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Purification, and some molecular and enzymatic features of a novel *ccb*-type cytochrome *c* oxidase from a microaerobic denitrifier, *Magnetospirillum magnetotacticum*

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Abstract

A novel *ccb*-type cytochrome *c* oxidase was purified from the magnetic bacterium, *Magnetospirillum magnetotacticum* MS-1. The enzyme was composed of three subunits with M_r's of 43,000, 34,000 and 28,000, respectively, and contained 0.91 mol of protoheme, 2.0 mol of heme *c* and 0.70 g atom of copper per mol of minimal structural unit. One mol of enzyme oxidized 187 mol of horse heart ferrocytochrome *c* and 34.4 mol of *M. magnetotacticum* ferrocytochrome c_{550}/s. The cytochrome *c* oxidase activity of the enzyme was 50% inhibited by 12 μM KCN. The enzyme seems to function as the terminal oxidase in microaerobic respiration.

Key words: Cytochrome *c* oxidase; Microaerobic respiration; Magnetic bacterium; *Magnetospirillum magnetotacticum*

1. Introduction

Magnetospirillum (formerly *Aquaspirillum*) *magnetotacticum* is a non-fermentative bacterium, which was isolated from freshwater sediments in 1979 [1]. The bacterium contains intracellular particles called magnetosomes, which are composed of magnetite (Fe_3O_4) [2] and enclosed by lipid bilayers [3]. Although *M. magnetotacticum* obtains energy for life processes by denitrification, the bacterium can not grow under strict anaerobic conditions with NO_3^- [1,4,5]. Furthermore, magnetite biosynthesis is depressed when the bacterium is cultured under high O_2 concentrations [6]. These results suggest that the bacterium appears to be a denitrifier with an absolute requirement for O_2, and the microaerobic denitrifying condition may be closely related to the physiological functions of the magnetosomes in *M. magnetotacticum*.

The terminal oxidase of the respiratory chain is a key enzyme which plays significant roles in providing organisms with energy for life processes. In the mitochondrial respiratory chain, the aa_3-type cytochrome *c* oxidase acts mainly as a terminal oxidase, while in the bacterial aerobic respiratory chain, many types of cytochrome *c* oxidases function as terminal oxidases. Cytochrome aa_3 [7], cytochrome caa_3 [8], cytochrome baa_3 [9], cytochrome ba_3 [10] cytochrome *co* [11] and cytochrome *cao* [12] have been purified from various prokaryotes, and some enzymatic and molecular properties of the enzymes have been studied.

O'Brien et al. have proposed, following spectrophotometric analyses, that *M. magnetotacticum* may contain two types of terminal oxidases, an a_1-type cytochrome and an *o*-type cytochrome [13]. Both types of the terminal oxidases are thought to be constantly expressed when the bacterium is cultivated under microaerobic conditions. Recently, we have purified the hemoprotein from *M. magnetotacticum* which shows similar spectral properties to those of 'cytochrome a_1' [14]. However, the hemoprotein did not contain heme *a* but two types of novel hemes, and hardly showed cytochrome *c* oxidase activity. On the basis of these results, we concluded that 'cytochrome a_1' is not the terminal oxidase in the respiratory chain of *M. magnetotacticum*.

In the present study, we tried to purify the terminal oxidase from *M. magnetotacticum*. We succeeded in purifying a novel *ccb*-type cytochrome *c* oxidase from the bacterium, and have characterized some of its enzymatic and molecular properties.

2. Materials and methods

2.1. Microorganism

M. magnetotacticum (ATCC 31632) was cultivated microaerobically in a chemically defined medium [1] with slight modifications as previously described [14].

2.2. Preparation of the membrane fraction from M. magnetotacticum

Frozen cells (about 20 g wet weight) were thawed, suspended in 10 mM Tris-HCl buffer (pH 8.0) and treated with a sonic oscillator (20 kHz, 250 W) for a total period of 20 min. Unbroken cells and magnetosomes were removed by centrifugation at 10,000 × *g* for 15 min. The supernatant obtained was centrifuged at 104,000 × *g* for 1 h and the pellet obtained was resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl. The suspension was gently stirred at 4°C for 1 h and centrifuged at 104,000 × *g* for 1 h to remove the soluble proteins weakly bound to the membrane. The pellet thus obtained was used as the membrane fraction.

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2.3. Reagents

Horse heart cytochrome *c* (Type VI) was purchased from Sigma Chemical (USA). *M. magnetotacticum* cytochrome c_{550} was purified by the method of Fukumori et al. [15]. Sucrose monocaprate was purchased from Mitsubishi-Kasei Food Corp. (Tokyo). DEAE-Toyopearl was a product of Tosoh Corp. (Tokyo). Tween 20 was a product of Wako Pure Chemical Industries (Osaka). All other chemicals were of the highest grade commercially available.

2.4. Physical and chemical measurements

Spectrophotometric measurements were carried out with a Shimadzu MPS-2000 spectrophotometer, using 1 cm light path cuvettes at room temperature. Polyacrylamide gel electrophoresis in the presence of Tween 20 was performed by the method of Davis [16]. The presence of hemes and the oxidase activity in the gel were detected by using heme staining reagents [17] and 'Nadi' reagents [18], respectively. Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli [19]. The sample was treated with 5% SDS in the presence of 5% β-mercaptoethanol at room temperature for 18 h. Protein concentration was determined by the method of Lowry et al. [20] with slight modifications [21]. The metal content was determined by inductively coupled plasma atomic emission spectrometry measurements using a Plasma Spectrometer SPS 1500 VR (Seiko Instruments Inc.). Hemes in the enzyme were extracted with a mixture of 0.25 N HCl and 80% acetone from the enzyme, extracted with ethylacetate and washed with 5% KCl. The contents of protoheme and heme *c* were determined on the basis of millimolar extinction coefficients at the α peaks of their pyridine ferrohemochromes and were 34.4 [22] and 29.1 [23] for the hemochromes of protoheme and heme *c*, respectively.

2.5. Enzymatic assay

Cytochrome *c* oxidase activity was determined spectrophotometrically by following the decrease in absorbance at 550 nm. The standard reaction mixture contained 20 μM horse heart ferrocytochrome *c* and 2 nM enzyme in 10 mM Tris-HCl buffer (pH 8.0) containing 0.2% Tween 20 in a total volume of 1 ml. In the determination of *M. magnetotacticum* ferrocytochrome c_{550} oxidase activity, the reaction mixture contained 8.0 μM *M. magnetotacticum* ferrocytochrome c_{550} and 2 nM enzyme in 10 mM sodium phosphate buffer (pH 6.0) containing 0.2% Tween 20 in a total volume of 1.0 ml. The reactions were initiated by adding 10 μl of 0.2 μM enzyme.

3. Results

3.1. Purification of the oxidase

The membrane fraction prepared from magnetic cells of *M. magnetotacticum*, as described in section 2, was suspended in 10 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer for 18 h at 4°C. In order to solubilize the enzyme, sucrose monocaprate was added to the suspension to give a final concentration of 1%. The suspension was gently stirred for 20 h at 4°C, and then centrifuged at 104,000 × *g* for 1 h. Cytochrome *c* oxidase was almost completely solubilized with this treatment. The supernatant thus obtained was subjected to chromatography on a DEAE-Toyopearl column (2 × 10 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.3% sucrose monocaprate. Cytochrome *c* oxidase passed through the column while all other membrane-bound cytochromes were adsorbed on the column. To the eluates containing cytochrome *c* oxidase, 20% Tween 20 was added to give a final concentration of 0.5%. The enzyme solution was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 0.2% Tween 20 for 3 h, and subjected to chromatography on a DEAE- Toyopearl column (2 × 4 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.2% Tween 20. Cytochrome *c* oxidase adsorbed on the column was eluted with a linear gradient of NaCl produced from 250 ml each of 10 mM Tris-HCl buffer (pH 8.0) containing 0.2% Tween 20 and buffer containing 0.2% Tween 20 and 0.15 M NaCl. The enzyme was eluted at about 20 mM NaCl. The fraction containing cytochrome *c* oxidase was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 0.2% Tween 20, and used as the purified enzyme preparation. The purification procedure of cytochrome *c* oxidase from *M. magnetotacticum* is summarized in Table 1. The purified enzyme contained 19 nmol of heme *c* per mg protein, and the total recovery of activity was approximately 35%.

When the preparation was subjected to polyacrylamide gel electrophoresis in the absence of SDS, one major band was stained with Coomassie brilliant blue (Fig. 1). Furthermore, this band was also stained with heme-staining reagents and Nadi reagents.

3.2. Spectral properties of the oxidase

Fig. 2 shows the absorption spectra of the oxidized and reduced forms of *M. magnetotacticum* cytochrome *c* oxidase. The enzyme showed absorption peaks at 410 nm in the oxidized form, and at 417, 520 and 550 nm, and a shoulder at 560 nm in the reduced form. In the oxidized form, a broad peak at around 830 nm was not

Table 1
Purification of cytochrome *c* oxidase from *M. magnetotacticum*

Experimental steps	Total volume (ml)	Protein concn. (mg/ml)	Total protein (mg)	Total activity (U)[a]	Purification[b]	Recovery[c] %	Cyt. *c* oxidase (nmol)[d]
Membrane fraction	50.0	2.99	150	236	1	100	n.d.[e]
Sucrose monocaprate extract	58.0	1.70	98.6	2.50	1.61	106	n.d.[e]
1st DEAE passed fraction	66.0	0.282	18.6	241	8.24	102	36.7
Purified enzyme	24.0	0.0533	1.28	83.1	41.3	35.3	13.6

[a] A unit of the enzyme was defined as 1 μmol of horse heart ferrocytochrome *c* oxidized per min.
[b] Calculated from the specific activity (units per mg protein).
[c] Calculated from the total activity (units).
[d] Determined on the basis of millimolar extinction coefficient at 550 nm of reduced enzyme, 19.0 per Fe (see text).
[e] n.d., not determined.

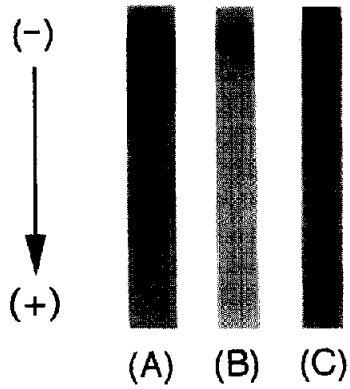

Fig. 1. Polyacrylamide gel electrophoresis of *M. magnetotacticum* cytochrome *c* oxidase in the presence of 0.2% Tween 20. After the electrophoresis, the gel was cut into three parts and these were separately stained with (A) Coomassie brilliant blue, (B) heme-staining reagents and (C) Nadi reagents, respectively.

observed (data not shown). To determine the prosthetic groups of the enzyme, the hemes were extracted from the enzyme by the method described in section 2. The pyridine ferrohemochrome of the HCl/acetone-extracted heme showed a single α peak at 556 nm, and that of the precipitate obtained when the enzyme was treated with HCl/acetone showed an α peak at 550 nm. Heme *o* and heme *a* were not detected in the HCl/acetone extract by reverse-phase HPLC analysis (data not shown). These results suggest that *M. magnetotacticum* cytochrome *c* oxidase contains two types of hemes, protoheme and heme *c*. Fig. 3 shows reduced + CO minus reduced difference spectrum of the enzyme. It showed absorption peaks at 415, 535 and 569 nm, and troughs at 429, 517 and 550 nm, and a shoulder at 560 nm. These spectral features are quite similar to those of the *cb*-type cytochrome *c* oxidase with a high spin protoheme [24].

3.3. Molecular and enzymatic properties of the oxidase

The subunit structure of *M. magnetotacticum* cytochrome *c* oxidase was studied by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 4). When the enzyme treated with 5% SDS in the presence of 5% β-mercaptoethanol was subjected to SDS-PAGE, three bands corresponding to M_r's of 43,000, 34,000 and 28,000, respectively, were observed on staining with Coomassie brilliant blue.

The metal content of the enzyme was estimated from the metal and protein contents. The purified enzyme contained 29 nmol of Fe per mg protein and 6.7 nmol Cu per mg protein. These results suggest that the enzyme contains 3.0 iron atoms and 0.70 copper atoms in the minimal structural unit, which is assumed to be composed of one molecule each of the three subunits with M_r's of 43,000, 34,000 and 28,000, respectively. The millimolar extinction coefficient at 550 nm of the reduced enzyme was calculated to be 19.0 per Fe on the basis of the iron content and the absorbance at 550 nm in the reduced form.

The heme content of the enzyme was determined by pyridine ferrohemochrome spectra of the HCl/acetone-extracted heme and that of the precipitate obtained when the enzyme was treated with HCl/acetone. 0.42 nmol of the enzyme, which was calculated on the basis of the millimolar extinction coefficient of the enzyme as described above, contained 0.38 nmol of protoheme and 0.83 nmol of heme *c*. The ratio of heme *c* to protoheme was calculated to be 2:1. These results suggests that the enzyme contained one molecule of protoheme and two molecules of heme *c* in the minimal structural unit.

M. magnetotacticum cytochrome *c* oxidase rapidly oxidized horse ferrocytochrome *c* and *M. magnetotacticum* ferrocytochrome c_{550}. The optimal pH for the cytochrome *c* oxidizing activity of the enzyme was found at pH 8.0 and 6.0 when horse heart ferrocytochrome *c* and *M. magnetotacticum* ferrocytochrome c_{550} were used as the electron donor, respectively. The V_{max} values (mol of ferrocytochromes *c* oxidized per s per mol of enzyme) were 187 with horse heart cytochrome *c* and 37.4 with *M. magnetotacticum* cytochrome c_{550}. The K_m value for horse heart cytochrome *c* was 12.2 μM, while that for *M. magnetotacticum* cytochrome c_{550} was 3.02 μM. The oxidation of horse heart ferrocytochrome *c* was about 50% inhibited by 12 μM KCN.

4. Discussion

A novel type of cytochrome *c* oxidase was purified from the microaerobic denitrifying magnetic bacterium, *M. magnetotacticum*, to an electrophoretically homoge-

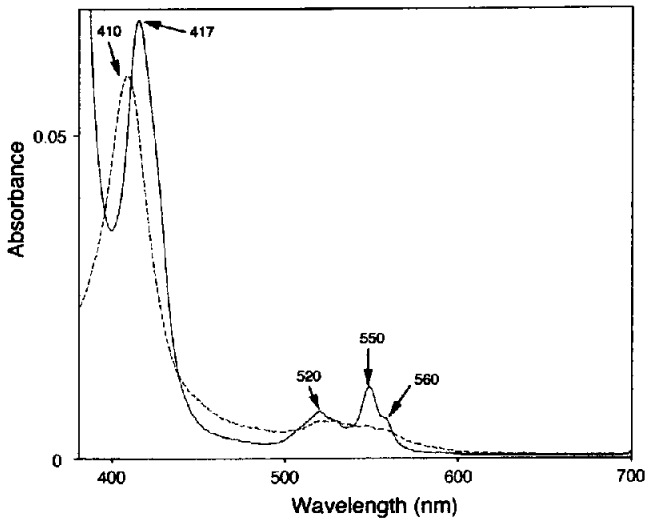

Fig. 2. Absorption spectra of *M. magnetotacticum* cytochrome *c* oxidase. The oxidase at a concentration of 0.185 μM was dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 0.2% Tween 20. (---) Oxidized form; (——) reduced form prepared by the addition of a small amount of $Na_2S_2O_4$

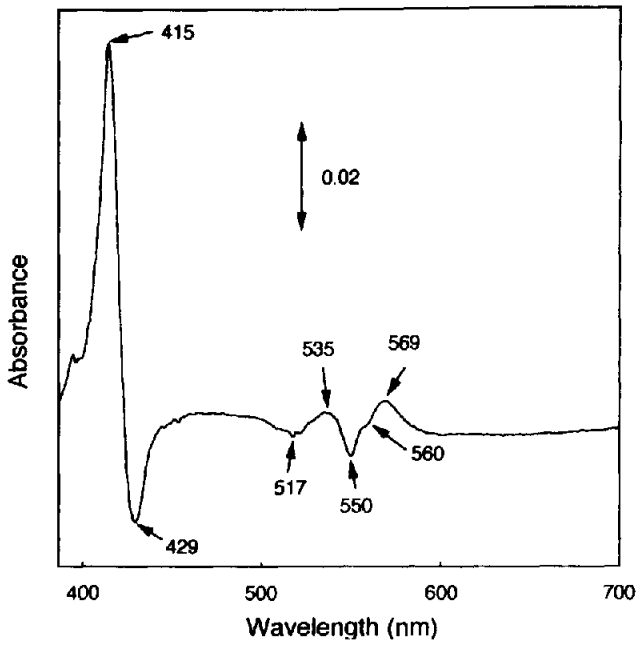

Fig. 3. Reduced+CO minus reduced difference spectrum of *M. magnetotacticum* cytochrome *c* oxidase. The enzyme was dissolved in 100 mM Tris-HCl buffer, pH 8.0, containing 0.2% Tween 20. The reduced form of the enzyme was prepared by the addition of a small amount of $Na_2S_2O_4$. CO was bubbled through the reduced form of the enzyme.

neous state. The enzyme has two molecules of heme *c*, one molecule of protoheme and approximately one atom of copper in the minimal structural unit which is composed of one molecule each of three kinds of subunits with M_r's of 43,000, 34,000 and 28,000, respectively. Further, the absorption spectrum in the near infrared region suggests that the enzyme seems to contain no Cu_A in the molecule.

The spectral properties of *M. magnetotacticum* cytochrome *c* oxidase closely resemble those of *co*-type cytochrome *c* oxidases purified from *Rhodopsudomonas palustris* [11], *Pseudomonas aeruginosa* [25], *Methylophilus methylotrophus* [26] and *Pseudomonas stutzeri* [27]. Further, the CO+reduced minus reduced difference spectrum of the enzyme is also similar to those of *co*-type cytochrome *c* oxidases [25–27]. However, the molecular properties of *M. magnetotacticum* cytochrome *c* oxidase purified in the present study are quite different from those of *co*-type cytochrome *c* oxidases. First, *M. magnetotacticum* cytochrome *c* oxidase is composed of three kinds of subunits of M_r's of 43,000, 34,000 and 28,000, respectively, while *co*-type cytochrome *c* oxidases of *R. palustris* and *P. aeruginosa* are composed of four kinds of subunits with M_r's of 30,500, 25,500, 12,200 and 9,500, and 29,000, 21,000, 15,000 and 9,500, respectively [11,25]. Further, cytochrome *c* oxidase of *M. methylotrophus* is composed of two kinds of subunits with M_r's of 31,500 and 23,800, respectively [26]. Secondly, *M. magnetotacticum* cytochrome *c* oxidase contains two molecules of heme *c*, one molecule of high spin protoheme and probably one atom of copper in the minimal structural unit. Although the heme composition of most of the *co*-type cytochrome *c* oxidases has not been studied stoichiometrically, cytochrome *c* oxidase of *M. methylotrophus* contains equal amounts of protoheme and heme *c* [26]. These results show that the cytochrome *c* oxidase of *M. magnetotacticum* purified in the present study is different from the *co*-type cytochrome *c* oxidase, and is classified as a *ccb*-type cytochrome *c* oxidase.

Recently, cytochrome *c* oxidases which contain heme *c* and protoheme in the molecule have been identified in *Bradyrhizobium japonicum* [28], *Rhodobacter sphaeroides* [29] and *Rhodobacter capsulatus*[24]. The spectral features and the subunit composition of these enzymes closely resemble those of *M. magnetotacticum* cytochrome *c* oxidase. Further, the enzymes are induced when the organisms are grown under microaerobic condition. *M. magnetotacticum* is a microaerophilic bacterium, and is associated with the a group of Proteobacteria [30]. Therefore, it seems likely that the cytochrome *c* oxidase purified in the present study is the same type as those enzymes, and heme *c*- and high spin protoheme-containing cytochrome *c* oxidase is the general type of the terminal oxidase participating under microaerobic conditions.

Recently we purified a cytochrome cd_1-type nitrite reductase from the soluble fraction of *M. magnetotacticum* [15]. Although the cytochrome cd_1 showed high TMPD-nitrite oxidoreductase activity and TMPD-oxidase activity, *M. magnetotacticum* ferrocytochrome c_{550} and *P. aeruginosa* ferrocytochrome c_{551} were not oxidized with cytochrome cd_1 in the presence of nitrite and/or oxygen. On the other hand, the cytochrome *c* oxidase purified in the present study shows high ferrocytochrome

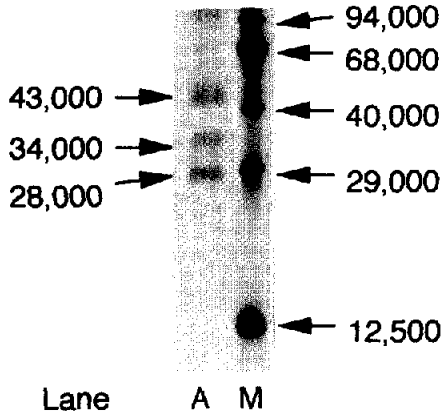

Fig. 4. Polyacrylamide gel electrophoresis of *M. magnetotacticum* cytochrome *c* oxidase in the presence of SDS. Electrophoresis was carried out as described in section 2. Lane A, the enzyme was treated as described in section 2. Lane M, marker proteins: phosphorylase *a* (M_r 94,000), bovine serum albumin (M_r 68,000), aldolase (M_r 40,000), carbonic anhydrase (M_r 29,000) and horse heart cytochrome *c* (M_r 12,500). Each lane was stained with Coomassie brilliant blue.

c oxidizing activity; 1 mol of the enzyme oxidizes 34.4 mol of *M. magnetotacticum* cytochrome c_{550}/s. Furthermore, *M. magnetotacticum* can not grow under strict anaerobic conditions with NO_3^- [1,4,5]; the bacterium requires oxygen for the growth. These results suggest that the bacterium may obtain energy for life process by aerobic respiration in which the cytochrome *c* oxidase purified in the present study functions as the terminal oxidase.

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