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Kinetics of CO binding to H^+-motive oxidases of the caa_3-type from *Bacillus FTU* and of the *o*-type from *Escherichia coli*

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The kinetics of CO rebinding with isolated *Bacillus FTU* caa_3-type oxidase and with solubilized *Escherichia coli* membranes (GO103 strain) containing the *o*-type oxidase as the main O_2-reducing enzyme were studied under reducing conditions by laser flash photolysis of the CO–oxidase complexes. The spectra of the optical absorbance changes upon photolysis were characteristic of CO–caa_3- and CO–*o*-oxidase complexes in *Bac. FTU* and *E. coli*, respectively. Small quantities of *d*-type oxidase in *E. coli* GO103 membranes were detected. The kinetics of CO reassociation with reduced caa_3- and *o*-type oxidases were monophasic with τ 25–30 ms in both cases.

Flash photolysis; caa_3-Type oxidase; *o*-Type oxidase; *Bacillus FTU*; *Escherichia coli*

1. INTRODUCTION

The terminal segment of the *E. coli* respiratory chain, like that in many other aerobic bacteria, is branched and contains at least two oxidases i.e.: *o*- and *d*-types. The former was shown to be a proton pump, like the mitochondrial cytochrome *c* oxidase, while the latter, in spite of taking part in energy coupling, does not pump H^+ [1]. According to data obtained in our laboratory, the *E. coli d*-type oxidase operates as a Na^+-pump when the cells are grown under low $\Delta\tilde{\mu}_H^+$ conditions [2]. A Na^+-motive oxidase was also described in this group in the alkalo- and halotolerant *Bac. FTU* [3].

Recently it has been shown by our group that *Bac. FTU* contains terminal oxidases of the caa_3- [4,5] and *o*-type [5,6]. However, in contrast to *E. coli*, an *o*-type oxidase seemed to be involved in the Na^+ translocation [3], whereas a caa_3-type oxidase was responsible for H^+ pumping like the *o*-type oxidase from *E. coli* [3,5]. Another common property of the *E. coli o*-type and *Bac. FTU* caa_3-type oxidases is that they seem to dominate in the beginning of cell growth and at high O_2 levels. In the present paper, we show that these two enzymes have the same kinetics of recombination with CO.

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Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine; PAG, polyacrylamide gel; $\Delta\tilde{\mu}_H^+$, the proton electrochemical gradient.

2. MATERIALS AND METHODS

2.1. *Preparations*

The *Bac. FTU* cells were grown aerobically in the medium previously employed by Semeykina et al. [7]. Cells taken at the exponential phase of growth were used. The *Bac. FTU* membrane particles were obtained as described elsewhere [5]. The caa_3-type oxidase was isolated and purified from the membrane particles [8]. The enzyme was stored as an ammonium sulfate precipitate at 4°C.

Escherichia coli strains GO103 (GO-103:GR70N,Δcyd::kan, str^r,kan^r) with a deletion in the *d*-type oxidase gene and GO102 (GO102/pFH 101-GO102:F^-;cyo 123, rps L,rel A,lon 100, thi,gal, Δcyd::kan,str^r, kan^r;pF 101), which overproduced the *d*- type oxidase and had a deletion in the *o*-type oxidase gene, were gifts from Prof. R.B. Gennis. The bacteria were grown in medium LB. Membrane particles were obtained as previously described [9] and stored in medium A, containing 50 mM Tricine-KOH (pH 8.1), 150 mM KCl, 2.5 mM Na_2SO_4, 1 mM EDTA, supplied with 20% glycerol, in liquid nitrogen.

Before the experiments, the particles were treated with 30 mM octyl glucoside. The supernatant of a 10,000 × *g* (10 min) centrifugation was used in the optical measurements. Control experiments showed that octyl glucoside was without effect on the kinetics of the laser flash-induced photolysis of the CO complexes.

Electrophoresis under non-denaturating conditions was performed according to the method of Davis [10] with slight modifications; to the polyacrylamide gels 0.3% Triton X-100 was added. The haem staining procedure was the same as described by Thomas et al. [11].

2.2. *Measurements*

The reduced-*minus*-oxidased and CO-difference spectra were measured as described elsewhere [5].

The kinetics of the laser flash-induced absorption changes of CO–oxidase complexes were measured at fixed wavelengths in semi-micro 1-cm optical cuvettes with a single-beam spectrophotometer interfaced to an IBM XT-286 computer via a DL-1080 transient recorder. The amplified transient flash-induced signals were filtered with a bandpass filter (the time constant, 10 μs). The CO–cytochrome complex reassociation kinetics were measured in the 400–650 nm range with 1 nm bandwidth using a 75-W KGM-9V halogen lamp as the source for the monitoring light. Photoexitation of CO–cytochrome complexes was achieved with a Quantel YG-481 neodymium laser (λ = 532 nm; pulse half-width, 15 ns; energy, 50 mJ per flash). The photomultiplier was

protected against the exciting light with a second monochromator and with cut-off filters. Usually, 10–25 curves were stored with 5 s intervals and averaged. Data storage, processing, and curve fitting were carried out using a set of programs developed in our laboratory by Dr. A.L. Drachev. The kinetic traces were treated as sums of several exponents to find $\tau(t_{1/e})$ values and amplitudes using the computer program DISCRETE developed by Provencher [12]. All measurements were performed at 23–25°C.

The respiratory activity of the *Bac. FTU* enzyme and *E. coli* membrane particles were measured with a Clark-type electrode at 25°C using TMPD and ascorbate as electron donors in medium A.

Protein was determined by the modified Lowry procedure with BSA as a standard [13].

3. RESULTS AND DISCUSSION

The spectra of the flash-induced optical absorbance changes of CO complexes with reduced *Bac. FTU* caa_3-type oxidase and *E. coli* GO103 membranes (Fig. 1A) were found to be nearly mirror-symmetrical to the respective CO-difference spectra (Fig. 2). In both cases, the flash- induced absorbance changes corresponded to about 80% of the absolute absorbency in the CO-difference spectra. The absorbance change–laser power relationship showed that the power used was equal to 80% of the saturating one. The flash photolysis spectra of absorbance changes proved to be consistent with the assumption that we are dealing with CO–caa_3-type and CO–*o*-type oxidase complexes in *Bac. FTU* and *E. coli* GO103, respectively.

The kinetics of CO reassociation with *Bac. FTU* caa_3-type oxidase (Fig. 1B) are well described by one exponential with τ 25–30 ms in the wavelength range 405–630 nm.

The flash-induced absorbance changes in *E. coli* GO103 samples were in part (no more than 5%) due to a very small amount of a *d*-type oxidase still present in the *E. coli* GO103 membranes. This amount was so small that it could not be seen in the difference spectra (Fig. 2B) and in the 4–9% gradient PAG after electrophoresis under non-denaturating conditions and haem staining (Fig. 3B). Nevertheless, it was detected after staining gels with TMPD (Fig. 3A, lanes 1,2). The *E. coli* GO102 membranes appeared to have *d*-type oxidase only (Fig. 3A, lanes 3–5). The kinetics of CO reassociation with reduced *E. coli* GO102 membranes (see the next paper [14]) were the same as in the case of the small fast component (τ < 5 ms) in the kinetic curves of *E. coli* GO103 membranes. Thus the small fast component in the kinetics from their τ and spectrum of absorbance changes was identified with the CO reassociation of *d*-type oxidase and was substracted from the spectral changes obtained in *E. coli* GO103 membranes.

The kinetics of CO reassociation with *E. coli o*-type

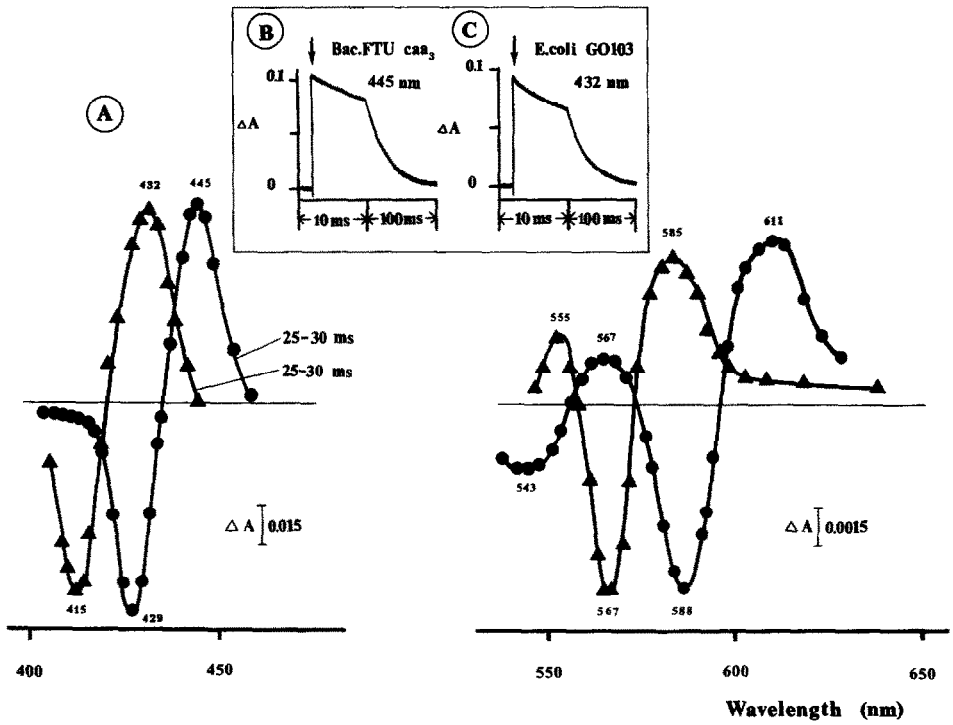

Fig. 1. Flash photolysis of CO complexes of the reduced *Bac. FTU* caa_3-type oxidase (●) and the solubilized *E. coli* GO103 reduced membranes (▲). (A) Laser flash-induced spectral changes. The samples were treated with argon for 15 min, reduced with sodium dithionite and then treated with CO for 5 min. Cuvettes contained 0.7 ml samples. The protein concentrations were 0.075 mg/ml (*Bac. FTU*), 0.33 mg/ml (*E. coli*). The samples were dissolved in buffer A (see section 2) supplemented, in the case of *E. coli*, with 30 mM oxtyl glucoside. (B,C) Kinetics of absorbance change decays upon laser flash photolysis. The CO complexes with *Bac. FTU* reduced caa_3-type oxidase at 445 nm (B) and solubilized *E. coli* GO103 reduced membranes at 432 nm (C). Arrows indicate addition of laser flash.

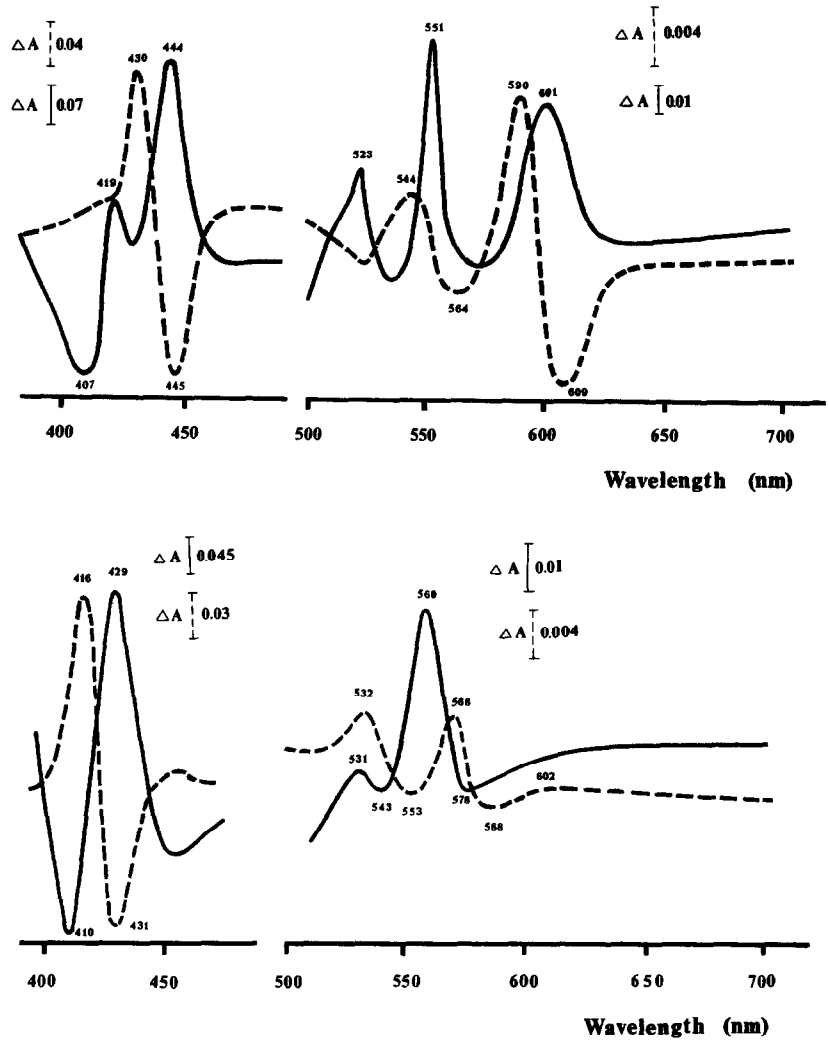

Fig. 2. Sodium dithionite-reduced *minus* O_2-oxidised (solid line) and CO-difference (dashed line) spectra of *Bac. FTU* caa_3-type oxidase (A) and solubilized *E. coli* GO103 membranes (B). Spectra were recorded with a Hitachi U-3400 spectrophotometer at 25°C. For conditions, see Fig. 1. Protein concentrations were 0.1 mg/ml (A) and 3.5 mg/ml (B).

oxidase was the same as in the case with *Bac. FTU* caa_3-type oxidase, i.e. monophasic with τ 25–30 ms.

Our results are in good agreement with the latest data on the structural homology between the *E. coli o*-type and a number of aa_3-type oxidases [15]. In fact, these enzymes and the *Bac. FTU* one belong to the same family [16]. The same pattern of recombination with CO seems to indicate the same affinity for each of the compound involved i.e. CO and O_2. It is noteworthy, that both *E. coli o*-type [17,18] and *Bac. FTU* caa_3-type oxidases [5] are induced in the exponential phase of growth. There are indications that they are induced when the O_2 level is high. This was shown for *PS3* aa_3-type [19] and *E. coli o*-type [20,21] oxidases. In this context it seems important that the oxidases in question are H^+ pumps. This was demonstrated for *E. coli o*-type [22], *PS3* caa_3-type [23] and *Paracoccus denitrificans* aa_3-type [24] oxidases. In the case of *Bac. FTU*, the caa_3-type oxidase apparently plays the same role of H^+ pump. This is confirmed by the same inhibitory effect of KCN (K_i = 2

Table I

The respiratory activities of the *E. coli* and *Bac. FTU* membranes. TMPD (0.05–10 mM) and ascorbate (10 mM) were used as electron donors.

The studied systems	V_{max}
E. coli GO103, membranes	2.0[a]
E. coli GO102, membranes	0.35[b]
Bac. FTU, beginning of log phase, membranes	11.2[c]
Bac. FTU, stationary phase, membranes	2.2[d]

V_{max} of the respiratory activities were expressed in:
[a] μmol $O_2 \times min^{-1} \times nmol^{-1}$ haem *o*;
[b] μmol $O_2 \times min^{-1} \times nmol^{-1}$ haem *d*;
[c] μmol $O_2 \times min^{-1} \times nmol^{-1}$ haem *a*;
[d] μmol $O_2 \times min^{-1} \times nmol^{-1}$ haem *o*.

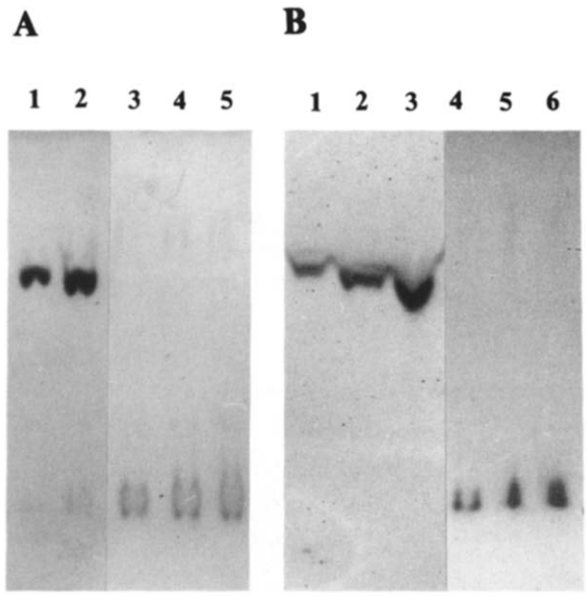

Fig. 3. Identification of oxidases in *E. coli* membranes. Electrophoresis under non-denaturating conditions was performed in 4–9% gradient PAG as described in section 2. The gels were stained for TMPD-oxidising activity (A) and for haems (B). The protein concentrations were for *E. coli* GO103 membranes: A, 50 and 80 μg for lanes 1 and 2; B, 50, 100 and 200 μg for lanes 1, 2 and 3. The protein concentrations were for *E. coli* GO102 membranes: A, 70, 100 and 150 μg for lanes 3, 4 and 5; B, 65, 130 and 185 μg for lanes 4, 5 and 6.

μM) on the H^+ transport of *Bac. FTU* membranes [3] and of the respiratory activity of the purified caa_3-type oxidase [5].

In both *E. coli* and *Bac. FTU*, the *o*-type and the caa_3-type oxidases are more sensitive to CN^- [5,20,25] and use more effectively the artificial electron donor, TMPD [5,20,25], than the alternative (putative Na^+-motive) oxidases from the same bacteria, (see also Table I and Fig. 3: compare panel A, lanes 1–5 with panel B, lanes 1–6).

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