BIOCHEMICAL ECOLOGY OF METAL SULFIDE OXIDIZING BACTERIA

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INTRODUCTION

Metal sulfides in the presence of oxygen will oxidize to their corresponding metal sulfates and sulfuric acid; these reactions are accelerated by the metabolic activities of acid-loving iron oxidizing bacteria. The various mechanisms of metal sulfate oxidations have been reviewed (20). Microbial oxidation of sulfide metals have harmfull effects by contributing to stream pollution. Pyrites and marcasites, both associated with coal mining, when in the presence of oxygen, water and iron-oxidizing bacteria oxidize to sulfuric acid and iron sulfate. The acid is corrosive to equipment, kills aquatic life, dissolves rocks and minerals, and contributes to the hardness of water. The iron sulfate, soluble under acid conditions, eventually hydrolyzes and precipitates from solution to form colored complexes of ferric sulfates and hydrated oxides.

Considerable thought has been given to the prevention of biological iron oxidation as a means of controlling water pollution (28). However, as this report demonstrates, any thought of microbiological control must consider the metabolism of the whole organism. Attempts to inhibit iron oxidation can result in the organism oxidizing sulfur and/or organic compounds. A complex biochemical relationship exists in these bacteria and a particular metabolic expression depends upon regulatory mechanisms (11); the latter are subject to influence by the environment.

It is now recognized that the iron-oxidizing thiobacilli can grow and metabolize iron, sulfur, or glucose. It is the purpose of this presentation to discuss aspects of the cells' metabolism when oxidizing the different substrates for their primary energy source; emphasis is given to sulfur oxidation.

MATERIALS AND METHODS

Cultures. Ferrobacillus ferrooxidans was grown in the following ways:

I. Growth of ferrous iron. Cells were grown under conditions as reported previously (13). The organism was propagated in 16-liter glass carboys on the ferrous sulfate-9K medium (9,000 ppm of Fe^{++}, pH 3.3) under forced aeration and was harvested after 48 to 54 hr by use of a Sharples Centrifuge.

II. Growth on elemental sulfur. Cells were grown in 2-liter Fernbach flasks containing 500 ml of the 9K salts solution (pH 3.3), 1.0 ppm of $FeSO_4$, and 5 g of precipitated sulfur. These flasks were autoclaved for 5 min at 121°C prior to inoculation and were cooled rapidly to prevent sulfur from coalescing. Flasks were agitated on a reciprocating shaker for 5 to 6 days at 28°C and cells were harvested, after the pH had dropped below 2.0, with a Sorvall RC-2 refrigerated centrifuge. The sulfur in the flasks was not depleted during this time. The inoculum consisted of cells trained to sulfur by repeated transfers on the sulfur medium.

III. Growth on glucose. Cells were first grown in the regular 9K medium containing 0.5% glucose. Subsequent transfers were made into fresh media containing decreasing concentrations of ferrous

sulfate. Cells trained to grow on glucose grew well in the absence of iron. Culturing was done in 250-ml Erlenmeyer flasks and the pH of the medium at the time of transfer was 3.2.

Cell-free extracts and enzyme purification. Washed cells were suspended in 0.5M Tris-HCl buffer, pH 7.8 (25-30%, w/v), and passed three times through a cold French-pressure cell (15,000-20,000 p.s.i.) or treated with sonic energy. All operations were performed in the presence of 0.01M sodium thiosulfate. The broken cells were treated with 300 µg each of RNase and DNase at room temperature for 20 min after which the whole cells and debris were removed by centrifugation at 13,000 x g for 20 min. The resulting supernatant, the crude extract, was adjusted to pH 5 by slow addition of cold 1 N acetic acid and stirred in an ice bath for 20 min. Precipitated protein was removed by centrifugation and discarded. Ammonium sulfate was added to 25% saturation and the pH adjusted to 5.0 with stirring for 30 min, after which the precipitate was removed and discarded. More ammonium sulfate was added until 90% saturation was obtained and again the pH was adjusted to 5.0 with stirring for 30 min. The precipitate was collected and suspended in Tris-HCl buffer in .01M sodium thiosulfate and dialyzed against 4 liters of the same buffer.

The dialyzed extract was treated with 20 ml of a slurry of CM-cellulose in 0.025 acetate buffer, pH 5.0, for 1 hr at 4°C. Cellulose with the enzyme absorbed was recovered by centrifugation, and washed with the same volume of buffer at 0.05, 0.1, 0.2 and 0.3 M in the same manner. The enzyme was finally eluted with the same buffer at 0.50 M.

Protein was determined colorimetrically (Lowry) with crystalline bovine albumin as the standard.

Rhodanese assay. The assay for rhodanese was essentially that described by Bowen, Butler and Happold (4) where thiocyanate formed is measured colorimetrically. The reaction mixture contains 500 µmoles of Tris-HCl buffer (pH 8.5), 50 µmoles of sodium thiosulfate, 50 µmoles of potassium cyanide and partially purified enzyme. The reaction vessel was a screw cap test tube and the time of the reaction was 10 min. The reaction was stopped by the addition of 0.5 ml of the ferric nitrate reagent. The tubes were centrifuged to remove protein and the absorbancy measured in a Klett-Summerson 800 colorimeter equipped with a blue filter. A value of 36 Klett units equals 0.1 µmole of thiocyanate.

Electron microscopy. Cells were washed with a low concentration of EDTA solution prior to fixation and then fixed in 1.5% glutaraldehyde in s-collidine buffer (pH 7.6) for 10 min at room temperature, washed once in the same buffer and finally fixed overnight in osmium tetroxide (1.0%) in distilled water. Fixed cells were enrobbed in agar, cut into cubes, dehydrated in an alcohol series, embedded in an epoxy resin contained in gelatin capsules, and following polymerization sectioned in an ultra microtome. Sections were first stained with uranyl acetate and post stained with lead citrate.

RESULTS

Electron microscopy. Figure 1 shows thin sections of *F. ferrooxidans* following growth on the different substrates. Certain structural differences are apparent which reflect metabolic changes associated with changes in substrates. Structural differences are not the major endeavor of this paper, but certain changes are obvious when cells are grown autotrophically (iron or sulfur) or heterotrophically

(glucose). Glucose-grown cells (Fig 1b) contain poly-beta-hydroxybutyrate (P), a bacterial storage product, which is not present in iron or sulfur-grown cells. Also, there are no electron dense bodies (g) in glucose-grown cells compared to autotrophically-grown cultures (Fig 1a). Sulfur-grown cells have a more localized nuclear area (n), rather than a diffuse nucleus of the other cells. We are still uncertain as regards structural changes in the cell envelope; this is being studied for the envelope is important in substrate oxidations.

Iron oxidation. The oxidation of ferrous iron has been studied more extensively than the oxidation of sulfur and glucose, and the status of iron oxidation has been reviewed (7, 15). In simplest terms the oxidation is described as involving:

$$Fe^{++} \longrightarrow \text{cytochrome c} \longrightarrow \text{cytochrome a} \longrightarrow O_2$$

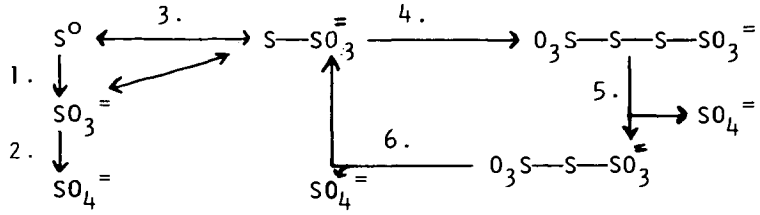
A model has been published for how oxidation might function in ferrobacilli (8).

Glucose oxidation. Iron oxidizing bacteria were once considered to be obligate autotrophs and therefore unable to grow heterotrophically. Results from our laboratory have shown that iron-grown cells could be trained to grow on glucose as the sole energy source (12, 16). How autotrophs might differ from heterotrophs as regards the utilization of simple organic compounds has been discussed (9,23). In iron-oxidizing bacteria, it has been shown that both a glycolytic pathway and a Krebs cycle are probably present (3). Since these are major metabolic routes in heterotrophy for energy and biosynthesis, any transition from autotrophic growth to a heterotrophic way of life for these bacteria should not be a difficult task.

Sulfur oxidation. Sulfur oxidation provides a link to iron oxidation through the $SO_4^=$ anion and its associated H^+ ions. The latter contributes to an acidophilic environment whereas the anion is required for iron oxidation (10); the mechanism by which $SO_4^=$ functions is not known. Earlier reports from this laboratory have described the organisms growth on sulfur (13) as well as the effects of glucose upon sulfur and iron oxidation (19).

The exact pathway for the oxidation of reduced sulfur by the sulfur oxidizing bacteria (Thiobacillus Spp.) is still in doubt, and it is possible that different species of organisms have different oxidative pathways. The status of inorganic sulfur oxidation has been reviewed (27).

For the iron-oxidizing bacteria, which are considered by us to be members of the Thiobacillus group, the below model has been constructed to serve as a guide for the study of sulfur oxidation.



Reaction 1 is catalyzed by the sulfur oxidizing enzyme with the formation of sulfite. This reaction is probably the central reaction for all pathways involving the oxidation of sulfur (24, 25, 15). The sulfur oxidizing enzyme has been found in iron oxidizing bacteria (18). It is possible that $SO_3^=$ exists bound rather than free (15). Sulfite can be oxidized to sulfate (reaction 2) via APS reductase (14) or

mediated by a cytochrome system (5). Both of these reactions can be coupled to energy generation. No energy is associated with reaction 1 for it is not coupled to oxidative phosphorylation. When elemental sulfur is oxidized, thiosulfate is formed enzymatically. Thiosulfate is the substrate for the thiosulfate splitting enzyme rhodanese, (reaction 3) which forms $SO_3^=$. This enzyme may function to keep the level of $SO_3^=$ high and counteract the nonenzymatic formation of thiosulfate. Thiosulfate may also be oxidized via the thiosulfate oxidizing enzyme (reaction 4). This reaction is found in other bacteria including thiobacilli (2, 6, 22) and may represent the major oxidative pathway for thiosulfate (27). Reactions 5 and 6 which involve tetrathionate and trithionate are suspected of being physiological reactions but enzymes catalyzing these reactions have yet to be found (21). It is known that both trithionate and pentathionate are formed non-biologically and are likely to be present whenever other forms of inorganic sulfur are present. In the proposed scheme $SO_4^=$, important for iron oxidation, would be a by-product.

Our present research effort is directed towards the demonstration of these reactions in iron oxidizing bacteria. The latest reaction demonstrated is that catalyzed by the enzyme rhodanese. The enzyme has been isolated and partially purified from both sulfur-grown and iron-grown cells. Rhodanese was purified about 50 fold over crude cell-free extracts, and all preparations are stable at room temperature for about 4 hr and can be stored at 0°C for 5 days with little loss in activity. Table 1 shows that enzyme, thiosulfate and cyanide are all required for the formation of thiocyanate. Neither cysteine, mercaptoethanol nor reduced glutathione (GSH) could replace thiosulfate.

TABLE 1. Requirements and specificity of rhodanese*

Deletions	Additions	μmoles SCN formed
---	---	1.06
KCN	---	0.08
S_2O_3	---	0.11
Enzyme	---	0.07
enzyme	boiled enzyme	0.38
S_2O_3	50 μmoles cysteine	0.12
S_2O_3	0.1% mercaptoethanol	0.14
S_2O_3	50 μmoles GSH	0.11

*The enzyme activity was determined under standard conditions outlined in the Methods. Additions and deletions are as indicated. 0.45 mg protein was used.

The pH optimum of the enzyme ranges from 7.5 to 9.0. Table 2 shows the effects of some inhibitors upon rhodanese activity. The thiol-alkylating agent, iodoacetamide, was the most effective inhibitor. Glucose, not shown in the table, had no effect upon the enzyme.

TABLE II. Effect of various inhibitors on rhodanese activity*

Addition	Concentration	Activity	% of control
None	---	0.87	100
p-hydroxymercuribenzoate	10^{-3}M	0.86	98.9
EDTA	10^{-2}M	0.88	101.1
arsenate	10^{-2}M	0.86	98.9
arsenite	10^{-2}M	0.88	101.1
o-phenanthroline	10^{-3}M	0.87	100
NaF	10^{-2}M	0.85	97.7
$HgCl_2$	10^{-3}M	0.62	71.3
GSH	10^{-2}M	0.83	95.4
N-ethylmaleimide	10^{-3}M	0.69	79.3
Na_2SO_3	10^{-3}M	0.55	63.2
iodoacetamide	10^{-3}M	0.14	16.1
mercaptoethanol	0.1%	0.86	98.9
NaN_3	10^{-3}M	0.88	101.1

*The enzyme activity was determined under standard contitions with additions as indicated. 0.45 mg protein was used. Activity is expressed as the number of μmoles of SCN^- formed per 10 min.

DISCUSSION

From a metabolic standpoint, the iron-oxidizing bacteria represent a very sophisticated group of organisms. They are biosynthetically complete and have evolved in an ecological niche where reduced iron and sulfur as well as acid (H^+) predominate. The organisms are able to use both reduced iron and sulfur for growth and since iron is the more soluble substrate probably prefers it to sulfur even though the energy yield per gram atom of substrate is lower. The requirement for $SO_4^=$ for iron oxidation is met through sulfur oxidation. Also, the acid from the oxidation maintains an acid environment. The organism is able to grow heterotrophically giving it an additional survival advantage. It is not known whether all members of the bacterial population adapt to glucose for an energy source or whether selective cells (mutants) adapt to heterotrophic growth and are selected out by the cultural procedures.

The metabolic-diversity possessed by these bacteria means that any attempt to control their growth with metabolic inhibitors will be difficult, and one must be cognizant of the whole metabolic potential before control is considered.

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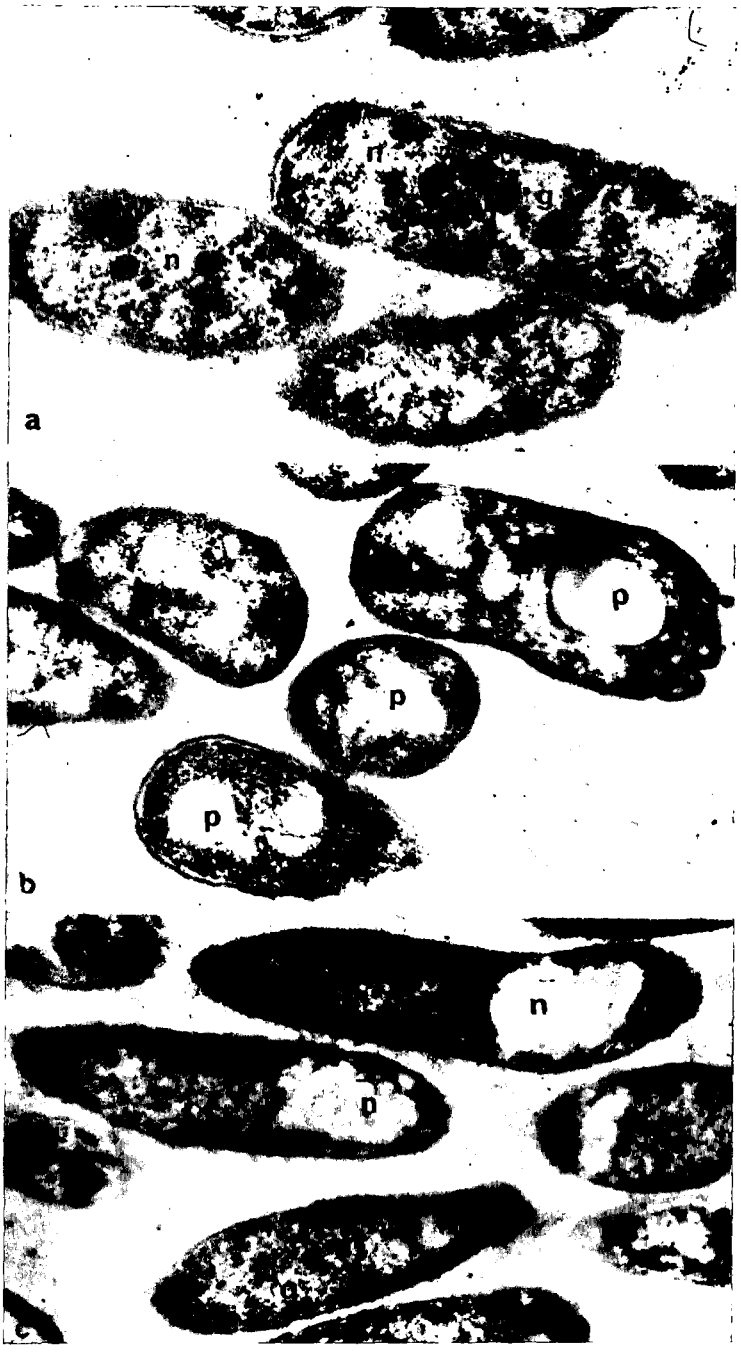

Fig 1. Sections of iron-oxidizing bacteria grown on (a) iron, (b) glucose, (c) sulfur. Label n, nucleus; g, granule; p, poly-beta-hydroxybutyrate. a, 58,000X; b, 30,000X; c, 29,000X.