Biochimica et Biophysica Acta, 547 (1979) 70—78

BBA 47683

THE TERMINAL OXIDASE OF *PHOTOBACTERIUM PHOSPHOREUM*

A NOVEL CYTOCHROME

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(Received November 24th, 1978)

Key words: Cytochrome bd; Terminal oxidase; (Photobacterium phosphoreum)

Summary

The terminal oxidase of *Photobacterium phosphoreum* has been purified to the electrophoretically homogeneous state and some of its properties have been studied.

The enzyme catalyses oxidation of ascorbate in the presence of phenazine methosulphate or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The reaction is inhibited by cyanide. Nitrite at comparatively high concentrations inhibits the enzyme, but the enzyme does not catalyse nitrite reduction with ascorbate plus the electron mediator as the electron donor.

The enzyme shows the absorption peaks at 632, 565, 534 and 436 nm in the reduced form. It has two kinds of haems: protohaem and haem *d*. Namely, the enzyme is a 'cytochrome *bd*'-type oxidase; a novel cytochrome.

Introduction

In 1928, Yaoi and Tamiya [1] found that some bacteria showed an absorption band in the red region of the spectrum. The band was attributed to the cytochrome component which was designated as cytochrome a_2 by Keilin [2,3]. The component, which is now called cytochrome *d*, is autoxidizable, and combines with cyanide and carbon monoxide. Therefore, the component has been believed to function as the terminal oxidase in the organisms which possess it. However, the 'true' cytochrome *d* which possesses only haem *d* as the prosthetic group has not yet been isolated.

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Nitrite reductase derived from some denitrifying bacteria is cytochrome *cd* which has not only haem *d* but also haem *c* as the prosthetic groups, and it acts also as a cytochrome oxidase [4]. Namely, it may be said to be true that the cytochrome which has haem *d* as the prosthetic group functions as a terminal oxidase in some bacteria.

During the study of the respiratory system of *Photobacterium phosphoreum*, we have found that the organism shows a distinct absorption band at 648 nm, and that the band shifts to 632 nm on reduction [5]. We expected that the microorganism might have the 'true' cytochrome *d* and tried to extract it. In the present investigation, we have purified a terminal oxidase from *P. phosphoreum* and determined some of its properties. The oxidase is a novel cytochrome which has protohaem and haem *d* in the molecule. The results obtained in the present investigation show that even when an absorption band around 640 nm is observed with a microorganism it does not necessarily mean that 'cytochrome *d*' exists in the organism.

Materials and Methods

Growth of the bacterium. P. phosphoreum strain 496 (wild) [5] was used. The mass cultivation of the organism was performed in 600 l culture medium with a stainless steel tank of 1000 l in volume. The medium [5] was inoculated with the seed culture (10 l) and incubated at 25°C. The cultivation was performed for 20 h with vigorous aeration, and continued for 5 h without aeration. Then, the cells of the organism were collected by a Sharples continuous flow centrifuge. About 2 kg of the packed cells were obtained.

Reagents. DEAE-cellulose was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.), Sephadex G-200 from Pharmacia (Uppsala, Sweden), and horse cytochrome *c*, DNAase and RNAase from Sigma Chemicals Company (U.S.A.). Cytochrome *c* (552, *Nitrosomonas europaea*) [6] and cytochrome *c* (550, *Thiobacillus novellus*) [7] were purified by the methods as previously established in our laboratory. Cytochrome c_3 (552, *Desulfovibrio vulgaris*) and cytochrome *c* (551, *Pseudomonas aeruginosa* P6009) were purified according to the methods of Yagi and Maruyama [8], and of Ambler [9], respectively. Cytochrome c_2 (550 *Rhodospirillum rubrum*) [10] and cytochrome *c* (*Saccharomyces oviformis*) [11] were kindly supplied by Dr. T. Horio (Institute for Protein Research, Osaka University, Japan) and Sankyo Co. (Tokyo, Japan), respectively.

Physical and chemical measurements. Spectrophotometric determinations were performed in a Cary spectrophotometer, model 16 using cuvettes of 1 cm light path. Determinations of molecular weight by gel filtration and polyacrylamide gel electrophoresis in the presence of SDS were performed by the method of Andrews [12] and by the method of Weber and Osborn [13] as modified by Wada and Snell [14], respectively.

Oxygen consumption was determined polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument Co., OH, U.S.A.). The reaction mixture contained 0.1 M phosphate buffer, pH 7.0, 1 mM ascorbate, 5 μM phenazine methosulphate or 166 μM tetramethyl phenylenediamine and 0.15 μM oxidase in a total volume of 3.0 ml. The phosphate buffer was saturated with air at the experimental temperature.

Purification of oxidase. Purification of the oxidase was usually performed with 100 g packed cells as the source material. The frozen cells were lysed in 400 ml of distilled water which contained 50 mg lysozyme, 5 mg DNAase and 5 mg of RNAase. The resulting lysate was allowed to stand for 1 h with stirring and then centrifuged at 13 000 × *g* for 20 min. The debris obtained was washed twice with 200 ml distilled water and suspended in 400 ml of 20 mM potassium phosphate buffer, pH 7.0, containing 0.5% deoxycholate, and 0.5% Triton X-100. The suspension was allowed to stand for 12 h with stirring and then centrifuged at 20 000 × *g* for 30 min. The supernatant thus obtained was fractionated by $(NH_4)_2SO_4$ and the precipitate formed between 30 and 70% saturation was collected by centrifugation at 20 000 × *g* for 30 min. The precipitate obtained was dissolved in the minimum volume of 20 mM potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100 and the resulting solution was dialysed overnight against the same buffer as used for the dissolution. The dialysed solution was charged on the DEAE-cellulose column (3 cm × 15 cm) which had been equilibrated with 20 mM potassium phosphate, pH 7.0, containing 0.5% Triton X-100. The enzyme adsorbed on the column was eluted by the linear gradient solution which was produced from 300 ml each of 20 mM potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100 and 50 mM NaCl, and 20 mM potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100 and 1 M NaCl. The eluate was collected as 5-ml fractions. The eluate from fraction numbers 9—20 (the first peak in the elution curve, Fig. 1) was pooled for further purification. Although the eluate included in the second peak of Fig. 1 also showed the oxidase activity, the further purification of the enzyme included in the peak was more difficult than that of the enzyme in the first peak. Therefore, in the present study, only the eluate included in the first peak was used for the further purification of the enzyme. The second chromatography with DEAE-cellulose column was performed in the same ways as for the first chromatography. In the second chromatography, a single peak

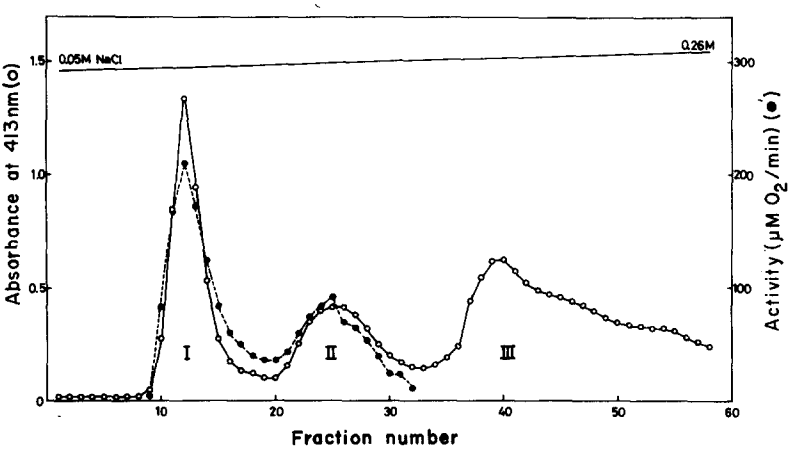

Fig. 1. Elution pattern of *P. phosphoreum* oxidase during chromatography on a DEAE-cellulose column. The oxidase content was monitored by the absorbance at 413 nm (○———○), while the activity by the oxygen consumption with ascorbate plus phenazine methosulphate as the electron donor (●- - - - -●). The eluate was collected as 5.0-ml fractions.

was observed in the elution curve as monitored by both the absorbance at 413 nm and the activity. The eluate thus obtained was used as the terminal oxidase preparation of *P. phosphoreum.*

The preparation obtained here was subjected to slab polyacrylamide gel (7.5%) electrophoresis in 50 mM Tris-HCl buffer, pH 8.5. After the electrophoresis the gel slab was cut into three strips, and these were separately stained by Coomassie brilliant blue, by use of the Nadi reaction [3] and by the haem-staining reagents [15], respectively. In each staining, a single band was observed at the same distance from the origin.

Results

Spectral properties

The purified enzyme showed absorption peaks at 413 and 648 nm in the oxidized form, and at 436, 534, 565 and 632 nm in the reduced form (Fig. 2). The pyridine ferrohaemochrome of the enzyme showed the peaks at 421, 527, 557 and 604 nm. When the enzyme was treated with acidic acetone (conc. HCl : acetone = 1 : 100, v/v), both the chromophores were extracted into the acetone layer. On neutralization of HCl in acetone layer with 2 N NaOH, a green layer appeared under the acetone layer. The pyridine ferrohaemochrome of the green layer obtained above showed the absorption peaks at 420, 527, 558 and 616 nm. These results described above seem to show that the enzyme has two kinds of haems: protohaem and haem *d* [16,17] although, at present, the two haems are not separated from each other. The isolation of each haem is now under progress in our laboratory, and the results will be published in future. The millimolar extinction coefficients (ϵ_{mM}) for the peaks at 565 and

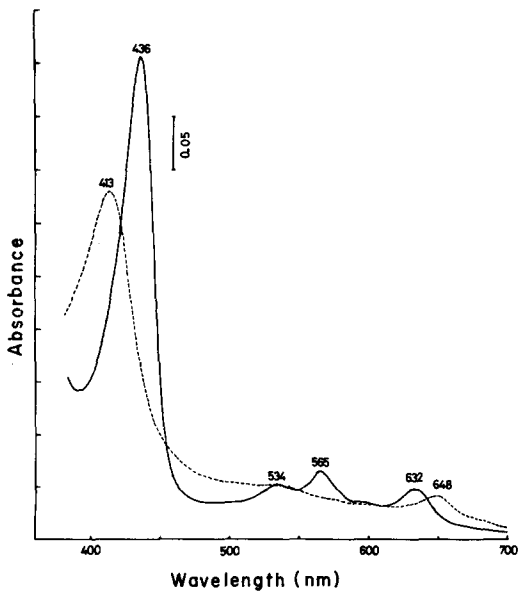

Fig. 2. Absorption spectra of purified *P. phosphoreum* oxidase. The enzyme was dissolved in 20 mM potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100. ------, oxidized; ———, reduced with $Na_2S_2O_4$.

632 nm were determined to be 20.0 and 14.7, respectively, on the basis of ϵ_{mM} at the α peak of the pyridine ferroprotohaemochrome [17].

Cyanide and carbon monoxide complexes

As will be described later, the enzyme was inhibited by cyanide. Therefore, the absorption spectra of the enzyme were expected to be affected by the compound. When cyanide was added to the oxidized enzyme, the Soret peak of the enzyme shifted from 413 nm to 415 nm, and the absorbance of the peak at 648 nm was lowered slightly. When the cyanide complex was reduced with $Na_2S_2O_4$, the peak in the red region of the spectrum appeared at 628 nm. When CO was bubbled through the $Na_2S_2O_4$-reduced enzyme, the peak at 632 nm of the intact enzyme shifted to 634 nm, while the peaks at 565, 534 and 436 nm, shifted to the shorter wavelengths by 2—4 nm. Thus the peak at 436 nm of the intact enzyme shifted to 432 nm. The effects of these ligands on the spectra of the enzyme are summarized in Table I. The absorption spectrum of the enzyme was not affected by azide at the concentrations of 1—10 mM.

Enzymatic activity

The enzyme oxidized ascorbate in the presence of phenazine methosulphate; as Fig. 3 shows, when the enzyme was added to the reaction mixture composed of 1 mM ascorbate and 5 μM phenazine methosulphate, the oxygen consumption occurred rapidly. K_m for oxygen of the enzyme was estimated to be less than 10 μM from the oxygen consumption curve as determined by the oxygen electrode. From the Lineweaver-Burk plot, V in the oxygen consumption by the enzyme was determined to be about 4000 mol of O_2/mol of enzyme per min. The enzyme was rapidly reduced by ascorbate in the presence of phenazine methosulphate or tetramethyl phenylenediamine, while it was not reduced by ascorbate in the absence of the electron mediator. The reduced enzyme was rapidly oxidized when it was shaken with air (Fig. 4). The oxidase reaction showed a maximum activity at pH 7.0 when ascorbate plus phenazine methosulphate or ascorbate plus tetramethyl phenylenediamine was used as the electron-donating system. The reaction catalysed by the enzyme was not

TABLE I

ABSORPTION PEAKS OF *P. PHOSPHOREUM* OXIDASE AND ITS DERIVATIVES

Addition	Absorption peaks (nm)			
None				
Oxidized	413			648
Reduced	436	534	565	632
+KCN				
Oxidized	415			648
Reduced	437—438	535—536	566—567	628
+CO				
Reduced	432	532—533	563—564	634
+Pyridine + NaOH (reduced)	421	527	557	604
HCl/acetone				
Extract + pyridine + NaOH (reduced)	420	527	558	616

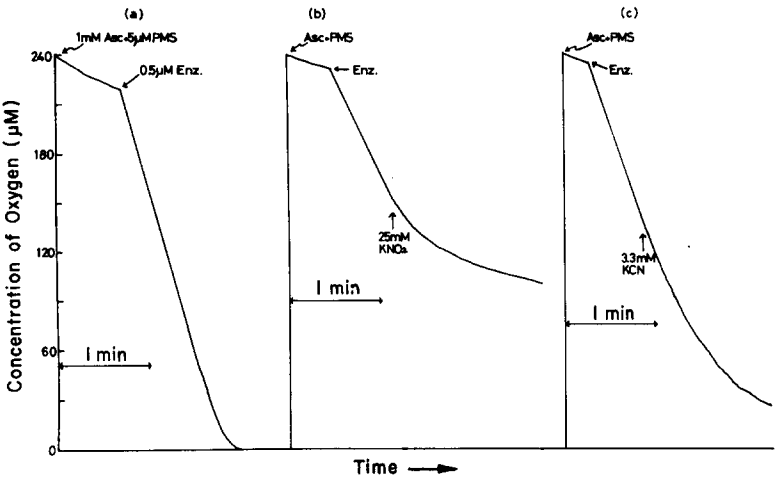

Fig. 3. Oxygen consumption catalysed by *P. phosphoreum* oxidase with ascorbate plus phenazine methosulphate as the electron donor. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100, 1 mM ascorbate, 5 μM phenazine methosulphate, 240 μM O_2 and 0.15 μM enzyme in a total volume of 3.0 ml. The reactions were performed at 20°C. (a) Complete system; (b) 25 mM KNO_2 added; (c) 3.3 mM KCN added. Asc, ascorbate; PMS, phenazine methosulphate; enz., oxidase.

affected by addition of catalase, and the enzyme did not show the peroxidase activity with guaiacol as the electron donor.

The oxidation of ascorbate catalysed by the enzyme was 50% inhibited by 60 μM cyanide but even in the presence of 10 mM cyanide about 10% of the activity was still observed. Therefore, the oxidase seemed to be comparatively insensitive to cyanide. The ascorbate-oxidizing activity of the enzyme seemed to be inhibited also by nitrite although the K_i value of the salt was as high as 56 mM. The inhibition by nitrite was competitive with oxygen. As Fig. 5 shows, when nitrite was anaerobically added to the oxidase which had been reduced by ascorbate plus tetramethyl phenylenediamine, haem *d* in the enzyme was oxidized but protohaem remained reduced; the peak at 632 nm disappeared, while the peaks at 565, 534 and 436 nm remained unchanged.

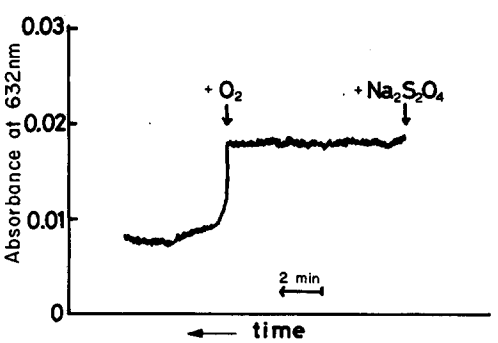

Fig. 4. Autooxidation of reduced *P. phosphoreum* oxidase. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100 and 0.9 μM enzyme in a total volume of 3.0 ml. Oxidation of the reduced enzyme was monitored by decrease in the absorbance at 632 nm.

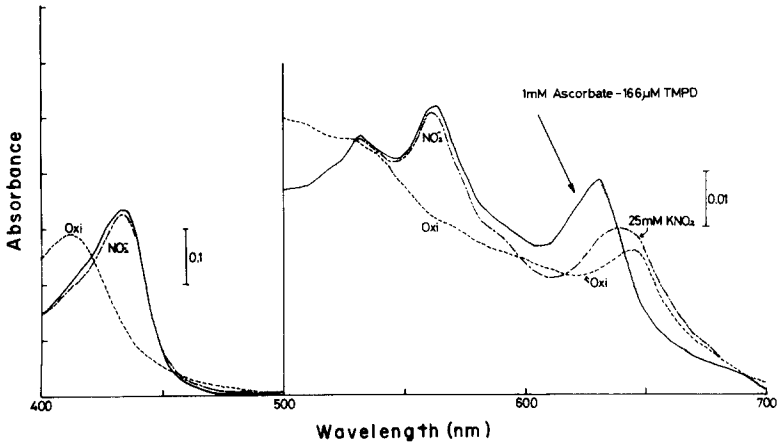

Fig. 5. Effect of nitrite on reduced *P. phosphoreum* oxidase. To the enzyme which had been anaerobically incubated for 15 min with 1 mM ascorbate plus tetramethyl phenylenediamine (TMPD) was added 25 mM KNO_2. The reactions were performed at 20°C in a Thunberg-type cuvette under nitrogen atmosphere. ------, oxidized; ———, reduced; ———, reduced + KNO_2.

However, appreciable nitrite reduction catalysed by the enzyme was not observed with ascrobate plus phenazine methosulphate. This means that the enzyme does not act as a nitrite reductase although electron can be transferred from haem *d* in the reduced enzyme to nitrite.

Electron donors

As already described, the enzyme oxidized ascorbate in the presence of phenazine methosulphate or tetramethyl phenylenediamine. Phenazine methosulphate of 5 μM was sufficient to mediate electrons from ascorbate to the enzyme, while tetramethyl phenylenediamine of about 160 μM was necessary to mediate electrons from ascorbate to the enzyme as efficiently as phenazine methosulphate at 5 μM did.

Various kinds of cytochromes *c* were tested if they reacted with the oxidase. Cytochromes *c* tested were horse cytochrome *c*, cytochrome *c* (*S. oviformis*), cytochrome *c*, (551, *P. aeruginosa* P6009), cytochrome *c* (550, *T. novellus*), cytochrome *c* (552, *N. europaea*), cytochrome c_2 (550, *R. rubrum*) and cytochrome c_3 (552, *D. vulgaris*). None of their reduced forms were not oxidized by the enzyme or stimulated the oxygen consumption catalysed by the enzyme with ascorbate plus phenazine methosulphate as the electron donor. Cytochrome *c*-552 of *P. phosphoreum* (Kamita, Y. and Yamanaka, T., unpublished results) did not react with the enzyme, either.

Molecular weight

The molecular weight of the enzyme was determined to be 115 000 by polyacrylamide gel electrophoresis in the presence of SDS. The same value of the molecular weight was obtained by the gel filtration with Sephadex G-200. Therefore, the ezyme molecule is composed of one peptide chain.

Discussion

The terminal oxidase of *P. phosphoreum* has been purified to the electrophoretically homogeneous state. It shows absorption peaks at 632, 565, 534 and 436 nm in the reduced form. Further, the pyridine haemochrome of the acid/acetone extract of the enzyme shows the peaks at 557 and 616 nm. These facts indicate that the oxidase possesses protohaem and haem *d*. As its molecule is composed of one peptide chain, the enzyme can be called 'cytochrome *bd*'-type oxidase; a novel cytochrome. Although the γ peak around 460 nm due to haem *d* is not observed with the *P. phosphoreum* oxidase unlike the cases of cytochrome *cd* [18] and cytochrome cd_1 [19], the fact seems to be attributable to the difference in the positions of the γ peaks due to the haem other than haem *d* or haem d_1 between cytochrome *bd* and cytochrome *cd* or cd_1. Thus, the γ peak due to protohaem of the *P. phosphoreum* oxidase is located at 436 nm, while that due to haem *c* in cytochrome *cd* or cd_1 is at 412—418 nm. Determination of the content of each haem in the enzyme is now in progress in our laboratory and the results will be published in future.

The *P. phosphoreum* enzyme oxidizes rapidly ascorbate in the presence of phenazine methosulphate or tetramethyl phenylenediamine, and the reduced enzyme is very autoxidizable. However, the enzyme does not oxidize reduced cytochromes *c* which are derived from various organisms. As we have already reported [20], the terminal oxidases such as cow cytochrome oxidase [21], *P. aeruginosa* nitrite reductase (=*Pseudomonas* cytochrome oxidase) [22], and *T. novellus* cytochrome oxidase [23] react with either the mammalian-type cytochrome *c* or with the 'bacterial-type' cytochrome *c*. Therefore, the fact that the oxidase derived from *P. phosphoreum* does not react with any of cytochromes *c* tested appear to show that the direct electron donor for the enzyme is not a *c*-type cytochrome. Thus, a *c*-type cytochrome purified from the organism differs from usual cytochrome *c*; e.g. its molecular weight is about 100 000. It does not react with the *P. phosphoreum* oxidase either (Yamanaka, T. and Kamita, Y., unpublished results).

The 'cytochrome *bd*'-type oxidase as the *P. phosphoreum* enzyme may be responsible for such electron transfer systems with cytochrome *b* and 'cytochrome-*d*' as reported with *Azotobacter vinelandii* [25]; the spectrophotometric observation can take the 'cytochrome *bd*'-type oxidase for cytochromes *b* and *d*.

Acknowledgements

The authors wish to thank Professor H. Matsubara for his interest in this work. They are also grateful to Mr. Y. Fukumori for mass cultivation of *P. phosphoreum* and to Miss Keiko Ejima for her valuable experimental assistance.

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