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EXPERIMENTAL ARTICLES

The Cytochrome *cbo* from the Obligate Methylotroph *Methylobacillus flagellatus* KT Is a Cytochrome *c* Oxidase

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Abstract—The *cbo*-type oxidase of *Methylobacillus flagellatus* KT was purified to homogeneity by preparative native gel electrophoresis, and the kinetic properties and substrate specificity of the enzyme were studied. Ascorbate and ascorbate/*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) were oxidized by cytochrome *cbo* with a pH optimum of 8.3. With TMPD as an electron donor for the *cbo*-type oxidase, the optimal pH (7.0 to 7.6) was determined from the difference between respiration rates in the presence of ascorbate/TMPD and only ascorbate. The kinetic constants determined at pH 7.0 were as follows: oxidation by the enzyme of reduced TMPD was characterized by K_M = 0.86 mM and V_{max} = 1.1 μmol O_2/(min mg protein), and oxidation of reduced horse heart cytochrome *c* was characterized by K_M = 0.09 mM and V_{max} = 0.9 μmol O_2/(min mg protein). Cyanide inhibited ascorbate/TMPD–oxidase activity (K_i = 4.5–5.0 μM). The soluble cytochrome c_H (12 kDa), partially purified from *M. flagellatus* KT, was found to serve as a natural electron donor for the *cbo*-type oxidase.

Key words: obligate methylotroph, *Methylobacillus flagellatus* KT, cytochrome *c*, cytochrome *c* oxidase, *cbo*-type oxidase.

Quinol and cytochrome *c* are the natural substrates for bacterial oxidases (quinol oxidases and cytochrome *c* oxidases, respectively). During methylotrophic bacterial growth, cytochrome *c* oxidases aa_3, *cbb*', or cytochrome *co* are at work [1, 2]. The latter class of cytochrome oxidases initially included oxidases containing *b*-type CO-binding cytochromes, the spectral characteristics of which were similar to those of cytochrome *o* from *Escherichia coli* [3]. The first oxidase of this type isolated and purified from methylotrophic bacteria was cytochrome *co* from the restricted facultative methylotroph *Methylophilus methylotrophus* [4]. Later, preparations of the oxidases *co* from the obligately methylotrophic strain 4025 [5] and the facultative acidophilic methylotroph *Acetobacter methanolicus* [6] were partially purified. In 1991, heme O was described, which contained a 17-carbon-atom side chain of hydroxyethylfarnesyl at position 2 of porphyrin and a methyl group at position 8 [7]. Based on the data of spectroscopic, biochemical, and molecular genetic analyses, it was inferred that the oxidase catalytic center is binuclear and consists either of a high-spin heme and a copper atom Cu_B or of two high-spin hemes [8]. Thus, both the structure of the catalytic center and the nature of the high-spin heme (B or O) of the cytochrome oxidase *co* still remain unclear.

Most of the studied bacterial oxidases contain a binuclear heme–copper center that binds and reduces oxygen [8]. These oxidases differ in subunit and heme composition, number of copper redox centers, and the natural substrate (cytochrome *c* or quinol). They form a superfamily of heme–copper oxidases. The recognized heme–heme oxidases currently include only a *bd* quinol oxidase [8], although more and more recent evidence suggests that the *bb*-type oxidases may also belong to this group of enzymes [9]. Thus, the biochemistry of cytochromes *co* of methylotrophic bacteria remains poorly studied. These oxidases are only known to contain CO-binding *b*- and *c*-type cytochromes and to oxidize substrates with a high redox potential (reduced *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine and cytochrome *c*); they are also known to be inhibited by low cyanide concentrations [1, 6].

In view of the aforesaid, it was of particular interest to study the terminal components of the respiratory chain in the obligate methylotroph *Methylobacillus flagellatus* KT. A kinetic analysis of the optical changes induced by laser flash photolysis in CO complexes of reduced membrane proteins showed that this methylotroph contains two oxidases, arbitrarily designated *o* and *o′* [10, 11]. Note that *o′*-type oxidase contains two high-spin *b*-type cytochromes and presumably belongs to the enzymes with a heme–heme binuclear oxygen-binding center. We have recently purified to a homogeneous state cytochrome *o* and presented its characterization [12]. Judging from the structural properties of this oxidase, it is a novel member of the superfamily of heme–copper oxidases, a cytochrome *cbo* complex

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with a subunit and heme composition of 4C : 1B : 1O. Cytochrome *cbo* is a heterotetrameric complex (57, 40, 35, and 30 kDa), the two small subunits of which are cytochromes *c*, homodimeric and dihemic, respectively, whereas subunit I (57 kDa) contains a binuclear heme O–Cu_B catalytic center and a low-spin heme B [12].

The aims of this study were to analyze the kinetic properties and the substrate specificity of the cytochrome *cbo* complex from the obligate methylotroph *M. flagellatus* KT and to determine the natural donor of electrons for this oxidase.

MATERIALS AND METHODS

The obligate methylotroph *Methylobacillus flagellatus* KT [13] from the collection of the Department of Microbiology, Moscow State University, was grown in the presence of 1 vol % methanol in mineral medium of the following composition (g/l): KH_2PO_4, 10.0; NaCl, 1.0; $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 4.0. Trace elements (5 ml/l) were added to the following final concentrations (mg/l): $FeSO_4 \cdot 7H_2O$, 3.0; $CaCl_2 \cdot 2H_2O$, 5.3; $Na_2MoO_4 \cdot 2H_2O$, 0.04; $MnSO_4 \cdot 5H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.2; $CoCl_2 \cdot 6H_2O$, 0.04; $CuSO_4 \cdot 5H_2O$, 0.04; H_3BO_3, 0.03. Methanol was sterilized by passing it through a 225G5 Chamberlain filter (Germany). Cultures were grown at 37°C in 250-ml flasks containing 150 ml of medium on a shaker at 250 rpm (New Brunswick Sci. Co., United States).

The soluble and membrane cell fractions were obtained from a culture harvested at the end of the logarithmic growth phase, as described previously [12]. Cytochrome oxidase was purified using preparative electrophoresis in a gradient of polyacrylamide gel (PAAG, 4–12%), followed by electroelution [12]. The preparation was stored at –20°C in a 30% glycerol solution.

To obtain a partially purified cytochrome c_H, proteins were precipitated from the soluble fraction at 60% saturation with ammonium sulfate, separated by centrifugation (10000 *g*, 10 min, 4°C), and redissolved in 25 mM Tris–glycine buffer (pH 8.3). The latter solution was filtered under nitrogen pressure through a YM100 membrane (Amicon, United States). The obtained filtrates were separated by electrophoresis in the same buffer (200 V, 40 mA, 8 h) using a Biotrap BT 1000 device (Schleicher & Schuell, Germany). The partially purified and concentrated preparation of cytochrome c_H was withdrawn from the cathode zone of the device. Since the pI of cytochrome c_H is higher than the pH of the eluting buffer (pI > 8.5), cytochrome c_H was positively charged under these conditions.

Electrophoresis in the presence of 0.2% sodium dodecyl sulfate (SDS) was conducted in 12.6% PAAG by the Laemmli method [14] for 1 h at 40 V and 2 h at 100 V. Nondenaturing analytic electrophoresis was conducted in the Laemmli buffer system [14] in a PAAG gradient (4–20%) for 6 h at 75 V. The marker proteins were from Amersham Biosciences (Germany). After electrophoresis, the proteins were stained with 0.1% Coomassie Blue in a methanol–acetic acid–water (4.5 : 1.0 : 4.5, v/v) mixture or with 0.2% $AgNO_3$ in 0.08% (v/v) formalin solution; hemes were stained with 3,3',5,5'-tetramethylbenzidine (TMB) [15]. Cytochrome oxidase activity was detected by protein staining with 1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD).

Absorption spectra of the *cbo*-type oxidase and cytochrome c_H were recorded on a single-beam Beckman DU-8B spectrophotometer (United States) in 25 mM Tris–glycine buffer (pH 8.3) at room temperature. The samples were reduced with dry sodium dithionite or oxidized with H_2O_2 at a final concentration of 0.03%. The preparation of the *cbo*-type oxidase for recording CO difference spectra was obtained as described previously [12]. The amount of cytochrome c_H was estimated from the absolute absorption spectrum of the reduced form, using a molar extinction coefficient of 31 mM^{-1} cm^{-1} in the α band [16].

The rate of oxygen consumption was measured on a polarograph using a Clark-type electrode (Rank Bros., United Kingdom) in a 2-ml cell at 40°C. The kinetic constants of the oxidase activity were determined in a reaction mixture containing 25 mM 3-(*N*-morpholino)propanesulfonic buffer (MOPS), pH 7.0, and 0.1–4.0 mM TMPD or 30–300 μM horse heart cytochrome *c*, which were reduced with 5 mM ascorbate. The kinetic constants were calculated from initial reaction rates in the Michaelis–Menten system of coordinates using the Enzfitter software (Sigma, United States). The effect of pH on the respiratory activity of purified *cbo*-type oxidase was determined in MOPS–KOH buffer (pH 6.5–7.6) and 50 mM Tris–HCl buffer (pH 7.3–9.0). The enzyme concentration was 7–13 μg/ml; 5 mM ascorbate and 2 mM TMPD served as substrates. The oxidase respiratory activity was measured in the presence of cytochrome c_H in a reaction mixture containing 25 mM MOPS buffer (pH 7.0), 1 mM ascorbate, 6 nM cytochrome c_H, 10 μM TMPD, and 0.32 mg/ml of enzyme. To calculate the respiration rates, it was assumed that 200 nmol of O_2 are dissolved in 1 ml of the buffer at 40°C [17].

The content of protein was determined by the method of Lowry *et al.* [18] using horse heart cytochrome *c* (type III, Sigma, United States) as a standard.

RESULTS AND DISCUSSION

To study the kinetic properties and substrate specificity of the *cbo*-type oxidase from *M. flagellatus* KT, it was purified using preparative nondenaturing gel electrophoresis. By this method, a single protein band with an apparent molecular weight of 170 kDa was detected (Fig. 1), testifying to the homogeneity of the obtained enzyme preparation.

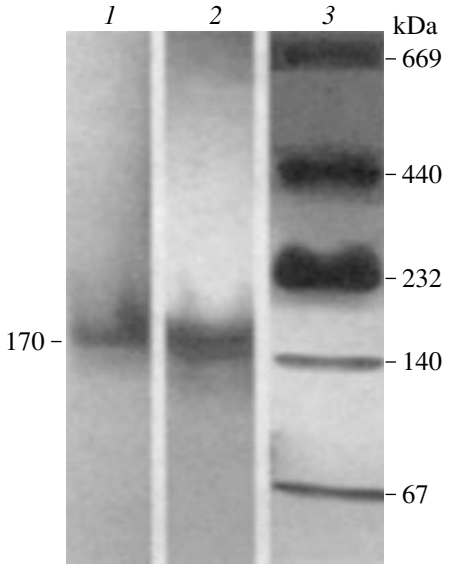

Fig. 1. Analytic nondenaturing electrophoresis in PAAG (4–20%). (*1*, *2*) *cbo*-type oxidase; *(3)* marker proteins (kDa): thyroglobulin, 669; ferritin, 440; catalase, 232; lactate dehydrogenase, 140; bovine serum albumin, 67. Lane *1* is stained with TMPD; lanes *2* and *3* are silver-stained.

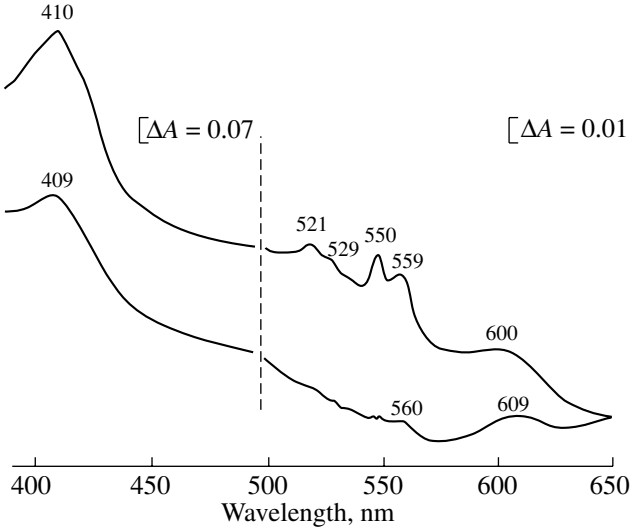

Fig. 2. Absolute absorption spectra of purified *cbo*-type oxidase (2.3 mg/ml): the upper spectrum, reduction with sodium dithionite; the lower one, oxidation with hydrogen peroxide.

Figure 2 shows absolute absorption spectra of the *cbo*-type oxidase. The absorption maxima of the reduced form of the *cbo*-type oxidase were at 550, 521, and 410 nm, which corresponded to the α, β, and γ bands of cytochromes *c*. The shoulder at 529 nm, the absorption maximum at 559 nm, and a broad low absorption peak at 600 nm belong to the cytochrome *bo* component. An oxidized form of the enzyme showed a peak of absorption at 409 nm and a broad maximum at 609 nm. In difference absorption spectrum, the cytochromes *c* exhibited maxima at 550, 521, and 415 nm, whereas *b*-type cytochromes exhibited absorption maxima at 560 and 529 nm [12].

Of special interest is the ability of cytochrome *cbo* to oxidize ascorbate. A pronounced maximum of the pH-dependent respiratory activity of the enzyme in the presence of ascorbate was observed at pH 8.3. The ascorbate oxidase activity seems to account for the same pH optimum at 8.3, which was characteristic of the ascorbate/TMPD–oxidase activity of the enzyme. The effect of pH on the rate of TMPD oxidation by the *cbo*-type oxidase was determined from the difference between oxidase activities toward ascorbate/TMPD and ascorbate alone. With TMPD as an electron donor, the *cbo*-type oxidase exhibited a broad pH optimum (7.0–7.6). Further kinetic analysis was performed at pH 7.0, where the level of ascorbate oxidase activity was lowest.

The *cbo*-type oxidase from *M. flagellatus* KT showed respiratory activity with reduced by ascorbate TMPD (K_M = 0.86 mM, V_{max} = 1.1 μmol O_2/(min mg protein)) and horse heart cytochrome *c* (K_M = 0.09 mM, V_{max} = 0.9 μmol O_2/(min mg protein)). The inhibition constant of the *cbo*-type oxidase activity by cyanide at pH 7.0 in the presence of ascorbate/TMPD ranged from 1.2 to 1.5 μM in membrane preparations and from 4.5 to 5 μM in purified enzyme preparation. The presence of autooxidizable cytochromes *c* in the oxidase preparation may account for the decrease in the sensitivity to cyanide. Indeed, CN^- and CO difference spectra of purified oxidase revealed cytochromes *c* that could bind cyanide and CO at low concentrations (Fig. 3). The absorption minima at 414 and 550 nm in the CN^- difference spectrum and those at 420 and 550 nm in the CO difference spectrum are characteristic of high-spin *c*-type cytochromes. Note that incubation of the *cbo*-type oxidase in the presence of carbon monoxide showed that 25% of the high-spin cytochrome *c* interacted with CO within 30 s (Fig. 3), whereas complete binding occurred after 5 min of incubation [12]. To all appearances, the presence of autooxidizable cytochromes *c* determines the ascorbate respiration of the enzyme. Thus, 5 mM ascorbate was oxidized by the enzyme at a specific rate of 0.29 μmol O_2/(min mg protein), and the oxidase activity was only 20% inhibited by 10 μM cyanide. Hence, 80% of ascorbate oxidation is not physiological; it is mediated by autooxidizable cytochromes *c*. Since membranes of *M. flagellatus* KT do not oxidize ascorbate, it is probable that cytochromes *c* of the *cbo*-type oxidase acquired their autooxidizable properties during enzyme purification.

Like other methylotrophs, *M. flagellatus* KT has two soluble periplasmic cytochromes *c*: c_L and c_H (Fig. 4, lanes *3* and *6*). Cytochrome c_L is characterized by a low isoelectric point and a relatively high molecular weight [1]. Conversely, cytochrome c_H has a high isoelectric point and a low molecular weight. Cytochrome c_L is the direct electron acceptor for methanol dehydrogenase,

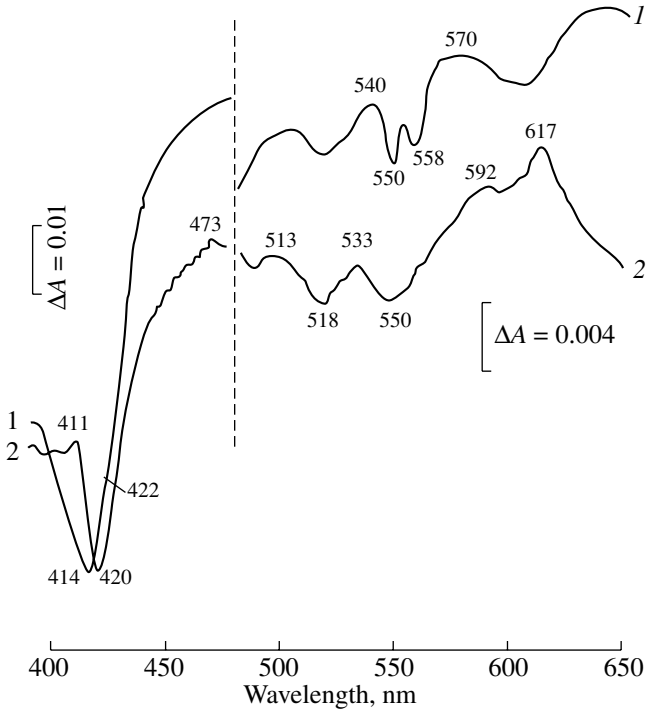

Fig. 3. Difference absorption spectra of purified *cbo*-type oxidase (2.3 mg/ml): *(1)* (reduction with dithionite + CN^-) minus reduction with dithionite; the KCN concentration was 20 μM; *(2)* (reduction with dithionite + CO) minus reduction with dithionite; the time of incubation with CO was 30 s.

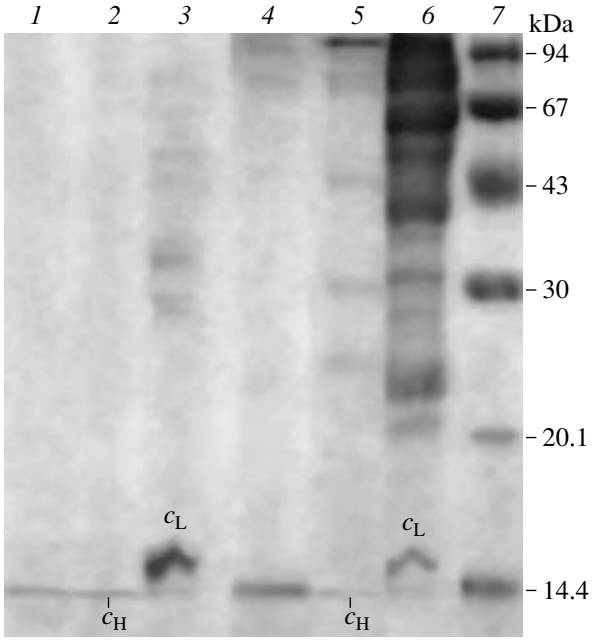

Fig. 4. SDS–PAAG (12.6%) electrophoresis. *(1, 4)* horse heart cytochrome *c*; *(2, 5)* partially purified preparation of cytochrome c_H; *(3, 6)* soluble fraction of *M. flagellatus* KT cell extract; *(7)* marker proteins (kDa): phosphorylase b, 94.0; bovine serum albumin, 67.0; ovalbumin, 43.0; carbonic anhydrase, 30.0; trypsin inhibitor, 20.1; α-lactalbumin, 14.4. Lanes *1*, *2*, and *3* are stained for heme with TMB; lanes *4–7* are stained for protein with Coomassie Blue.

whereas cytochrome c_H is believed to be the natural substrate for terminal cytochrome *c* oxidases [1]. Separation of the *M. flagellatus* KT cytochromes c_H and c_L was based on a significant difference between the electric charges of these proteins (see Materials and Methods). Thus, after electrophoresic separation of soluble proteins from *M. flagellatus* KT, the partially purified cytochrome c_H (12 kDa), which contained no cytochrome c_L, was concentrated in the cathodal space of the device (Fig. 4, lanes *2* and *5*). The dithionite-reduced cytochrome c_H from *M. flagellatus* KT exhibited absorption maxima at 551.7, 521.7, and 416.7 nm in the α, β, and γ bands, respectively (Fig. 5). The spectra of cytochromes c_H from *M. flagellatus* KT and from the facultative methylotroph *Methylobacterium extorquens* AM1 were surprisingly similar. Because of this, the concentration of reduced cytochrome c_H of *M. flagellatus* KT was determined from the absolute absorption spectrum using the molar extinction coefficient known for the α band of cytochrome c_H from *Methylobacterium extorquens* AM1 [16]. Cytochrome c_H from *M. flagellatus* KT (100 nM) exhibited a respiratory activity with 1 mM ascorbate (65 nmol O_2/(min mg protein)). To preclude endogenous ascorbate oxidation by cytochromes c_H and *cbo* in the reaction mixture, 1 mM ascorbate and 6 nM cytochrome c_H were used to study cytochrome c_H oxidation by the *cbo*-type oxidase. The ascorbate-reduced cytochrome c_H was oxidized by the *cbo*-type oxidase at a rate of 0.26 μmol O_2/(min mg protein). This implies that cytochrome *cbo* is a cytochrome *c* oxidase and that soluble cytochrome c_H serves as the natural electron donor for this enzyme.

The structural and functional properties of the *cbo*-type oxidase from *M. flagellatus* KT are in some respect similar to the properties of the *cbb'*-type oxidases from *Rhodobacter capsulatus, Rhodobacter sphaeroides*, and *Bradyrhizobium japonicum*, which were summarized by Sorokin *et al.* [19]. Like the *cbb'*-type oxidases, cytochrome *cbo* from *M. flagellatus* KT includes a dihemic cytochrome *c* as subunit III and oxidizes reduced TMPD and horse heart cytochrome *c*. However, unlike the *cbb'* complex, subunit II of *cbo*-type oxidase from *M. flagellatus* KT is a homodimeric, not a monohemic, cytochrome *c*. The catalytic center of the cytochrome *cbo* complex, which is located in subunit I, contains a high-spin heme O but not a heme B. Also, the *cbo*-type oxidase from *M. flagellatus* KT has an additional subunit IV (40 kDa) deprived of redox centers. The above structural features of the two types cytochrome *c* oxidases confer, presumably, specific functional properties. Thus, *cbb'*-type oxidases usually provide for bacterial growth under microaerobic conditions [19]. Conversely, cytochrome *cbo* is usually predominant in *M. flagellatus* KT at a high partial pressure of oxygen, whereas an additional *bb*-type (*o'*) cyto-

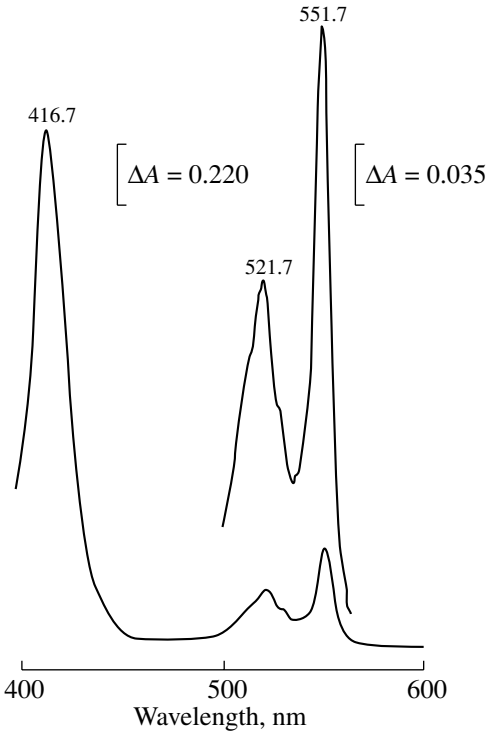

Fig. 5. Absolute absorption spectrum of dithionite-reduced partially purified cytochrome c_H from *M. flagellatus* KT. Protein concentration in the sample was 2.84 mg/ml.

chrome oxidase is involved in the respiratory chain at low p_{O_2} [11].

Taken together, the previously obtained data and the results of this study suggest that, in carbon-limited cells [11], as well as in the early logarithmic growth phase [10, 11], the methanol-oxidizing respiratory chain of *M. flagellatus* KT has the following structure: methanol dehydrogenase $\longrightarrow$ cyt. $c_L \longrightarrow$ cyt. $c_H \longrightarrow$ cyt. *cbo* $\longrightarrow O_2$.

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