Methylobacillus flagellatus KT contains a novel *cbo*-type cytochrome oxidase

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Abstract The *o*-type oxidase from the methanol-grown obligate methylotroph *Methylobacillus flagellatus* KT has been purified to homogeneity. The complex is composed of four subunits (57, 40, 35 and 30 kDa). It contains six haems (4C:1B:1O) and one copper atom per molecule. It is proposed that the haem O-Cu_B binuclear centre and a low-spin haem B are located in subunit I (57 kDa), two haems C reside in the cytochrome *c* homodimer (35 kDa), two haems C belong to the dihaem cytochrome *c* (30 kDa). The presented data provide evidence that cytochrome *cbo* is a novel representative of the haem–copper oxidase superfamily. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cytochrome oxidase; Cytochrome *c*; Cytochrome *co*; Methylotroph; *Methylobacillus*

1. Introduction

Methylobacillus flagellatus KT is an obligate methylotroph able to use only methanol and methylamine as the sole carbon and energy sources [1]. *M. flagellatus* KT synthesises two oxidases of the *o* type, the relative amounts of each depending on the growth phase. This has been demonstrated by kinetic analysis of the laser flash-induced optical absorbance changes of CO-oxidase complexes in membranes of the methanol-grown bacterium [2]. One oxidase (*o*) revealed slow monophasic CO-recombination kinetics and was predominant in the early exponential phase of growth, whereas the other (*o*′) had rapid three-phasic kinetics of CO-rebinding and was induced in the late exponential growth phase. The inhibitory analysis showed that oxidase *o* was more sensitive to cyanide than oxidase *o*′ and that they were both involved in horse heart cytochrome *c* oxidation in membrane preparations [2,3].

Cytochrome *c* oxidases of the *o* type seem to be widely distributed in methylotrophs, they contain haem *c* as their second prosthetic group and are referred to as cytochromes *co*. One of the first to be purified was cytochrome *co* from the restricted facultative methylotroph *Methylophilus methylotrophus* [4]. The partially purified *co*-type oxidases were obtained from the obligate methylotrophic strain 4025 [5] and the acidophilic methylotroph *Acetobacter methanolicus* [6]. Oxidase *co* has been purified from a number of heterotrophic bacteria as well, including *Azotobacter vinelandii* [7], *Rhodopseudomonas palustris* [8] and *Pseudomonas aeruginosa* [9]. Cytochromes *co* oxidise the high-potential substrates ascorbate/*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and exogenous cytochrome *c*, but not the low-potential ubiquinol. In the early literature oxidase was termed cytochrome *o* if it was a CO-binding *b*-type cytochrome with spectral characteristics similar to cytochrome *o* first described by Castor and Chance [10]. Since haem O was shown by reverse-phase high-performance liquid chromatography (HPLC) to be a distinct type [11], this technique is considered to be a powerful tool in determination of the nature of oxidase. This implies that the above-mentioned *co* oxidases may vary not only in haem composition but in oxygen-reactive centre organisation as well. In this respect two cytochrome *o*-type oxidases of *M. flagellatus* KT are very intriguing, as their quite different CO-binding properties suggest a different nature of the redox centres involved in oxygen activation and reduction. The present paper reports the purification and characterisation of cytochrome oxidase *o* from *M. flagellatus* KT, which is of the *cbo* type. The finding that *cbo* oxidase has a haem composition distinct from the cytochrome oxidases described so far makes it a novel cytochrome *c* oxidase.

2. Materials and methods

2.1. Bacteria and growth conditions

The obligate methylotroph *M. flagellatus* KT was isolated from an activated sludge of the wastewater treatment station in Moscow [1]. The growth medium [12] contained methanol (1% v/v). Batch cultures were grown at pH 7.0 in 250 ml flasks with 150 ml of the medium at 37°C and 250 rpm on G24 environmental incubator shaker (New Brunswick Co., NJ, USA) for 18–20 h.

Escherichia coli GO103 (Cyd^-) and GO104 (Cyo^-) were grown in a medium composed of (g/l): peptone, 10; yeast extract, 5; NaCl, 10, supplemented with kanamycin sulphate, 50 μg/ml and streptomycin sulphate, 100 μg/ml at pH 7.0 and 37°C. *Methylobacterium extorquens* AM1 was grown in a medium composed of (g/l): KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 2.0; NaCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.025; $FeSO_4$, 0.01; yeast extract, 0.1 and methanol 0.3% (v/v) at pH 7.5 and 28°C. Bacteria were grown in batch cultures at 250 rpm.

2.2. Preparation of membrane fractions

Cells of the late exponential growth phase were harvested by centrifugation at 5000×*g* for 30 min, washed twice with 50 mM potassium phosphate buffer (pH 7.0), resuspended in 50 mM Tris–HCl

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Abbreviations: DM, *n*-dodecyl-β-D-maltoside; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TMBZ, 3,3′,5,5′-tetramethylbenzidine; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

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buffer (pH 8.0), containing 1 mM phenylmethylsulphonyl fluoride, and disrupted by sonication (6×30 s). The resulting homogenates were centrifuged at 20 000×*g* for 30 min to remove cell debris and then at 180 000×*g* for 1.5 h to separate the membrane and soluble fractions. The membranes were washed in 20 mM Tris–HCl buffer (pH 8.0), resuspended in it and stored at −20°C.

2.3. Purification of the oxidase

Oxidase was solubilised by stirring membranes of *M. flagellatus* KT (25–30 mg protein/ml, final concentration) with 1% (w/v) DM in 20 mM Tris–HCl buffer (pH 8.0) on ice for 30 min; insoluble membranes were removed by ultracentrifugation at 180 000×*g* for 3 h at 4°C. The supernatant (10–12 mg protein/ml) was incubated with *N*,*N*-*bis*-(3-D-gluconeamidopropyl)-deoxycholamide (2% w/v) for 1 h on ice, followed by preparative native PAGE (4–10%, 3% C) in the buffer system of Laemmli [13] in the presence of 5 mM ethylenediaminetetraacetic acid (EDTA) using Pharmacia gel electrophoresis apparatus GE-4 (Sweden) at 4°C. Gels were stained for oxidase activity with 1 mM TMPD; stained protein bands were cut out, followed by electroelution in the same buffer using Biotrap BT 1000 (Schleicher and Schuell, Germany). The purified oxidase was stored at −20°C.

2.4. PAGE of the purified oxidase

SDS–PAGE (12.6% T, 3% C) or analytical native PAGE (4–20% T, 2% C) was performed in the buffer system of Laemmli [13]. Gels were stained for protein with Coomassie brilliant blue G-250, for haem using 3,3′,5,5′-tetramethylbenzidine (TMBZ) [14] and for oxidase activity with 1 mM TMPD. Molecular masses were determined with Pharmacia electrophoresis high and low molecular weight calibration kits. The best resolution for SDS–PAGE was achieved when purified oxidase was precipitated in acetone overnight at −20°C.

2.5. Haem analysis

Purified oxidase was extracted three times with ice-cold 5% (v/v) HCl in acetone and centrifuged. Supernatants, containing non-covalently bound haems, were combined and dried under a stream of N_2; the residue was dissolved in 20% (v/v) pyridine in 100 mM KOH. The pellet with haem C was air-dried and resuspended in the same basic pyridine.

Alternatively, non-covalently bound haems were reextracted twice from the HCl–acetone extracts with diethyl ether as described previously [15] and washed twice with water. Ether-extracted haems were dried under N_2, dissolved in acetonitrile and separated by HPLC on a Beckman Ultrasphere ODS reverse-phase column (4.6×250 mm, USA) using acetonitrile/water gradients in the presence of 0.1% trifluoroacetic acid. Haem-containing fractions were detected at 406 nm. The haem standards were prepared from membranes of *E. coli* GO103 (haems B, O), *E. coli* GO104 (haems B, D) and *M. extorquens* AM1 (haems B, A) according the above mentioned procedures.

2.6. Measurement of absorption spectra

Absorption spectra were recorded using a Beckman DU-8B single-beam spectrophotometer (USA) at room temperature. The scan speed was 100 nm/min, the bandwidth was 0.5 nm and the light path was 10 mm. The difference spectra were recorded using samples reduced with a few grains of sodium dithionite and oxidised with a few grains of potassium ferricyanide or ammonium persulphate. For the measurement of the CO difference spectra the purified oxidase was made anaerobic in a stoppered cuvette by purging with N_2 and then reduced with a concentrated solution of sodium dithionite. An absorption spectrum was recorded and stored as the baseline, CO was then bubbled through the sample for 5 min in the dark, a dithionite solution was added again and the spectrum of the CO-bound form was recorded.

Cytochrome and haem contents were calculated from difference spectra using the molar extinction coefficients (mM^{-1} cm^{-1}): cytochrome *c*, 550–535 nm, 19.1; cytochrome *b*, 563–577 nm, 28.5; haemochrome *b*, 556–575 nm, 30.0 [17]. Protein was assayed by the Lowry method [16] with bovine serum albumin as a standard.

2.7. Metal analysis

Iron and copper contents were determined in purified oxidase, dialysed against 10 mM EDTA in milli-Q water (MilliQ UFplus, Millipore, USA) at 4°C. The aliquots of samples (0.2–1.0 ml) were boiled in 2 ml of concentrated HNO_3 in thermostable tubes in hot sand until evaporation of nitrogen oxides, then 1 ml of a mixture of concentrated acids HNO_3:H_2SO_4:HCl (2:1:1) was added, followed by boiling; the residue was dried by heating. The resulting mineral salt pellet was dissolved in 1 ml of 0.5 M HCl. Metal analysis was carried out with an atomic absorption spectrometer AAS-3 (Carl Zeiss, Jena, Germany). Blank values were obtained with the dialysis buffer. As a control the same manipulations were performed on horse heart cytochrome *c* (type II-A, Sigma Co., USA), and the Cu/Fe contents of 0.01/1.25 atoms per molecule were obtained.

3. Results and discussion

The oxidase solubilised with DM was purified in a single-step procedure by preparative native PAGE (4–10%). The gels were stained for oxidase activity using TMPD. A most intensively stained protein band was cut out and after subsequent electroelution its molecular and spectral properties were studied.

SDS–PAGE analysis of the purified oxidase revealed four components of apparent molecular masses of 57, 40, 35 and 30 kDa (Fig. 1, lane 2). The two smaller subunits (35 and 30 kDa) stained for haem with TMBZ (Fig. 1, lane 3), indicating that they are *c*-type cytochromes. A band of 57 kDa retained traces of haem peroxidase activity with TMBZ on some gels, supporting the presence of non-covalently bound haems in it. In some cases a band of 35 kDa was absent on SDS–PAGE, instead a band of about 14 kDa appeared. This band stained for haem, indicating a component of 35 kDa is a homodimer. The TMBZ haem staining for a cytochrome *c* of 30 kDa was of the same intensity as that for the homodimeric cytochrome *c* of 35 kDa, suggesting the former might contain two haems C as well. The native molecular mass of the oxidase, estimated from molecular masses of subunits, is about 162 kDa. Native PAGE yielded one protein band with oxidase activity of an apparent molecular mass of 170 kDa (Fig. 1, lanes 4, 5), justifying the homogeneity of the enzyme preparation obtained.

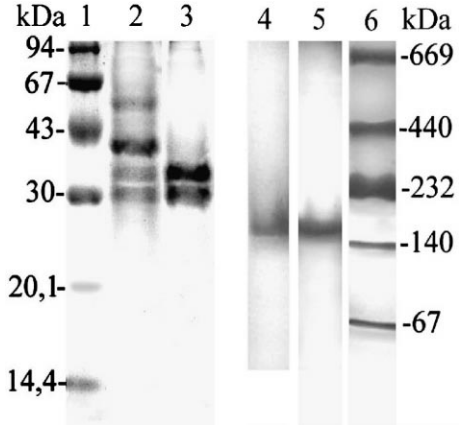

Fig. 1. SDS–PAGE (12.6% T, 3% C; lanes 1–3) and analytical native PAGE (4–20% T, 2% C; lanes 4–6) of purified oxidase of *M. flagellatus* KT. Lane 1, molecular mass standards (kDa): phosphorylase b, 94.0; albumin, 67.0; ovalbumin, 43.0; carbonic anhydrase, 30.0; trypsin inhibitor, 20.1; α-lactalbumin, 14.4. Lane 6, molecular mass standards (kDa): thyroglobulin, 669; ferritin, 440; catalase, 232; lactate dehydrogenase, 140; albumin, 67. Oxidase was incubated for 20 min at room temperature in the presence of 2% SDS and 3 mM mercaptoethanol prior to loading on the gel (lanes 2 and 3 contain 70 μg of protein). Lanes 4 and 5, untreated oxidase (45 μg of protein). The gels were stained for protein with Coomassie brilliant blue G-250 (lanes 1, 2, 5, 6), for haem using TMBZ (lane 3) and for activity with TMPD (lane 4).

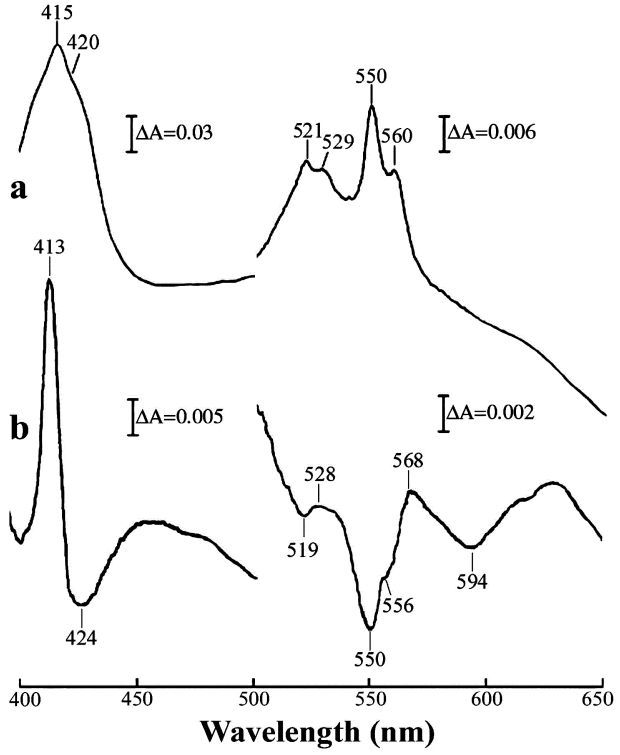

Fig. 2. Absorption spectra of the purified *o*-type oxidase of *M. flagellatus* KT. a: Dithionite-reduced minus ammonium persulphate-oxidised difference spectrum; b: (dithionite-reduced+CO) minus dithionite-reduced difference spectrum recorded after 5 min exposure to CO. Spectra were measured at room temperature in 20 mM Tris–HCl buffer (pH 8.0) in the presence of 0.1% DM; the protein concentration was 2.3 mg/ml and the light path was 10 mm.

The difference spectrum of the purified oxidase (Fig. 2a) shows peaks at 550, 521 and 415 nm corresponding to α, β and γ bands of *c*-type cytochromes respectively, while the peaks at 560 and 529 nm indicate the presence of *b*-type cytochromes. If the usual extinction coefficients for these cytochromes are assumed (see Section 2), the spectrum shows that the molar ratio of cytochrome *c* to cytochrome *b* is 4.5 to 1.9 in the complex, suggesting that the pure oxidase contains about four *c*-type and two *b*-type cytochromes per molecule. The CO difference spectrum of the oxidase (Fig. 2b) revealed the CO-binding *o*- and *c*-type cytochromes. A shoulder at 556 nm and a trough at 594 nm are consistent with the presence of a cytochrome *o*–CO complex. The presence of a high-spin cytochrome *c* is shown by a trough at 550 nm in the α band, by a trough at 519 nm in the β band, by an absorption peak at 413 nm and an associated trough at 424 nm in the Soret region.

The presence of cytochrome *b* and *c* types was further confirmed by preparing the pyridine haemochrome derivatives of the oxidase. Pyridine haemochrome spectra revealed about four *c*-type haems and two *b*-type haems per molecule of the oxidase complex (measured values of 3.9 and 2.3); neither haem A nor haem D was present.

Alternatively, the nature of non-covalently bound haems was determined by analytical HPLC (Fig. 3). The haems have different retention times depending on their hydrophobicities. The reverse-phase column was calibrated with haems B, O, D and A extracted from membranes of *E. coli* and *M. extorquens* AM1 respectively. Two types of non-covalently bound haems were detected in the acid–acetone extract of purified cytochrome oxidase, their retention times correspond to those of haem B and haem O.

Determination of the metal content by atomic absorption spectroscopy yielded a stoichiometry of six iron atoms and one copper atom per enzyme complex (measured values of 5.95 and 1.14 respectively). The six iron atoms can be assigned to six haems (4C:1B:1O). This ratio is in good agreement with the haem analysis data, indicating that the oxidase under study is of the *cbo* type. The value of one copper suggests that the oxidase complex contains Cu_B in the binuclear centre and lacks a Cu_A centre. Two non-covalently bound haems (B and O) together with a copper atom must be located in subunit I (57 kDa). Haem O, being usually high-spin, is most likely to form a binuclear centre with Cu_B. A low-spin haem B must be the direct electron donor of the haem–copper centre. Two haems C reside in the cytochrome *c* homodimer (35 kDa), another two haems C belong to the dihaem cytochrome *c* (30 kDa).

The above data provide evidence that cytochrome oxidase *cbo* of the obligate methylotroph *M. flagellatus* KT is a novel representative of the haem–copper oxidase superfamily.

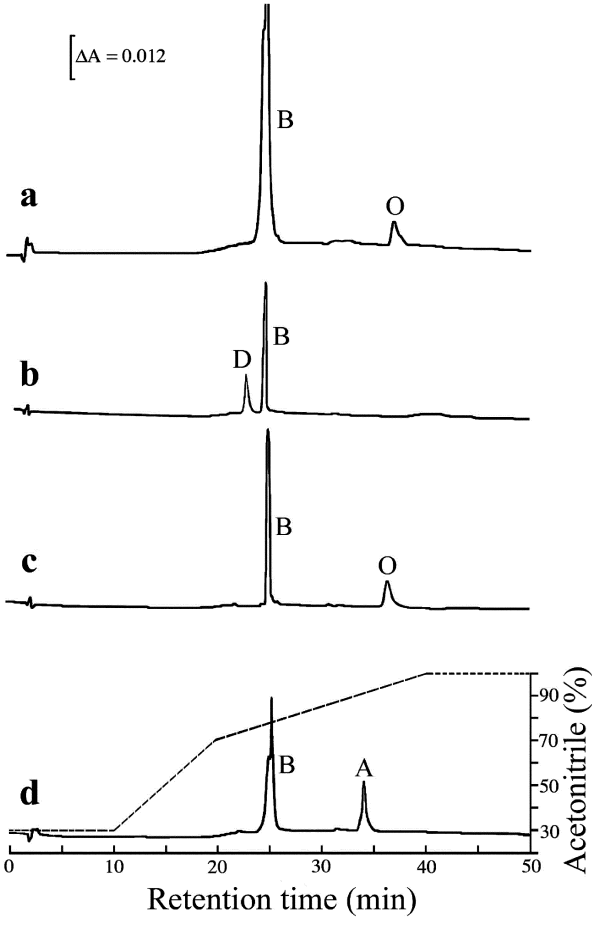

Fig. 3. Analytical HPLC of the non-covalently bound haems extracted from the purified *o*-type oxidase of *M. flagellatus* KT (a) and from control membranes of *E. coli* GO104 (b), *E. coli* GO103 (c) and *M. extorquens* AM1 (d). The detection wavelength was 406 nm. The dashed line (in d) shows an elution gradient of acetonitrile (30–100%) in water containing 0.1% trifluoroacetic acid. The retention times (min) of haem standards were: 23.6, haem D; 25.2, haem B; 33.7, haem A; 37.1, haem O.

Having a haem O–Cu_B binuclear centre, it seems to be responsible for the slow monophasic kinetics of CO rebinding of cytochrome *o* described in our previous work [2]. This implies that the *cbo* oxidase of *M. flagellatus* KT reveals a remarkable similarity in the kinetic behaviour during CO recombination with other haem–copper oxidases: quinol oxidase *bo* of *E. coli* and cytochrome *c* oxidase caa_3 of *Bacillus* sp. FTU [18]. On the other hand, the *cbo* oxidase complex of *M. flagellatus* KT shares some common structural and functional properties with haem–copper oxidases of the *cbb* type. These are: the presence of few haems C in the molecule, some of which bind CO, the absence of a Cu_A centre and the ability to oxidise exogenous cytochrome *c*.

To the best of our knowledge the enzyme studied in the present work is the first *cbo*-type oxidase characterised so far. In spite of this fact, *cbo* oxidases may be widely distributed in bacteria, especially in Gram-negative methylotrophs, since they involve *c*-type cytochromes in methanol and methylamine oxidation [19], but not ubiquinol.

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References

[1] Govorukhina, N.I., Kletsova, L.V., Tsygankov, Y.D., Trotsenko, Y.A. and Netrusov, A.I. (1987) Microbiology (Russia) 56, 849–854.
[2] Muntyan, M.S., Bloch, D.A., Dinarieva, T.Y., Drachev, L.A. and Netrusov, A.I. (1994) Biochem. Biophys. Res. Commun. 204, 428–435.
[3] Strom, E.V., Dinarieva, T.Y. and Netrusov, A.I. (1997) Microbiology (Russia) 66, 386–390.
[4] Froud, S.J. and Anthony, C. (1984) J. Gen. Microbiol. 130, 2201–2212.
[5] Auton, K.A. and Anthony, C. (1989) Biochem. J. 260, 75–79.
[6] Chan, H.T.C. and Anthony, C. (1991) J. Gen. Microbiol. 137, 693–704.
[7] Jurtshuk, P., Mueller, T.M. and Wong, T.Y. (1981) Biochim. Biophys. Acta 637, 374–382.
[8] King, M. and Drews, G. (1976) Eur. J. Biochem. 68, 5–12.
[9] Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1982) FEBS Lett. 139, 255–258.
[10] Castor, L.N. and Chance, B. (1959) J. Biol. Chem. 234, 1587–1592.
[11] Puustinen, A. and Wikstrom, M. (1991) Proc. Natl. Acad. Sci. USA 88, 6122–6126.
[12] Dinarieva, T.Y. and Netrusov, A.I. (1989) FEBS Lett. 259, 47–49.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] De Vrij, W., Azzi, A. and Konings, W.N. (1983) Eur. J. Biochem. 131, 97–103.
[15] Lubben, M. and Morand, K. (1994) J. Biol. Chem. 269, 21473–21479.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.P. (1951) J. Biol. Chem. 193, 265–275.
[17] Rieske, J.S. (1967) Methods Enzymol. 10, 488–493.
[18] Muntyan, M.S., Bloch, D.A., Ustiyan, V.S. and Drachev, L.A. (1993) FEBS Lett. 327, 351–354.
[19] Anthony, C. (1992) Biochim. Biophys. Acta 1099, 1–15.