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

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The kinetics of CO reassociation with isolated *Bacillus FTU* *o*-type oxidase and with solubilized membranes of *Escherichia coli* (GO102 strain) containing the *d*-type oxidase only, upon laser flash photolysis under reducing conditions, were studied. In both cases, kinetics are shown to be composed of three phases (τ 35–70 μs, 0.25–0.5 ms and 2–5 ms). The spectra of the flash-induced absorbance changes of the first kinetic components proved to be characteristic of CO–*o*- and CO–b_{595} *d*-cytochrome complexes in *Bac. FTU* and *E. coli*, respectively. The spectra of the second and the third components appeared to be nearly the same in *Bac. FTU* and *E. coli* with peaks for the former at 436–437 and 590 nm and troughs at 419–420 and 569 nm; and for the latter with peaks at 436–437 and 558–560 nm and troughs at 419–420 and 575–578 nm. The similarity between the putative Na^+-pumping *Bac. FTU* *o*- and *E. coli* *d*-type oxidases and their difference from the H^+-motive *Bac. FTU* caa_3- and *E. coli* *o*-type oxidases are discussed.

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

1. INTRODUCTION

E. coli *d*-type oxidase seems to be peculiar among haem-containing terminal oxidases [1]. The *d*-haem prosthetic group of this oxidase differs greatly from *a*- and *o*-haems. An unusually high affinity for O_2 [2,3] as well as resistance to low cyanide concentrations [3,4] have been noted for this oxidase. Detailed studies on its recombination with CO have not been previously reported.

In this paper, we analyzed the CO-binding properties of *E. coli* *d*-type oxidase in detail and compared this enzyme with the *Bac. FTU* *o*-type oxidase recently isolated in our group. Such a comparison seemed interesting since both oxidases were assumed to be Na^+ pumps. As shown in our laboratory, membrane vesicles from the *E. coli* mutant containing only the *d*-type oxidase [5] and those from *Bac. FTU* grown under low $\Delta\tilde{\mu}_{H^+}$ conditions [6] were competent in Na^+ transport when TMPD and ascorbate were used as electron donors. The process was resistant to low concentrations of KCN [5,7,8]. The present data reveal a high degree of similarity between these oxidases.

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylendiamine; $\Delta\tilde{\mu}_{H^+}$, the proton electrochemical gradient.

2. MATERIALS AND METHODS

2.1. *Preparations*

Bac. FTU cells were grown aerobically in a medium described elsewhere [9]. The *Bac. FTU* membrane particles were obtained from stationary cells by the method developed in this group [8]. The *o*-type oxidase was isolated and purified from membrane particles by extraction with octyl glucoside. The *o*-type oxidase fraction was salted out at 47–55% $(NH_4)_2SO_4$. The pellet was then dissolved in medium A containing 50 mM Tricine-KOH (pH 8.1), 150 mM KCl, 2.5 mM Na_2SO_4, and 1 mM EDTA and loaded onto a DEAE-Toyo-Pearl column equilibrated with medium containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA, and 30 mM octyl glucoside. Elution was performed by a step gradient of NaCl (50, 100, 200, 300, 400, 500 mM) in the same medium. The *o*-type oxidase was eluted with 400 mM NaCl. The fractions, containing the *o*-type oxidase, were combined and concentrated in an ultrafiltration cell. The enzyme was stored in medium A containing 25% glycerol and 30 mM octyl glucoside in liquid nitrogen. Before the experiments, the enzyme was diluted with medium A supplemented with 30 mM octyl glucoside.

E. coli strain GO102 (GO102/pFH 101-GO102:F^-;cyo 123,rps L,rel A,lon 100,thi,gall,Δcyd::kan,str^r,kan^r;pF 101), which overproduced the *d*-type oxidase and had a deletion in the *o*-type oxidase gene, was a gift of Prof. R.B. Gennis. Bacteria were grown in medium LB. Membrane particles were obtained as previously described [8]. The V_{max} of the respiratory activity of the membranes with TMPD and ascorbate was of 0.35 μmol $O_2 \times min^{-1} \times mg^{-1}$ protein. Membrane particles were stored in medium A containing 20% glycerol in liquid nitrogen.

2.2. *Measurements*

Measurements were performed as described in the preceding paper [11]. The kinetic curves were obtained as the result of the decay of the laser flash-induced optical changes of CO–oxidase complexes. The difference was concerned with the data storage. Usually, 50–100 curves were stored with 5 s intervals and averaged.

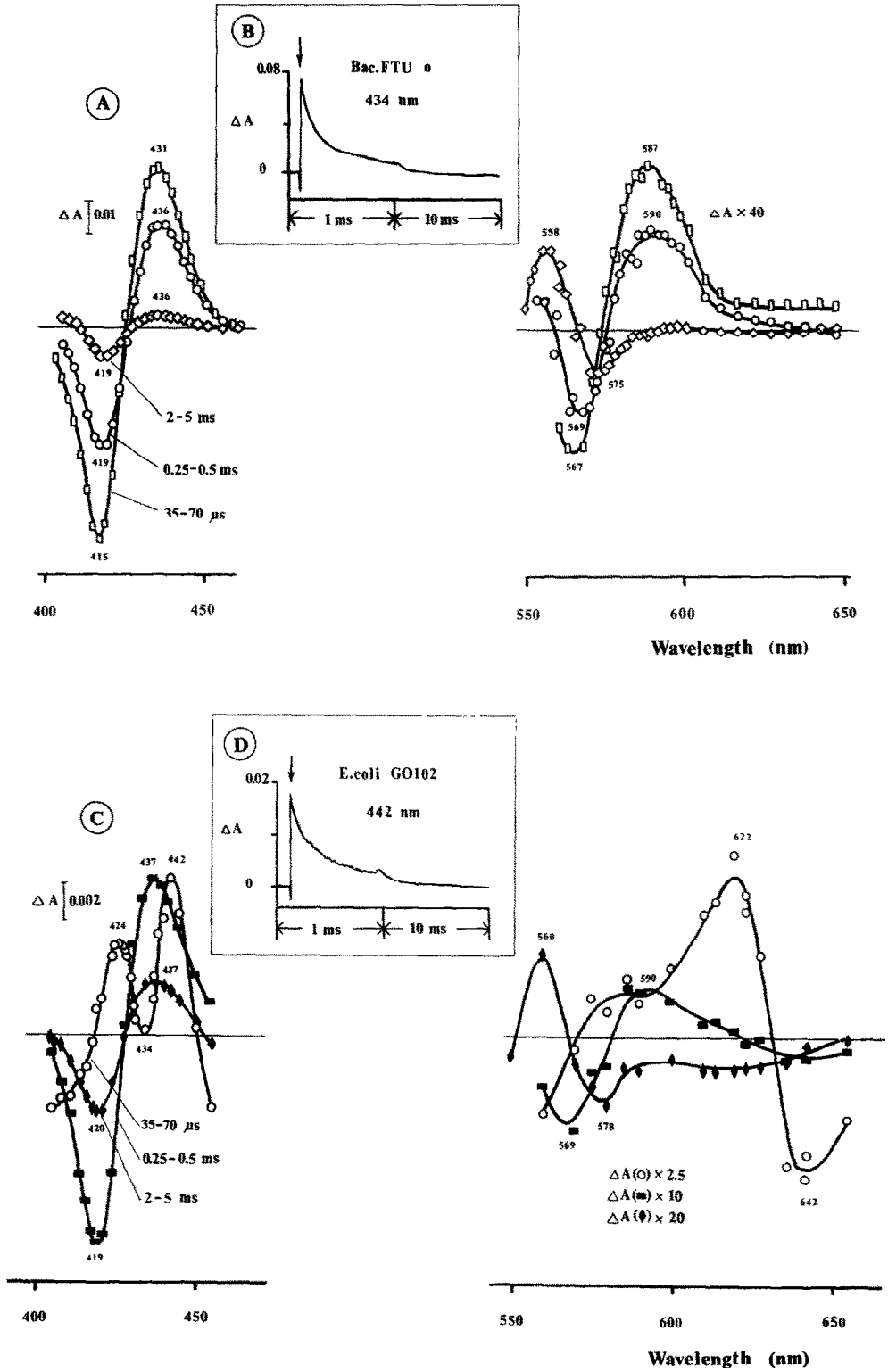

Fig. 1. Flash photolysis of CO complexes of reduced *Bac. FTU* o-type oxidase (A,B) and solubilized *E. coli* GO102 membranes (C,D). (A,C) Laser flash-induced spectral changes. The samples were treated with argon for 15 min, reduced with sodium dithionite and then treated with CO for 5 min. Cuvettes contained 0.7 ml samples; the protein concentrations, 0.2 mg/ml (A,B) and 3 mg/ml (C,D). The samples were diluted with buffer A (see section 2) containing 30 mM octyl glucoside. (B,D) Kinetics of absorbance change decays upon laser flash photolysis. The CO complexes with *Bac. FTU* reduced o-type oxidase at 434 nm (B) and solubilized *E. coli* GO102 reduced membranes at 442 nm (D). Arrows indicate the moments of laser flashes.

3. RESULTS AND DISCUSSION

As we reported elsewhere, in *Bac. FTU* both the respiratory activity (see Table I in [11]) and sensitivity for KCN are one order higher for the caa_3-type oxidase than for the *o*-type oxidase [10]. The KCN titration curves of respiration showed that the samples of isolated *Bac. FTU o*-type oxidase did not contain the caa_3-type oxidase. In fact, the highly KCN-sensitive component was not detected. *E. coli* GO102 membranes were shown to contain the *d*-type oxidase only [11]. The kinetics of CO reassociation and the spectra of absorption changes upon laser flash photolysis of CO complexes with the reduced *Bac. FTU o*-type oxidase and *E. coli* GO102 membranes were constant during 6 h at room temperature. The shapes of the laser flash-induced absorbance change spectra (Fig. 1A,C) as well as of the CO difference spectra (Fig. 2A,B) clearly indicated that we were dealing with the CO-*o*- and CO-*d*-oxidase complexes in *Bac. FTU* and *E. coli*, respectively.

The magnitudes of the flash photolysis absorbance changes were 80% of the corresponding absorbances in CO difference spectra in the case of both *Bac. FTU* and *E. coli* systems.

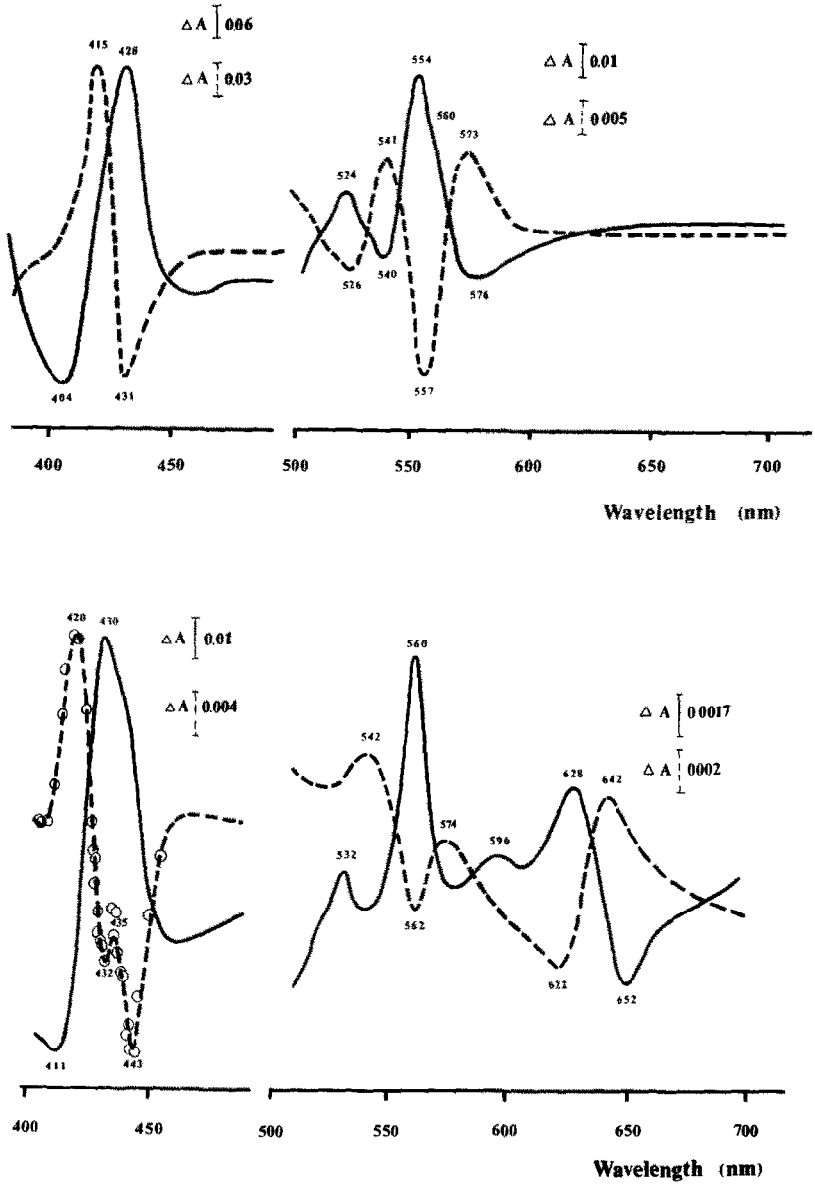

Fig. 2. Sodium dithionite-reduced minus O_2-oxidised (solid line) and CO (dashed line) difference spectra of *Bac. FTU o*-type oxidase (A) and solubilised *E. coli* GO102 membranes (B). Spectra were recorded with a Hitachi U-3400 spectrophotometer at 25°C. For conditions see Fig. 1. The protein concentrations, A, 0.2 mg/ml, B, 3 mg/ml. The open circles show the laser flash-induced optical absorbance changes (the upside-down presentation). The values of absorbances were calculated for the saturating laser power.

Analysis of the kinetic curves of the absorbance change decays upon laser flash photolysis of CO–oxidase complexes showed that they were composed of the three components in both cases (Fig. 1A,C). Their kinetics were similar for the *Bac. FTU* and *E. coli* enzymes (τ 35–70 μs, 0.25–0.5 ms and 2–5 ms). The spectra of the first kinetic components proved to be characteristic of the CO–*o*- and CO–b_{595} *d*-cytochrome complexes in *Bac. FTU* and *E. coli* enzymes, respectively. The second and the third components appeared to be nearly the same in both systems. The second component had peaks at 436–437 and 590 nm and troughs at 419–420 and 569 nm and seemed to indicate the high-spin *b*-haem presence in both oxidases. The third component had peaks at 436–437 and 558–560 nm and troughs at 419–420 and 575–578 nm. Perhaps the slowest components were due to the presence of the low-spin b_{559}-haems in both enzymes, which seems to become CO-reactive upon partial denaturation of the corresponding proteins. All three components were seen both in the presence and in the absence of octyl glucoside (membrane preparations of *Bac. FTU* as well as of *E. coli* were used).

It is clear from the presented results that the two terminal oxidases of the *o*- and *d*-type from *Bac. FTU* and *E. coli*, respectively, have much in common. On the other hand, they clearly differ from the *Bac. FTU* caa_3-type and the *E. coli* *o*-type oxidases [11].

1. The half-time of CO recombination with the reduced *Bac. FTU* *o*-type and *E. coli* *d*-type oxidases is, at least, two orders shorter than with *Bac. FTU* caa_3-type and *E. coli* *o*-type oxidases. This suggests that the two former oxidases should have much higher affinity for CO and O_2 than the two latter oxidases. It can be mentioned in this context that the former oxidases dominate at the stationary phase of growth when the O_2 concentration is lower. This was demonstrated for *Bac. FTU* *o*-type [10], *E. coli* *d*-type [12,13], and bacterium *PS3* *o*-type oxidases [14].

2. *Bac. FTU* *o*-type and *E. coli* *d*-type oxidases have a lower sensitivity to CN^- and lower activity with TMPD and ascorbate as electron donors. (Compare K_i for KCN in the case of (i) *Bac. FTU* caa_3- and *o*-type oxidases [10] and (ii) *E. coli* *o*- and *d*-type oxidases [4,15]. For specific activities of the four oxidases, see the preceding paper, Table I and Fig. 3 [11]).

Because *Bac. FTU* *o*-type and *E. coli* *d*-type oxidases are supposed to comprise one family of enzymes, we may deduce some features for one of them using the properties of the other. It is interesting that *E. coli* *d*-type oxidase does not contain Cu^{2+} ions [4]. There are some other bacterial oxidases which seem to belong to the same family and do not contain Cu^{2+} ions, i.e. bacterium *PS3* *o*-type [16] and *Pseudomonas aeruginosa* *o*-type [17] oxidases. EPR-invisible Cu^{2+} is known to form a binuclear center with haem in the aa_3-type cytochrome *c* oxidases and in the *o*-type oxidase from *E. coli* [18–21]. The center in question is responsible for the O_2 reduction catalysis. Apparently in the former oxidase family this process is catalyzed in some other way.

It should be stressed that *E. coli* *d*-type [22] and bacterium *PS3* *o*-type [16] oxidases do not transport H^+ across membranes. On the other hand, there are indications that the former enzyme and *Bac. FTU* *o*-type oxidase can operate as Na^+-pumps [5–9]. This is in contrast to the aa_3-type oxidases of mitochondria and various bacteria and to the *E. coli* *o*-type oxidase which are known to function as H^+ pumps [20].

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