cells/ml (190 days). No increase was noted even after the onset of sulfur oxidation. Thus it appears that resting cells were oxidizing sulfur and reducing ferric iron. In an increasingly acid environment the cells may use the energy of the sulfur oxidation to maintain the cytoplasm in a neutral pH-range. Vian et al. (1986) demonstrated with another strain that the growth rate of ferrous iron oxidizing *T. ferrooxidans* decreased with pH-values below 1.55. The results suggest that resting cells of *T. ferrooxidans*, while oxidizing sulfur, are able to reduce aerobically ferric to ferrous iron. The ferrous iron is not reoxidized at pH-values below 1.2. The result is in contrast to Brock & Gustafson (1976), as these workers stated that under aerobic conditions ferrous iron would be reoxidized immediately by *T. ferrooxidans*.

The inhibition of the reoxidation is probably caused by a represssion of the iron oxidizing system due to the low pH-values. This assumption is in contrast to results of Kulpa et al. (1986), who stated that the substrate sulfur inhibits the iron oxidizing system of *T. ferooxidans*. It is evident from Fig. 1 that the cells oxidized ferrous iron in the presence of sulfur (after the pH-adjustment). Furthermore the assumption is supported by the result that at an initial pH-value of 1.1 ferrous iron was not oxidized.

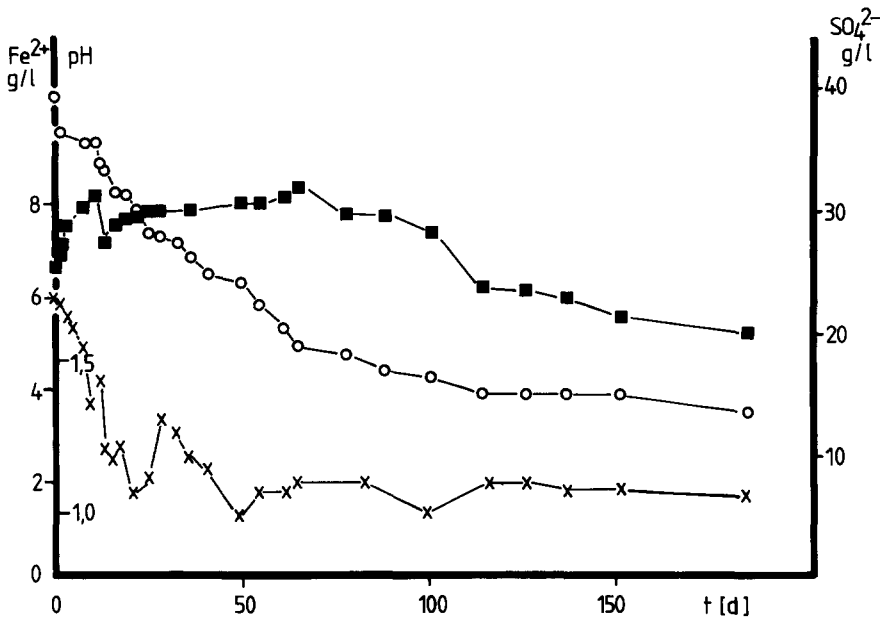

Fig. 2. Typical alterations of the ferrous iron content, the sulfate content, and the pH-value in an uninoculated blank.
○ = ferrous iron content, X = sulfate content, □ = pH-value, standard deviation ± 2%. Shake culture 250 rpm, 30 °C, dark.

Two different ways of sulfur oxidation have been proposed: sulfur–oxygen oxidoreductase (Silver & Lundgren 1968; Suzuki 1965) and sulfur–ferric iron oxidoreductase (Sugio et al. 1985). Based on my results a scheme for the linkage of ferric iron reduction and sulfur oxidation is outlined in Fig. 3. Under favourable conditions ferrous iron is readily oxidized by the iron oxidizing system. If ferrous iron and sulfur are simultaneously supplied, ferrous iron is used prior to sulfur. If sulfur is oxidized, the pH-value decreases. At pH-values below 1.3 the electrons of the sulfur oxidation may flow to the ferric iron reducing system. Ferrous iron will be generated. Additionally the ferrous iron oxidizing system will become inhibited by the proton concentration. Whether this system is only marginally affected cannot be answered by the data. This hypothesis is in agreement with Sugio et al. (1985). The view is complicated by the possibility to adapt ferrous iron oxidizing *T. ferrooxidans* to growth at low pH-values (Tuovinen & Kelly 1974). As is evident the iron oxidizing system is inhibited by pH-values below or above the (variable) pH-optimum. Nevertheless, the pathway of sulfur oxidation coupled to iron reduction in *T. ferrooxidans* remains uncertain.

The results have some economic importance. For the profitableness of leaching operations a high concentration of metal ions in the pregnant solution is essential. The metal yield is dependent on the ferric iron con-

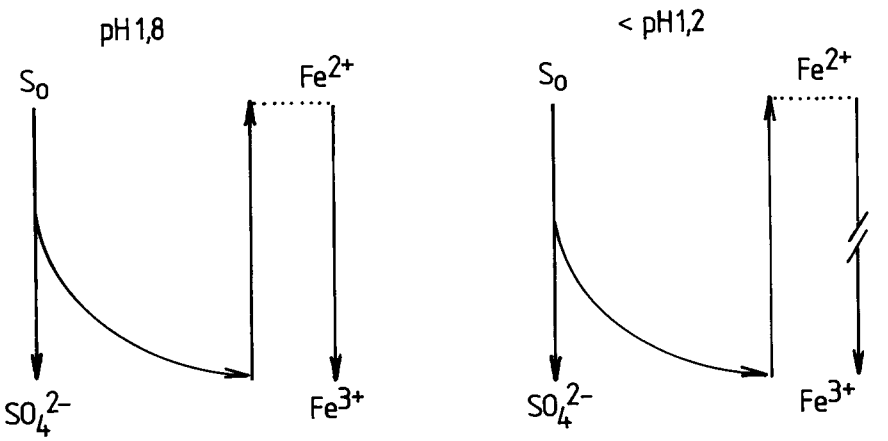

Fig. 3. Hypothetical scheme for the linkage of sulfur oxidation and ferrous iron oxidation/ferric iron reduction as a function of the pH-value.
S_0 = sulfur, SO_4^{2-} = sulfate, Fe^{2+} = ferrous iron, Fe^{3+} = ferric iron. $\|$ = inhibition of the ferrous iron reoxidation.

centration in leaching liquor (Brierley 1982). If the pH-value decreases below 1.3, which often is noted for leaching operations or acid mine drainage situations, the predominant bacterial population of *T. ferrooxidans* and *T. thiooxidans* (Schröter et al. In: Workshop on Biotechnology for the Mining Industries, Rensselaer Polytechnic Institute, Troy, NY, 1985) may start to reduce ferric iron and accumulate ferrous iron while oxidizing metal sulfides (sulfur is an intermediate of this process). Due to the reduced ferric iron concentration–ferric iron is the main oxidizing agent in the indirect leaching cycle–and the decreased redox potential a reduction of the metal output may result. Similar observations have been reported for moderately thermophiles (Marsh & Norris 1983). The physiological background of these phenomena is still uncertain. Further work is in progress.

References

Brierley CL (1982) Microbiological mining. Scientific American 247: 44–53

Brock TD & Gustafson J (1976) Ferric iron reduction by sulfur and iron oxidizing bacteria. Applied and Environmental Microbiology 32: 567–571

Anonymous (1984) Bestimmung von Eisen. In: Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung, E1. Verlag Chemie, Weinheim

Dugan PR & Apel WA (1978) Microbiological desulfurization of coal (pp 223–250) In: Murr LA, Torma AE & Brierley JA (Eds) Metallurgical Applications of Bacterial Leaching and Related Microbiological Phenomena. Academic Press, New York

Eccleston M & Kelly DP (1978) Oxidation kinetics and chemostat growth kinetics of Thiobacillus ferrooxidans on tetrathionate and thiosulfate. Journal of Bacteriology 134: 718–727

Harrison AP Jr (1981) Acidophilium cryptum gen. nov., sp. nov., heterotrophic bacterium from acidic mineral environments. International Journal of Systematic Bacteriology 31: 327–332

Kelly DP (1982) Biochemistry of the chemolithotrophic oxidation of inorganic sulphur. Philosophic Transactions of the Royal Society, London Series B. 298: 499–528

Kino K & Usami S (1982) Biological reduction of ferric iron by iron and sulfur oxidizing bacteria. Agricultural Biology and Biochemistry 46: 803–805

Kulpa CF Mjoli N & Roskey MT (1986) Comparison of iron and sulfur oxidation in Thiobacillus ferroxidans: Inhibition of iron oxidation by growth on sulfur. In: Ehrlich HL & Holmes DS (Eds) Workshop on Biotechnology for the Mining, Metal-refining and Fossil Fuel Processing Industries. Biotechnology and Bioengineering Symposium No. 16, Interscience Publication. J. Wiley, New York

Lawrence RW & Gunn JD (1985) Biological preoxidation of a pyrite gold concentrate. In: Spisak JK & Jergensen GV (Eds) (pp 13–17) Frontier Technology in Mineral Processing. AIME, New York, NY

Lundgren DG, Boucheron J & Mahony W (1983) Geomicrobiology of iron: mechanisms of ferric iron reduction, In: Rossi G & Torma AE (Eds) Recent Progress in Biohydrometallurgy (pp 55–69) Associazione Mineraria Sarda, Italy

Mackintosh, M.W., 1978. Nitrogen fixation by Thiobacillus ferrooxidans. Journal of General Microbiology 105: 215–218

Marsh, R.M. and Norris P.R., 1983. Mineral sulphide oxidation by moderately thermophilic acidophilic bacteria. Biotechnology Letters 5: 585–590

Murr, L.E. and A.P. Metha, 1982. Coal desulfurization by leaching involving acidophilic and thermophilic microorganisms. Biotechnology Bioengineering 24: 743–748

Myerson, A.S., 1981. Oxygen mass transfer requirements during the growth of Thiobacillus ferrooxidans on iron pyrite. Biotechnology Bioengineering 23: 1413–1416

Silver, M. and D.G. Lundgren, 1968. Sulfur oxidizing enzyme of Thiobacillus ferrooxidans. Canadian Journal of Biochemistry 46: 457–461

Sugio, T., C. Domatsu, O. Munakata, T. Tano, and K. Imai, 1985. Role of a ferric iron reducing system in sulfur oxidation of Thiobacillus ferrooxidans. Applied and Environmental Microbiology 49: 1401–1406

Sundermeyer-Klinger, H., W. Meyer, B. Warninghoff, and E. Bock, 1984. Membrane-bound nitrite oxidoreductase of Nitrobacter: evidence for a nitrate reductase system. Archives of Microbiology 140: 153–158

Suzuki, I., 1965. Oxidation of elemental sulfur by an enzyme system of Thiobacillus ferrooxidans. Biochimica et Biophysica Acta 104: 359–371

Tuovinen, O.H. and D.P. Kelly, 1974. Studies on the growth of Thiobacillus ferrooxidans. Archives of Microbiology 95: 165–180.

Vian M, Creo C, Dalmastri C, Gionni A, Palazzolo P & Levi G (1986) Thiobacillus ferrooxidans selection in continuous culture. In: Lawrence RW, Branion RMR & Ebner HG (Eds) Fundamental and Applied Biohydrometallurgy, (pp 395–401) Elsevier, Netherlands