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Two *o*-type oxidases in *Methylobacillus flagellatum* KT

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Two oxidases of the *o*-type in membranes of the methanol-grown obligate methylotroph *Methylobacillus flagellatum* KT were distinguished. For this purpose the kinetic analysis of the laser flash-induced optical absorbance changes of CO-oxidase complexes under reducing conditions was used. The ratio of these oxidases in membranes greatly depended on the phases of bacterial growth. One of the oxidases appeared to belong to the *Escherichia coli* *o*-type oxidase family being more sensitive to KCN (K_i = 1 μM). It showed monophasic CO recombination kinetics with τ 25-30 ms and was expressed in the early exponential phase of growth. The other oxidase seemed to be similar to the *Bacillus sp.FTU o-type* oxidase being less sensitive to KCN (K_i =6 μM), having three-phasic CO reassociation kinetics with τ 35-70 μs, 0.25-0.5 ms and 2-4 ms and dominating in the stationary growth phase. Pyridine haemochrome spectra showed haems A and D to be absent from the bacterial membranes.

CO flash photolysis of haemoproteins is the indicative tool for the presence of terminal oxidases (1,2). This method led Castor and Chance to the discovery of the *o*-type oxidase in microorganisms, the α-band of which was usually masked by the α-bands of *b*-type cytochromes in redox spectra (3,4). Being combined with the fast-resolving technique and computer analysis of the kinetic data this method has acquired a new importance (5-7). Recently this approach for fully reduced CO-oxidase complexes has been used by our group (8,9). It was shown that among a variety of bacterial terminal oxidases two general types could be distinguished. While one of them translocates H^+ across the membrane (8), the other does not pump H^+, presumably translocating Na^+(9). Representatives of each of the families are charac-

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Abbreviations: octyl glucoside, *n*-octyl-β-D-glucopyranoside; TMPD, N,N,N',N'-tetramethyl-*p*-phenylendiamine.

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terized by a number of common features: the same manner and kinetics of CO reassociation, CN^--sensitivity, TMPD oxidation patterns and physiological role. Interestingly, the two *o*-type oxidases found in *Escherichia coli* and *Bacillus* sp. FTU showed different properties after investigation according to the mentioned scheme: the former appeared to belong to the first family while the latter - to the second one. Matsushita and collegues (10) were the first to purify from *Acetobacter methanolicus* two different oxidases, *bo* and *co*. These oxidases terminate two independent respiratory chains oxidizing methanol or ethanol. Previously, it was shown that the restricted facultative methylotroph *Methylophilus methylotrophus* which used methanol as the sole energy and carbon source, contained two terminal oxidases of the aa_3- and *co*-type (11,12). In this paper we show that the obligate methylotroph *M.flagellatum* KT possesses two different *o*-type oxidases when grown on methanol.

MATERIALS AND METHODS

Growth conditions. The obligate methylotroph *Methylobacillus flagellatum* KT used in this work was isolated from activated sludge of the waste-water treatment station in Moscow (13). Bacteria were grown in batch culture in a mineral medium with 1% (v/v) methanol as the sole energy and carbon source (14). The culture was grown in a G24 orbital shaker (New Brunswick Co.) at 37°C and 300 rpm in 250 ml flasks containing 150 ml of the medium. To obtain cells in different growth phases optical density during the culture growth was photometrically monitored.

Preparation of the membrane fraction. Cells were harvested by centrifugation at 5,000×*g* for 15 min, washed twice with ice-cold medium A (25 mM MOPS-KOH buffer, pH 6.8), resuspended in medium A and disrupted by sonication (22kHz, 50μA, 6×30s periods) with cooling. The resulting homogenates were centrifuged at 135,000×*g* for 2 h to separate the membrane and soluble fractions. The membrane fraction was washed once and resuspended in buffer A.

Measurements. The reduced-*minus*-oxidized and CO-difference spectra were obtained as described earlier (15) using a Hitachi U-3400 spectrophotometer. For optical measurements semi-micro optical cuvettes with 1 cm-light path (usually, with 0.7 ml samples) were used. The membrane samples were suspended in the medium A used for membrane respiratory activity registration (see below). In case octyl glucoside was used the samples were incubated for 5 min in the medium with the detergent (1mg/1mg of protein) and then clarified by centrifuging (16,000×*g*, 10 min). Laser flash photolysis experiments with CO-oxidase complexes were performed as previously described (8). In case bacterial membranes were used with or without the detergent 50-100 or 500, respectively, kinetic curves obtained as the laser flash-induced optical absorbance changes were stored with 1 s intervals and averaged. As a rule a bandpass filter with the time constant of 3 μs was used to filter the amplified transient flash-induced signals. Special experiments showed no significant change in the

results when a filter with the time constant of 0.4 μs was used. All the optical experiments were performed at room temperature.

The respiratory activity of the membrane particles was measured with a Clark-type electrode at 25°C using 1 mM TMPD and 10 mM ascorbate as electron donors in a medium B containing 50 mM Tricine-KOH (pH 8.0), 0.15 M K_2SO_4, 5 mM $MgSO_4$, 2.5 mM Na_2SO_4 and 0.5 mM EDTA.

Protein was determined by the modified Lowry procedure with BSA as a standard (16). Pyridine haemochrome spectra were obtained according to Pappenheimer and Hendee (17).

RESULTS

M.flagellatum KT membranes obtained from the cells in the early exponential and in the stationary growth phases had the same shapes of reduced-*minus*-oxidized spectra, as in case of CO-difference spectra (Fig.1). The presence of cytochromes *b*, *o* and *c* could be detected. The pyridine haemochrome method did not reveal the presence of haems A and D in the membranes (data not shown). However, the cyanide titration curves of the respiratory activity with TMPD and ascorbate were distinctly different for the two species of membranes (Fig.2 A,B). Those from the early exponential growth phase were more sensitive to cyanide (K_i=1 μM) than the membranes from the stationary one (K_i=6 μM).

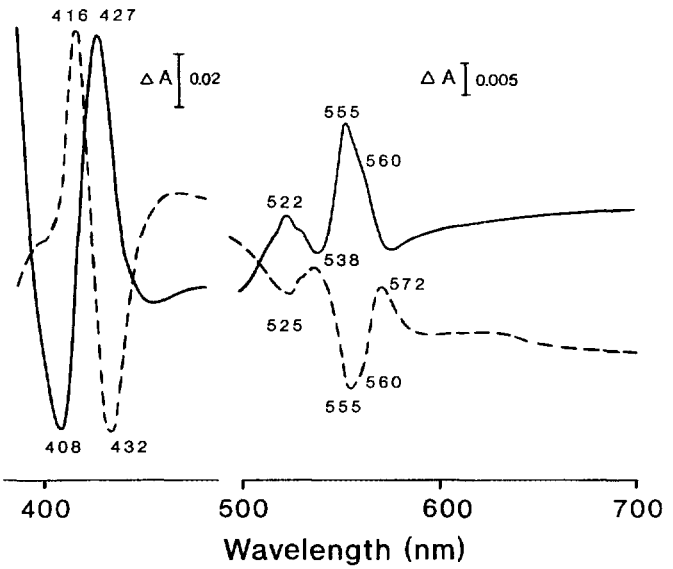

Fig.1. Sodium dithionite-reduced *minus* O_2-oxidized (solid line) and CO-difference (dashed line) spectra of *M.flagellatum* KT membranes at protein concentrations of 1 or 3 mg/ml, respectively. Membranes were isolated from the cells in the stationary growth phase and suspended in medium B (see 'Methods').

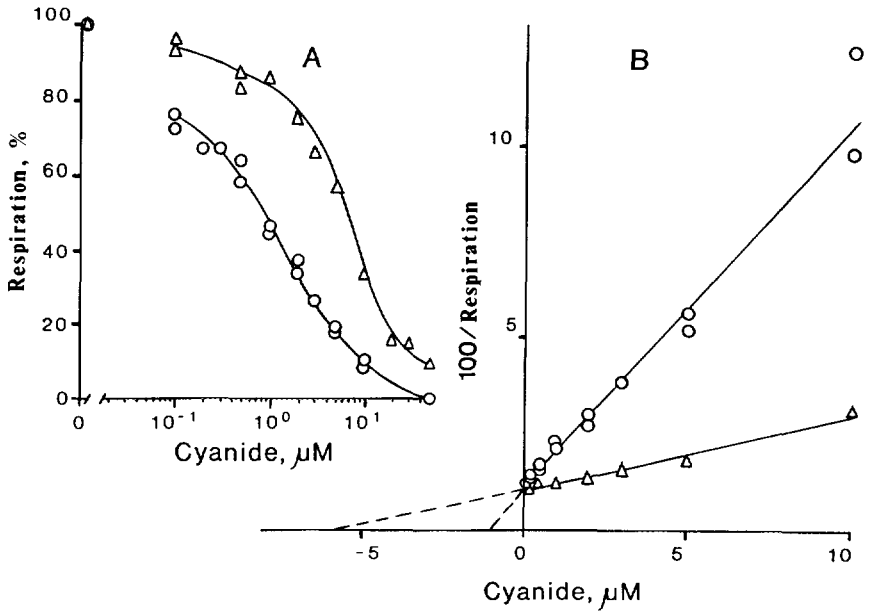

Fig.2. Cyanide inhibition of respiratory activity of membranes obtained from *M.flagellatum* KT cells in the early exponential (○) and in the stationary (△) growth phases. For conditions see 'Methods'. 100% activity corresponds to 480 ng atom O×min^{-1}×mg^{-1} of protein. A: inhibitory patterns. B: semireciprocal plots.

Kinetic analysis of the optical absorbance changes and the spectra of kinetic components upon laser flash photolysis of CO complexes with solubilized membrane enzymes under reducing conditions revealed both similarity and difference between the samples. The optical absorbance changes in both samples (Fig.3 A-C) consisted of four components with τ 35-70 μs, 0.25-0.5 ms, 2-4 ms and 25-30 ms. These components had the same spectral characteristics in both samples, correspondingly. The fastest (τ 35-70 μs) and the slowest (τ 25-30 ms) components seem to have the spectra of the *o*-type cytochromes (Fig.3 B,C) while the other two (τ 0.25-0.5 ms and 2-4 ms) are likely to be formed by the *b*-type cytochromes (Fig.3 C). The saturation laser light power for all the components appeared to be the same. Special experiments showed that the kinetic characteristics of the optical absorbance changes upon laser flash photolysis of CO complexes and the amplitude ratio of the kinetic components were the same for the samples with and without octyl glucoside. It is worth mentioning that the amplitude ratio of the kinetic components in the freshly obtained membranes and in those stored during half a year at -20°C was the same.

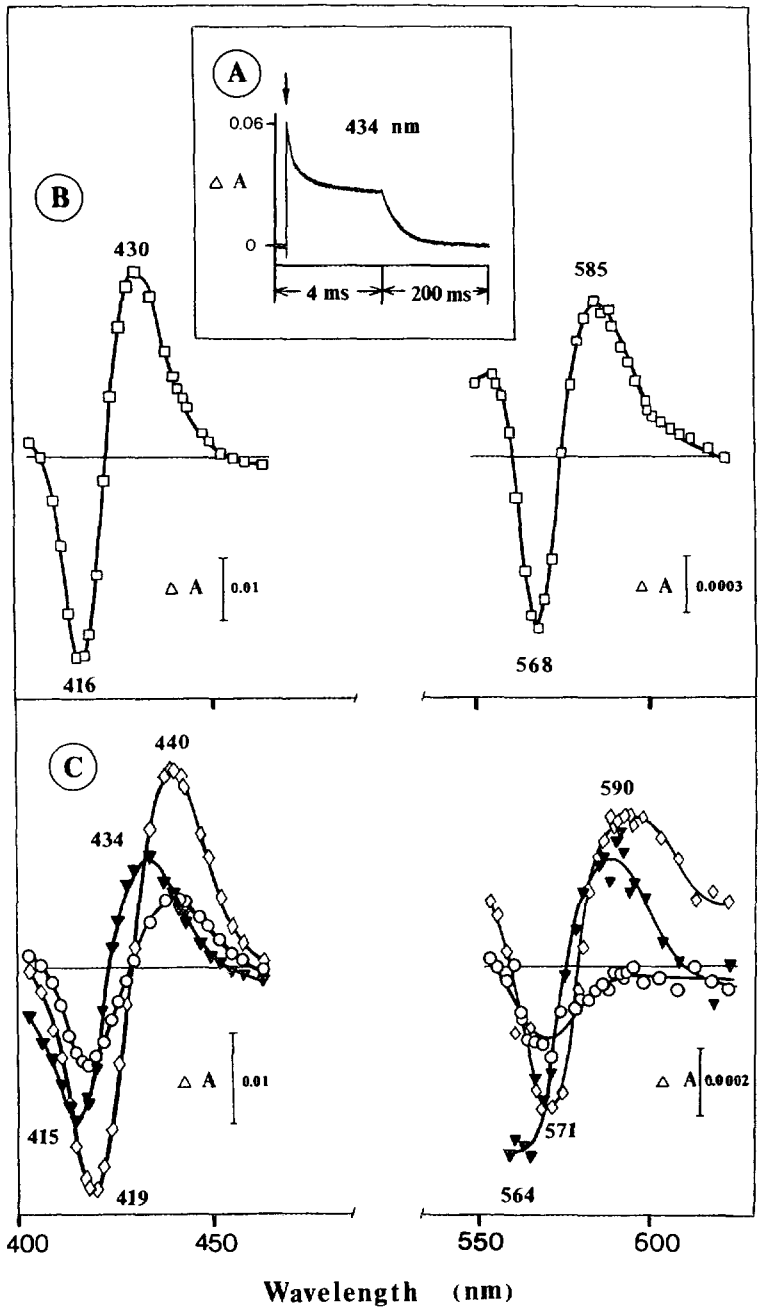

Fig.3. Flash photolysis of CO complexes of the solubilized *M.flagellatum* KT reduced membranes obtained from the stationary cells. 0.38ml of the membranes (5.6mg of protein) were dissolved in 1ml of medium B containing octyl glucoside (1mg/mg of protein) and clarified by centrifuging. 0.7ml samples were used in semi-micro optical cuvettes with 1cm-light path. (A) Kinetics of absorbance change decays upon laser flash photolysis at 434 nm. Arrow indicates the moment of the laser flash. (B,C) The spectra were obtained upon kinetic analysis of the laser flash-induced optical absorbance changes. τ of the kinetic curves were of 25-30 ms (□) in B, and 35-70μs (▼), 0.25-0.5ms (◇) and 2-4ms (○) in C.

The amplitude ratio of the three fastest components was the same in both membrane samples and did not correlate with the amplitude of the slowest component (τ 25-30 ms) (Table 1). Thus the slowest component dominating in the membranes from the cells in the early exponential growth phase might be associated with the presence of the *o*-type oxidase with a low affinity for O_2 and a high CN^--sensitivity like the one from the *E.coli* membranes. The three fast components formed the main part of the optical absorbance changes in membranes from the cells in the stationary growth phase. They might be due to the presence of another *o*-type oxidase with a high affinity for O_2 and a lower CN^--sensitivity like the one from the *Bacillus* sp. FTU membranes.

DISCUSSION

M.flagellatum KT terminal oxidases seem to present a difficult example for identification and estimation. In contrast, the *E.coli* terminal oxidases as well as those from *Bacillus* sp. FTU can be easily detected and their content can be estimated from the absorbance in γ- or α-band of the corresponding cytochromes in the reduced-*minus*-oxidized and CO-difference spectra. The method used by our group in this and in the previous works (8,9) allows to recognize yet indistinguishable *M.flagellatum* KT oxidases and, moreover, it gives the possibility to estimate the content of oxidases in membranes without purification.

Two *o*-type oxidases of *M.flagellatum* KT cells displayed during CO recombination a remarkable similarity in the kinetic behaviour with purified two *Bacillus* sp. FTU oxidases (caa_3- and *bo*-type) and with two *E.coli* oxidases (*bo*- and *bd*-type). Mono-

Table 1. The influence of the *M.flagellatum* KT growth phase on amplitudes (in the γ-band) of optical absorbance changes provided by various kinetic components

τ of the kinetic component	Amplitudes of kinetic components (in conventional units)	
	early exponential phase	stationary phase
25-30 ms	8.7 ± 0.05	3.1 ± 0.05
2-4 ms	1 ± 0.05	1 ± 0.05
0.25-0.5 ms	2.3 ± 0.05	2.3 ± 0.05
35-70 μs	1.5 ± 0.05	1.6 ± 0.05

phasicity of kinetics characteristic of the slowest component (τ 25-30 ms), the independence of this component of the three fast components and its domination in the early exponential growth phase suggest that we deal with an *o*-type oxidase which closely resembles the *E.coli bo*-type and *Bacillus* sp. FTU caa_3-type oxidases (8). The very close coincidence of the kinetic curves of the three fast components with those of the *Bacillus* sp. FTU *bo*- and *E.coli bd*-type oxidases (9) and their domination in the stationary growth phase show that they are provided by the other *o*-type oxidase.

The two triplets of oxidases, slowly and rapidly reacting with CO, respectively, from the three studied bacteria appear to share another common feature: the former group of oxidases is more sensitive to cyanide than the latter one.

Our results on CO recombination with a variety of oxidases are in good agreement with the recent report of Basu et al.(18). This team showed the existence of two groups of oxidases. The rate constants of CO recombination at -70°C with representatives of one group (*E.coli bo*-type, *S.cerevisiae* aa_3-type, *T.thermophilus* aa_3-, caa_3-types and *P.denitrificans* aa_3-type) were around 10^{-3}-10^{-2} s^{-1}, being about two orders of magnitude lower than those with the other one (*E.coli bd*-type and *T.thermophilus* ba_3-type).

According to our data, the oxidases with the fast kinetics of CO reassociation [*Bacillus* sp. FTU *bo*-type, *E.coli bd*-type (9) and *M.flagellatum* KT *bo*-type (Fig.3 B,C)] appear to have an additional high-spin haem, i.e. of the *b*-type with τ 0.25-0.5 ms, except of the obvious high-spin one of the *o*- or *d*-type. This observation is consistent with the latest data obtained by Hill et al.(19) on *E.coli bd*-type oxidase. Using Fourier transform infrared spectroscopy, the second high-spin haem b_{595}, existing in this oxidase was shown to react with the CO molecule more slowly under low temperature than the *d*-haem did (19). The *E.coli bd*-oxidase is known to have no copper (20), and the high-spin *d*- and *b*-haems according to Hill et al.(19) form the haem-haem binuclear center for O_2 reduction. The nature of the *b*-haem response with τ 2-4 ms is just unclear. It might be explained by partial denaturation of the low-spin *b*-haems and obtaining the CO combining property by them.

Oppositely, the other family of oxidases (*E.coli bo*, *M.flagellatum* KT *o* and *Bacillus* sp.FTU caa_3) with slow CO reassociation

kinetics appears to have just one high-spin haem, that is, correspondingly, *o*- or a_3-haem, while the second haem is in the low-spin form (*b* in *E.coli*, *b* or maybe *c* in *M.flagellatum* KT and *a* in *Bacillus* sp. FTU). In this group of oxidases the binuclear haem-Cu center seems to reduce O_2 to H_2O, translocating additional H^+ across the membrane during the catalytic cycle (the $H^+/\bar{e}$ ratio=2).

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