o-TYPE CYTOCHROME OXIDASE IN THE MEMBRANE OF AEROBICALLY GROWN *PSEUDOMONAS AERUGINOSA*

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1. Introduction

A variety of cytochrome oxidases, including the aa_3-type, a_1-type, *d*-type and *o*-type, have been identified in bacteria. Many aa_3-type bacterial cytochrome oxidases have been purified and characterized [1–7]. These studies have contributed much to the analysis of cytochrome oxidase; but, except for the soluble *Vitreoscilla* cytochrome *o*, no *o*-type cytochrome oxidase has been characterized completely in spite of its extensive distribution in bacteria [8–10]. Membrane-bound *o*-type cytochrome oxidases have been studied in *Azotobacter vinelandii* [11–13] and *Rhodopseudomonas palustris* [14]. In the case where these oxidases show high activity, they contain a *c*-type cytochrome the role of which is not known.

In *Pseudomonas aeruginosa* grown aerobically, *o*-type cytochrome may have function as the only terminal oxidase in the membrane-bound, electron-transport system [15]. Thus, we solubilized and purified the *o*-type cytochrome oxidase from the membrane of aerobically grown *P. aeruginosa*. This oxidase consists of 4 polypeptides that include cytochrome *o* and cytochrome *c*, both of which react with carbon monoxide.

2. Materials and methods

2.1. *Strain, growth and preparation of membranes*

Pseudomonas aeruginosa IFO 3445 was used throughout this study. Growth conditions and the preparation of membranes have been described in [16].

2.2. *Purification of cytochrome oxidase from* P. aeruginosa

All operations were performed at 0–4°C. Membranes were treated with cholate plus deoxycholate [16], then washed with 1% cholate plus 1 M KCl. The residual precipitate (600–800 mg protein) was extracted with 2% Triton X-100 overnight in 0.1 M Tris–HCl buffer (pH 7.5 at 20°C) at 10 mg protein/ml. The extract was diluted with the same volume of cold distilled water and immediately applied to a DEAE-cellulose column (2.4 × 15 cm) pre-equilibrated with 0.05 M Tris–HCl buffer (pH 7.5). The column was washed with 150 ml the same buffer containing 0.05% Brij 58, after which the oxidase was eluted with 150 ml of the same buffer containing 0.15 M KCl. Fractions which were eluted first that had high specific activity were combined, then diluted 4-fold with cold water after which they were applied to a DEAE-cellulose column (2.4 × 7 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.05% Brij 58. After washing the column with 150 ml of the same buffer, the oxidase was eluted with a linear gradient of 150 ml each of the above buffer and of the same buffer containing 0.3 M KCl. At ~0.15 M KCl, the oxidase activity was eluted as a single symmetrical peak that coincided with the peaks of protein and cytochrome. The orange–yellow active fraction was concentrated with an ultrafilter then used as the purified oxidase.

2.3. *Analytical procedures*

Cytochrome oxidase activity was assayed by measuring *N,N,N′,N′*-tetramethyl-*p*-phenylene diamine (TMPD) oxidase activity. The reaction mixture consisted of 33 mM potassium phosphate buffer (pH 7.0), 1.8 mM TMPD, 0.067% Tween 20 and the enzyme. Activity was measured by following the increase in absorption at 520 nm at 25°C, and was expressed as μmol oxidized TMPD, based on 6.1 as the mM extinction coefficient. Heme and protein contents were

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determined as in [15]. Iron and copper contents were estimated with a Shimadzu AA-640 atomic absorption spectrophotometer for 3 different enzyme preparations dialyzed overnight against 0.01 M Tris–HCl buffer (pH 7.5) containing 1 mM EDTA.

3. Results

Cytochrome oxidase can be solubilized directly from the membrane of *P. aeruginosa* with 1–2% Triton X-100 in the presence of 1 M KCl. To purify the oxidase, however, it was extracted with 2% Triton X-100 alone after being treated with cholate–deoxycholate followed by cholate–KCl treatment. Purification of the solubilized oxidase was achieved by repeating the chromatography on DEAE-cellulose in the presence of 0.05% Brij 58. This gave a reproducible result although, in the final step, it produced a decrease in the specific activity of the TMPD oxidase. Results of a typical purification are presented in table 1. Purification was 15-fold for TMPD oxidase activity and 34-fold for the CO-binding activity in the final step. The activity was increased by changing the detergent system used in the purification process (not shown). Further chromatographic purification on hydroxyapatite or gel filtration resulted in a decrease in enzyme activity with no change in the peptide and cytochrome compositions of the enzyme preparation. Fig.1 shows the electrophoretic pattern of the purified oxidase on urea–SDS–polyacrylamide gel. Four major bands were detected together with a small portion of minor peptide with higher M_r-values, when the oxidase was treated at <60°C. The oxidase heated at 90°C appeared to be aggregated. The app. M_r-values of the major proteins were 29 000, 21 000, 11 500 and 9500. The 29 000 and 21 000-dalton bands were stained for heme-catalyzing peroxidase activity [17].

Fig.1. Electrophoretic pattern of purified *Pseudomonas o*-type cytochrome oxidase. The purified oxidase (30 μg) was heated at 30°C, 60°C or 90°C (left to right) for 30 min in 2% SDS and 2% β-mercaptoethanol, then it was subjected to electrophoresis on a slab gel containing 8% acrylamide, 0.5% SDS and 8 M urea.

The purified oxidase had an absorption spectrum consisting of *c*- and *b*-type cytochromes (fig.2). The absorption maxima were at 419, 522, 552 and 560 nm

Table 1
Purification of *Pseudomonas aeruginosa o*-type cytochrome oxidase

Fraction	Total protein	TMPD oxidase[a]		CO-binding[b]	
	(mg)	Total act. (units)	Spec. act. (units/mg)	Total cont. (nmol)	Spec. cont. (nmol/mg)
Initial membrane	972	3890	4.0	146	0.15
Washed membrane	711	3840	5.4	128	0.18
Triton extract	83.6	3860	46.2	92.8	1.11
1st DEAE cellulose	12.8	1420	111.0	58.6	4.58
2nd DEAE cellulose	6.7	417	62.2	34.6	5.16

[a] TMPD oxidase activity was measured as in the text
[b] CO-binding activity was estimated from the CO-reduced minus the reduced difference spectrum in 0.05 M Tris–HCl buffer (pH 7.5) containing 1% Tween 20 based on a mM extinction coefficient of 170 [19] at 415–429 nm

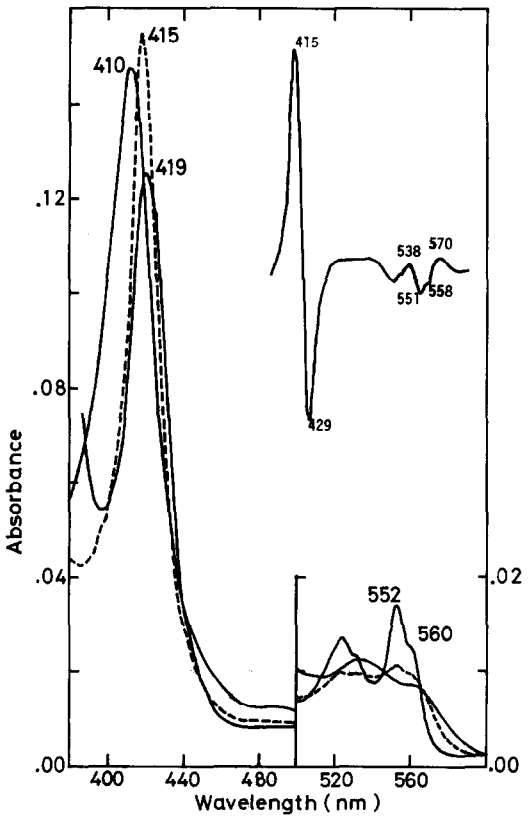

Fig.2. Absorption spectra of purified *Pseudomonas o*-type cytochrome oxidase. Spectra were recorded at room temperature and at 0.1 mg protein/ml in 0.05 M Tris–HCl buffer (pH 7.5) containing 0.05% Brij 58. (——) oxidized and dithionite-reduced forms; (– – –) the sample was reduced with dithionite after which CO was bubbled through the solution for 1 min. The insertion shows the CO + reduced minus reduced difference spectrum.

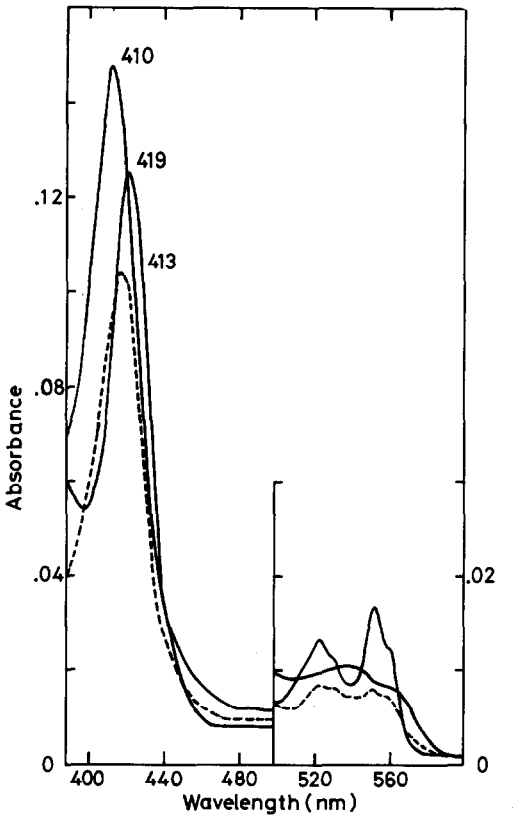

Fig.3. Absorption spectra of purified *Pseudomonas o*-type cytochrome oxidase: (——) the same oxidized and reduced forms as in fig.2; (– – –) the sample reduced with dithionite was shaken intensely and its spectrum recorded.

in the reduced form, and at 410 nm in the oxidized form. On addition of CO to the reduced form, the peak of the γ-band shifted to 415 nm and absorbance increased, but the absorbances at 552 and 560 nm in the α-band decreased. The CO + reduced minus reduced difference spectrum showed absorption maxima at 415, 538 and 570 nm, and troughs at 429, 551 and 558 nm (fig.2). Cyanide (10 mM) reacted with the oxidized form of the oxidase (but not with the reduced form), shifting the γ peak to 412 nm. When purified oxidase that had been reduced with a small amount of dithionite was aerated by shaking it, an oxygenated form was obtained, which showed a decreased γ peak at 413 nm and very low α and β peaks (fig.3). The cytochrome components of the oxidase were reduced almost completely with ascorbate–TMPD, an indication that the sample contained little cytochrome *b*. Heme components of the oxidase were separated at almost the same ratio with HCl–acetone into heme *c* and heme *b*. But, as this treatment caused the loss of hemes, the exact heme content has not yet been determined. The iron contents of the purified oxidase ranged from 26.0–36.5 and the copper contents from 2.8–3.8 nmol/mg protein.

4. Discussion

Cytochrome *o*-type oxidase was purified in a spectrally pure form from the membrane of aerobically grown *P. aeruginosa*. This oxidase was clearly constructed of *b*- and *c*-type cytochromes. The *b*-type cytochrome was judged to be a cytochrome *o* from

its CO-binding property. The oxidase had a high TMPD oxidizing activity like that of *Azotobacter vinelandii* which also contains cytochrome $c + o$ [11]. But, unlike *Azotobacter* enzyme [18], the *c*-type cytochrome contained in the purified oxidase could bind with CO as judged from its CO difference spectrum, which suggests that the oxidase may function as a cytochrome *co*. The oxidase reacted with CO and KCN, and it produced an oxygenated form by reacting with oxygen. This indicates that this oxidase is a functional terminal oxidase in the membrane-bound respiratory chain of *P. aeruginosa*.

The purified oxidase consisted of 4 polypeptides, although which polypeptides are essential has yet to be determined. Because the total M_r of the 4 major peptides in the purified oxidase was 71 000 and the iron content was 26–36 nmol/mg protein, the oxidase would contain 2 iron atoms/1 copy of each subunit, and these 2 iron atoms may correspond to heme *b* and heme *c*. If so, the oxidase must contain 2 copies of each subunit to function as a 4 electron carrier. In any case, establishment of polypeptide species and the location of the hemes must now be made.

The purified cytochrome oxidase oxidized horse and yeast cytochromes *c* in addition to an artificial electron donor. A cytochrome *c*-551 obtained from *Pseudomonas* membrane also was oxidized by the oxidase as was eukaryotic cytochrome *c*. To determine the functions of the *o*-type cytochrome oxidase found extensively in bacteria, we are further purifying our oxidase and are making a more detailed analysis of its properties.

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