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Terminal Oxidases of the *bb*- and caa_3-Types in *Bacillus* sp. FTU

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We previously identified two oxidases in the membranes of bacterium *Bacillus* sp. FTU. One of them slowly (caa_3) and the other rapidly (*bo*) recombines with carbon monoxide (CO) after laser flash photolysis, in this respect resembling the *Escherichia coli bo*- and *bd*-type oxidases, respectively. In the present study we found three copper atoms in the slowly CO-recombining oxidase from *Bacillus* sp. FTU. In the other oxidase, the copper content is very low and clearly substoichiometric. Reversed-phase chromatography revealed the presence of haems A and C in the *Bacillus* sp. FTU copper-containing oxidase and haems B and C in the non-copper-containing one. We thus suggest that the *Bacillus* sp. FTU oxidase rapidly reacting with CO previously attributed to *bo*-type by analogy in redox spectrum with the *E.coli* enzyme be redefined as *bb*-type oxidase.

A terminal oxidase capable of reversible CO binding, having (i) a cytochrome *b*-like redox spectrum, and (ii) CO-difference spectrum with absorption maximum at 416 nm, was first described in *Staphylococcus albus* (1,2) and was later defined as the *o*-type oxidase (3). Subsequently, all cytochromes reducing O_2 to H_2O, binding CO and cyanide, and showing CO-difference spectra like that of the *St.albus* oxidase were called the *o*-type oxidases (for review, see ref. 4). Until 1991, it was assumed that *o*-type oxidases contained haem *b* because of their spectral properties. However, Puustinen et al. (5,6) showed that the *E.coli* *o*-type oxidase contains, in addition to haem B, a new haem possessing hydroxyl and farnesyl substituents, like haem A, but retaining methyl group at position 8, like haem B. This haem was named O (6).

Further studies (7-9) revealed great diversity of properties among various oxidases traditionally attributed to the *o*-type

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according to their spectral properties and reactivity towards O_2 and CO. Some of them proved to be similar to the *E.coli bo*-type oxidase, the others resembled the *E.coli bd*-type oxidase. In this paper, we show that the *Bacillus* sp. FTU terminal oxidase previously defined due to above mentioned spectral features, as *bo*, contains, in fact, haems B but neither haem O nor copper.

MATERIALS AND METHODS

Bacteria and growth conditions. *Bacillus* sp. FTU cells were grown to early exponential or to stationary phase of growth. Membranes were prepared as described elsewhere (10). *E.coli* strains GO 103 (GO103:GR70N, Δcyd::kan, str^r, kan^r) with a deletion in the *bd*-type oxidase gene and GO 104 (GO104:GR70N, Δcyo::kan, str^r, kan^r) with a deletion in the *bo*-type oxidase gene were gifts from Dr. R.B.Gennis. The *E.coli* strains were grown at 37° C in a rotary shaker (200 rpm) to the late exponential growth phase in Luria broth containing streptomycin (100 mg/l) and kanamycin (50 mg/l). *E.coli* membranes were prepared following the procedure of Avetisyan et al. (11).

Enzymes. Horse heart cytochrome *c* was purchased from Sigma (USA). Bovine heart cytochrome *c* oxidase was a gift from Dr. A.A.Konstantinov. The *Bacillus* sp. FTU *bb*-type oxidase, previously named *bo*-type oxidase, was purified as described elsewhere (7). The caa_3-type oxidase from this bacterium was prepared according to the procedure reported by Muntyan et al. (12). Cytochromes were extracted from the late exponential cell membranes with 30 mM octyl glucoside. The extract was then fractionated with $(NH_4)_2SO_4$. The protein fraction obtained within the interval of 50-55% $(NH_4)_2SO_4$ saturation was enriched with the *bb*-type oxidase, and of 65-72% $(NH_4)_2SO_4$, with the caa_3-type oxidase. The next steps of purification of the two *Bacillus* oxidases were performed by chromatography on two ion-exchange columns in each case (12). To purify *bd*-type oxidase, cytochromes were extracted with 30 mM octyl glucoside from the *E.coli* GO 104 membranes. Then the floating cytochrome *bd*-enriched protein was obtained by salting out with 30-35% $(NH_4)_2SO_4$ saturation. The dialyzed protein was twice fractionated by chromatography on DEAE-Sephacel (Sigma, USA) and DEAE-Toyo-Pearl (Toyo-Soda, Japan).

Haem contents were determined by measuring the reduced-*minus*-oxidized difference spectra using a U-3400 spectrophotometer (Hitachi). The following extinction coefficients were used: haem *a*, 20 $mM^{-1}cm^{-1}$ at 601-625 nm for *Bacillus* sp. FTU oxidase [the coefficient was determined from pyridine haemochrome-*minus*-haemichrome difference spectrum using extinction coefficient 25.02 $mM^{-1}cm^{-1}$ at 588-630 nm (13)] and 13.5 $mM^{-1}cm^{-1}$ at 605-630 nm for bovine heart oxidase (14); haem *b*, 17.5 $mM^{-1}cm^{-1}$ at 560-575 nm (15) and haem *d*, 18.8 $mM^{-1}cm^{-1}$ at 628-649 nm (16).

Extraction and HPLC analysis of haems were performed as described by Sone and Fujiwara (17). For each separation, heams were extracted from about 2-4 mg of protein of the *Bacillus* or of the *E.coli* GO 103 membranes, 10-15 mg of protein of the *E.coli* GO 104 membranes, 1-2 mg of protein of cytochrome *c*, and 0.1-0.25 mg of protein of purified enzymes. A Diasorb C-16 column (3 x 250 mm) from Elsico (Russia) and a PU 4110 chromatograph (Philips) were used to separate haems. The eluted compounds were detected by absorbance at 406 nm. Flow rate was of 1 ml/min.

Copper in oxidase samples was measured by inductively-coupled plasma (ICP) atomic emission spectroscopy (18) using an ICAP 9000 spectrophotometer (Thermo Jarrell Ash, USA). Before the analysis, the samples were dialyzed overnight against 1000 volumes of 20 mM Tris-HCl (pH 7.8) containing 10 mM EDTA.
Protein was determined by a modification of the Lowry method (19) with BSA as a standard.

RESULTS

By spectroscopy in the near infrared region, copper was detected in the purified *Bacillus* sp. FTU caa_3-type oxidase but not in the so-called *bo*-type oxidase (data not shown). ICP-atomic-emission spectroscopy detected three copper atoms per molecule of the caa_3-type oxidase (Table I), like those in the bovine heart cytochrome oxidase, and a very low, clearly substoichiometric amount of copper in the "*bo*-type" oxidase, resembling in this respect the *E.coli bd*-type oxidase.

Comparative haem analysis of membrane fractions revealed the absence of haems O and D in the *Bacillus* sp. FTU early exponential and stationary phase cells (Fig.1 a,b). Both of these samples contain haems A, B and C (Fig.1, compare a,b and e-h). The difference between the two kinds of *Bacillus* sp. FTU membrane fractions is in the three-fold greater amount of haem A in the exponential phase membranes. The caa_3-type oxidase obtained from

Table I. Copper content in various oxidases, determined by the ICP-atomic emission spectroscopy

Oxidase	Haem content, nmol/mg protein	Copper, mol/mol enzyme
Bovine heart aa_3-type	9.4*	2.7
E.coli bd-type	5.8**	≤ 0.2
Bacillus sp. FTU caa_3-type:		
partly purified enzyme (after ammonium sulfate fractionation)	4.1*	3.3
the purified enzyme	19*	2.7
Bacillus sp. FTU *bb*-type:		
partly purified enzyme (after ammonium sulfate fractionation)	5***	0.4
the purified enzyme	15***	≤ 0.2

Contents of haems *a* (*), *d* (**) and *b*(***) were determined. The enzyme content was estimated on the assumption that two moles of haem *a* are present in the bovine heart and *Bacillus* sp. FTU caa_3-type oxidases, one mole of haem *d* - in the *E.coli bd*-type oxidase and three moles of haem *b* - in the *Bacillus* sp. FTU *bb*-type oxidase.

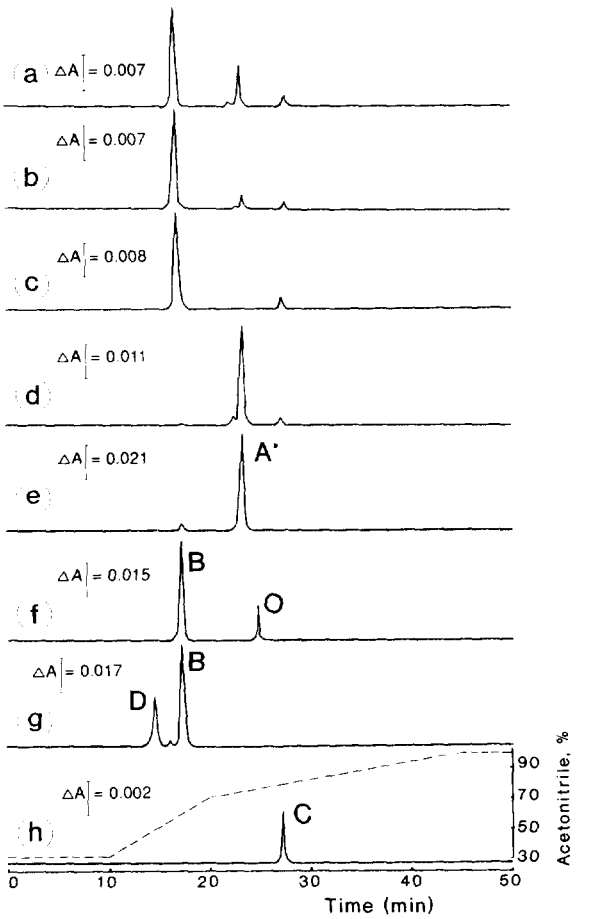

Fig.1. Elution profiles for the reversed-phase HPLC of the haem compounds. The absorbance was monitored at 406 nm. The flow rate was 1 ml/min. The dashed line (Fig.1h) shows an elution gradient of acetonitrile concentration (30-100%) in water containing 0.05% trifluoroacetic acid. A, B, O, D and C indicate the elution peaks of standard haems A, B, O, D and C, respectively. Haems were extracted from the *Bacillus* sp. FTU early exponential phase (a) and stationary phase (b) membranes, purified *Bacillus* sp. FTU *bb*-type (c) and caa_3-type (d) oxidases, purified bovine heart aa_3-type oxidase (e), *E.coli* GO 103 (f) and GO 104 (g) membranes, and horse heart cytochrome *c* (h).

the exponential phase cells appeared to contain haems A and C, whereas the other oxidase from *Bacillus* sp. FTU, previously named *bo*-type, showed the presence of haems B and C (Fig.1 c,d). Haems O or D were detected in neither oxidase. It should be mentioned that the method used results in complete extraction of haems A, B, O and D, but only traces of haem C were extracted. As a result, haem C content was underestimated. Observations indicating that haem C is present in the aa_3-type oxidase from *Bacillus* sp. FTU are summarized elsewhere (12).

DISCUSSION

In the presented study, we have shown that the so-called *o*-type oxidases from *E.coli* and *Bacillus* sp. FTU, besides the already described differences in the cyanide sensitivity and CO recombination pattern (7,8), also differ in the content of copper. The amount of copper in the oxidases rapidly reassociating with CO, i.e. of the *E.coli bd*-type and the *Bacillus* sp. FTU "*bo*-type" (7), were far below that stoichiometric to the amount of the enzymes (Table I). Evidently, so-called *o*-type oxidase from thermophilic *Bacillus* PS3 belongs to the same group of enzymes; it does not contain copper (20) and, like the two above mentioned oxidases, has lower cyanide sensitivity and is induced at the end of exponential growth phase or at low O_2 level (21). The non-copper-containing *o*-type oxidase from *Pseudomonas aeruginosa* (22) may also be a representative of this group.

Another group of oxidases, including the *E.coli bo*-type and *Bacillus* sp. FTU *caa*$_3$-type, slowly reassociating with CO (8), appeared to contain copper (Table I). This suggests that in the *Bacillus* sp. FTU *caa*$_3$-type oxidase there are (i) one Cu_B and (ii) two Cu_A forming the binuclear copper-copper center, as in *Bacillus subtilis* (23). The presence of Cu_B in oxidases had been associated with vectorial proton translocation by the enzymes (24-26). Recently, this suggestion gained support in a study on the *E.coli bo*-type oxidase mutants (27).

In the copper-containing *bo*-type oxidase, it is HCN rather than CN^- that binds to the O_2-reducing center. Interestingly, the absence of Cu_B accelerates the rate of CO reassociation with the *E.coli bo*-type oxidase (28) and makes it unable to bind HCN (27), making the oxidase similar to the *E.coli* alternative oxidase of the *bd*-type. These data are in good agreement with our results on the more rapid CO reassociation with non-copper-containing oxidases than with copper-containing oxidases.

The presence of high-spin haem(s) B but not O appeared to be another characteristic feature of the *Bacillus* sp. FTU "*bo*-type" oxidase. Similar haem content was shown in the *Bacillus* PS3 and *Bacillus stearothermophilus o*-type oxidases (17). In this context, it should be noted that no haem O was found in the membranes of the *Bacillus subtilis* wild strain either (29), though *Bac.subtilis* mutants defective in *ctaA*-gene which contained no haem A but produced haem O were obtained. The wild strain of *Bac.stearothermophilus*, as well as of *Bacillus* PS3,

was able to synthesize haem O but only under specific conditions (17). In all the mentioned cases, *Bacillus* oxidases containing the haem O appeared to be homologous to aa_3-type oxidase in that they pumped H^+.

Thus, the copper-containing oxidases, slowly recombining with CO, sensitive to low cyanide concentrations and pumping H^+, contain the high-spin haem A or O. As to non-copper-containing oxidases, rapidly recombining with CO, sensitive to higher cyanide concentrations and showing no H^+ pumping, haems D and/or B are high-spin. Obviously, haems A and O containing farnesyl chain, and non-farnesyl-containing haems B and D, comprise two groups of chemically related porphyrin compounds.

The *Thermus thermophilus* ba_3-type oxidase might be an exception from this rule. It contains copper (30), and seems to have fast CO recombination kinetics similar to that in the *E.coli bd*-type oxidase (31). However, kinetics of the CO recombination was studied in intact *T.thermophilus* cells using only two wavelengths. It is not clear therefore whether the measured transient response upon laser flash photolysis was due to the ba_3-type oxidase or to a rapidly CO-recombining *bb*-type oxidase resembling that described in *Bacillus* sp. FTU.

In any case, direct measurement of the haem composition of an oxidase seems absolutely necessary to attribute it to the *o*-type. It seems reasonable to define as *o*-type oxidases only those respiratory enzymes which contain haem O.

The bioenergetic function of the *bb*- and *bd*-type oxidases is an intriguing problem. As mentioned above, they cannot operate as H^+ pumps. On the other hand, *E.coli* cytochrome *bd* proved to be of vital importance for the bacterium to survive under low proton motive force conditions (32). Indications have been obtained that the *E.coli bd*-type oxidase and *Bacillus* sp. FTU *bb*-type oxidase can pump Na^+ rather than H^+, accumulating the energy as the sodium motive force (11).

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