Reconstitution of Iron Oxidase from Sulfur-Grown *Acidithiobacillus ferrooxidans*^{∇†}

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The iron oxidation system from sulfur-grown *Acidithiobacillus ferrooxidans* ATCC 23270 cells was reconstituted in vitro. Purified rusticyanin, cytochrome *c*, and *aa*₃-type cytochrome oxidase were essential for reconstitution. The iron-oxidizing activity of the reconstituted system was 3.3-fold higher than that of the cell extract from which these components were purified.

Acidithiobacillus ferrooxidans has enzyme systems that can oxidize not only ferrous iron but also reduced sulfur compounds. One big controversy in the study of energy generation in *A. ferrooxidans* is whether the iron oxidation enzyme system, containing rusticyanin, cytochrome *c*, and *aa*₃-type cytochrome *c* oxidase, is involved in sulfur oxidation. Sugio et al. (9, 10) proposed the involvement of an iron oxidation enzyme system in sulfur oxidation of iron-grown *A. ferrooxidans* cells by the oxidation of ferrous iron produced from the reduction of ferric iron by sulfide:ferric ion oxidoreductase ($\text{H}_2\text{S} + 6\text{Fe}^{3+} + 3\text{H}_2\text{O} \rightarrow \text{H}_2\text{SO}_3 + 6\text{Fe}^{2+} + 6\text{H}^+$) and sulfite:ferric ion oxidoreductase ($\text{H}_2\text{SO}_3 + 2\text{Fe}^{3+} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{SO}_4 + 2\text{Fe}^{2+} + 2\text{H}^+$). In contrast, some other researchers suggest the involvement of sulfide:quinone oxidoreductase, from *A. ferrooxidans* NASF-1 (15), and thiosulfate:quinone oxidoreductase, from *A. ferrooxidans* ATCC 19859 (2), in electron transport from sulfide and thiosulfate, respectively, to the quinone pool, and then the electrons may be transported directly from the quinone pool to *bd* quinol oxidase or to *ba*₃ cytochrome *c* oxidase via the *bc*₁ II complex and *c*₄ cytochrome (2). In this model, *bd* quinol oxidase and *ba*₃ cytochrome oxidase, but not *aa*₃-type cytochrome *c* oxidase, are working as the terminal oxidases in sulfur oxidation.

In this work we partially purified rusticyanin and cytochrome *c* as well as *aa*₃-type cytochrome *c* oxidase from sulfur-grown *A. ferrooxidans* ATCC 23270 cells and show for the first time that ferrous iron oxidation activity, higher than that observed in the cell extract, could be detected with the combination of all of these three components.

In brief (detailed methods can be found in the supplemental material), *A. ferrooxidans* ATCC 23270 was grown in sulfur medium in the absence of iron for 4 days and then harvested as described by Taha et al. (12). Crude cell extract was prepared by solubilization of the resting cells in 0.1 M β -alanine- SO_4^{2-} buffer (pH 2) containing 2% 1-*O*-*n*-octyl- β -D-glucopyranoside

(OGL) and 1 M Na_2SO_4 for 60 min. Rusticyanin, *aa*₃-type cytochrome *c* oxidase, and cytochrome *c* were purified from the solubilized crude extract using ammonium sulfate precipitation and Phenyl-650 M and Superdex 75 column chromatographies. Ferrous iron oxidation activity was measured by the *o*-phenanthroline method (8, 11). Protein concentration was measured by the method of Lowry et al. (6). The spectra of cytochrome *c*, *aa*₃-type cytochrome oxidase, and rusticyanin were measured with a Shimadzu UV-1700 spectrophotometer. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was done by the method of Laemmli (5). After electrophoresis, gels were stained by Coomassie blue or the protein bands on the gel were transferred to a polyvinylidene difluoride membrane by following the technique described by Ausubel et al. (1). The intended bands were excised, dried at room temperature, and kept at -20°C until sequencing by an Applied Biosystems 491 protein sequencer.

The genes involved in ferrous iron oxidation by *A. ferrooxidans* cells form the *rus* operon which encodes two cytochromes *c*, *aa*₃-type cytochrome *c* oxidase, and rusticyanin (17). Therefore, we tried to purify these components from sulfur-grown *A. ferrooxidans* ATCC 23270 cells. SDS-PAGE of the partially purified components (Fig. 1) showed only one band at 18.0 kDa in the rusticyanin fraction and three bands with molecular sizes of 57.2 (α -band), 25.1 (β -band), and 20.1 (γ -band) kDa in *aa*₃-type cytochrome *c* oxidase. Two bands at 47.0 and 25.0 kDa were detected in the cytochrome *c* fraction, suggesting the existence of the two cytochromes *c* encoded by *rus* operon genes *Cyc2* and *Cyc1*, respectively (16, 17). Spectral analysis of partially purified rusticyanin, cytochrome *c*, and *aa*₃-type cytochrome *c* oxidase fractions were in complete accordance with the literature (3, 4, 7, 11) (data not shown). N-terminal amino acid sequencing of 10 residues of the 18-kDa band of the rusticyanin fraction, the 20.1-kDa band of the *aa*₃-type cytochrome *c* oxidase fraction (γ -band), and the 25.0-kDa and 47.0-kDa bands of the cytochrome *c* fraction were carried out. The obtained sequences were GTLDTTWKEA, AAKKGMT TVL, AVGSADAPAP, and LPSFARQTGW, respectively. Searching for these sequences using the websites of The Institute for Genomic Research (<http://tigrblast.tigr.org/cmrb-blast/>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and the GENETYX

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FIG. 1. Coomassie blue-stained SDS-PAGE of partially purified rusticyanin (left), aa_3 -type cytochrome c oxidase (middle), and cytochrome c (right) from sulfur-grown *A. ferrooxidans* ATCC 23270. The molecular masses of the protein size marker and the partially purified rusticyanin, aa_3 -type cytochrome oxidase, and cytochrome c are expressed in kDa. Five micrograms from each fraction was applied to the gel. In the case of rusticyanin, 15% polyacrylamide gel was used, while in the case of aa_3 -type cytochrome c oxidase and cytochrome c , 12.5% polyacrylamide gel was used.

software showed that the above sequences are the same as those of rusticyanin, the γ -band of aa_3 -type cytochrome c oxidase, Cyc1 cytochrome, and Cyc2 cytochrome of *A. ferrooxidans* ATCC 23270, respectively.

The abilities of these partially purified proteins from sulfur-grown *A. ferrooxidans* ATCC 23270 cells to oxidize ferrous iron were studied either separately or in combinations. The combination of these three fractions showed higher activity (3.9 nmol Fe^{2+} oxidized $\text{mg protein}^{-1} \text{ min}^{-1}$) than that of the cell extract, from which these components were purified (1.2 nmol Fe^{2+} oxidized $\text{mg protein}^{-1} \text{ min}^{-1}$) (Table 1). These results indicate that rusticyanin, two cytochromes c (Cyc1 and Cyc2), and aa_3 -type cytochrome c oxidase are the essential components of the iron oxidation system in the sulfur-grown cells. The low enrichment factor (3.3-fold) may be due to the very complicated nature of the iron oxidation enzyme system of *A. ferrooxidans* because (i) it is composed of at least four protein components, rusticyanin, two cytochromes c (Cyc1 and Cyc2), and aa_3 -type cytochrome c oxidase, and (ii) these components

TABLE 1. Reconstitution of ferrous iron oxidation activity from the partially purified rusticyanin, cytochrome c , and aa_3 -type cytochrome c oxidase from sulfur-grown *A. ferrooxidans* ATCC 23270 cells separately or in combination^a

Protein combination ^b	Iron oxidase activity (nmol oxidized Fe^{2+} $\text{mg protein}^{-1} \text{ min}^{-1}$)
A.....	0.0
B.....	0.0
C.....	0.0
D.....	0.0
E.....	0.28
F.....	0.17
G.....	3.9
H.....	1.2

^a 0.1 mg protein of each component was used separately or in combination.

^b Protein combinations used: A, cytochrome c ; B, aa_3 -type cytochrome c oxidase; C, rusticyanin; D, cytochrome c + aa_3 -type cytochrome c oxidase; E, cytochrome c + rusticyanin; F, rusticyanin + aa_3 -type cytochrome c oxidase; G, rusticyanin + aa_3 -type cytochrome c oxidase + cytochrome c ; H, cell extract.

are localized in different cell loci (outer membrane, periplasmic space, and inner membrane) with proper configurations in the intact cells of *A. ferrooxidans*. We observed that a high iron-oxidizing activity of the intact cells decreased dramatically when a cell-free iron oxidation system was prepared. These results suggest that the functional localizations and/or configurations of the four components cannot be achieved in an in vitro assay system, neither with the crude cell extract nor with the purified reconstituted system. This explanation is strongly supported by the observation that the iron oxidation activity of the resting cells of *A. ferrooxidans* ATCC 23270 dramatically decreased compared to that of strain TI-1, a moderately thermophilic iron-oxidizing bacterium with a simple iron oxidation system composing only aa_3 -type cytochrome c oxidase but not rusticyanin and cytochrome c (13, 14). The direct extraction method, used in this study, gave approximately one-five hundredth of the iron oxidation activity of not only sulfur-grown but also iron-grown ATCC 23270 cells. Although an improvement should be done to prepare a more-active cell-free system, we are thinking that the dramatic decrease of iron-oxidizing activity after disrupting the intact cells is an intrinsic property of the elegant and complicated iron oxidation system of *A. ferrooxidans*. The possibility that our reconstituted system might miss some important components for iron oxidation seems to be very weak, because in the absence of any of the four essential components, activity could not be detected (Table 1).

To date there is no report of the reconstitution of the ferrous iron oxidation system from sulfur-grown *A. ferrooxidans* ATCC 23270 cells. In this report, all the components needed for iron oxidation by iron-grown *A. ferrooxidans* ATCC 23270 cells, such as rusticyanin, cytochromes c , and aa_3 -type cytochrome c oxidase, were purified from sulfur-grown *A. ferrooxidans* ATCC 23270 cells. We showed that iron oxidation activity approximately 3.3-fold higher than that observed in the cell extract was detected when all three fractions purified from sulfur-grown ATCC 23270 cells were added together to the reaction mixture. However, the addition of these fractions separately or in combinations of two did not show any iron oxidation activity. These results indicate that the iron oxidation enzyme system of sulfur-grown *A. ferrooxidans* ATCC 23270 cells was reconstituted for the first time with rusticyanin, two cytochromes c (Cyc1 and Cyc2), and aa_3 -type cytochrome c oxidase from sulfur-grown cells.

To clarify the sulfur oxidation mechanism in an industrially important bacterium more precisely, we are now studying whether both the cytochrome c and aa_3 -type cytochrome c oxidase purified from sulfur-grown *A. ferrooxidans* ATCC 23270 cells can be reduced enzymatically in the presence of reduced sulfur compounds.

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