A High-Affinity cbb₃-Type Cytochrome Oxidase Terminates the Symbiosis-Specific Respiratory Chain of Bradyrhizobium japonicum

OLIVER PREISIG,‡ RACHEL ZUFFEREY, LINDA THÖNY-MEYER, CYRIL A. APPLEBY,‡ AND HAUKE HENNECKE*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, CH-8092 Zürich, Switzerland

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It has been a long-standing hypothesis that the endosymbiotic rhizobia (bacteroids) cope with a concentration of 10 to 20 nM free O₂ in legume root nodules by the use of a specialized respiratory electron transport chain terminating with an oxidase that ought to have a high affinity for O₂. Previously, we suggested that the microaerobically and anaerobically induced fixNOQP operon of Bradyrhizobium japonicum might code for such a special oxidase. Here we report the biochemical characteristics of this terminal oxidase after a 27-fold enrichment from membranes of anaerobically grown B. japonicum wild-type cells. The purified oxidase has TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) oxidase activity as well as cytochrome c oxidase activity. N-terminal amino acid sequencing of its major constituent subunits confirmed the presence of the fixN, fixO, and fixP gene products. FixN is a highly hydrophobic, heme B-binding protein. FixO and FixP are membrane-anchored c-type cytochromes (apparent Mr's of 29,000 and 31,000, respectively), as shown by their peroxidase activities in sodium dodecyl sulfate-polyacrylamide gels. All oxidase properties are diagnostic for it to be a member of the cbb₃-subfamily of the heme-copper oxidases. The FixP protein was immunologically detectable in membranes isolated from root nodule bacteroids, and 85% of the total cytochrome c oxidase activity in bacteroid membranes was contributed by the cbb₃-type oxidase. The Kₘ values for O₂ of the purified enzyme and of membranes from different B. japonicum wild-type and mutant strains were determined by a spectrophotometric method with oxygenated soybean leghemoglobin as the sole O₂ delivery system. The derived Kₘ value for O₂ of the cbb₃-type oxidase in membranes was 7 nM, which is six- to eightfold lower than that determined for the aerobic aa₃-type cytochrome c oxidase. We conclude that the cbb₃-type oxidase supports microaerobic respiration in endosymbiotic bacteroids.

The free O₂ concentration in legume root nodules is extremely low (∼3 to 22 nM) (18). This condition, termed microaerobiosis, creates an energetic challenge for the endosymbiotic rhizobia (called bacteroids), which depend on respiratory ATP production for the costly N₂ fixation reaction. According to an almost 40-year-old hypothesis (2), bacteroids use a terminal oxidase having a high affinity for O₂. The Kₘ [O₂] of such an oxidase was expected to be in the range of the free O₂ concentration prevailing in root nodules, or at least below the equilibrium dissociation constant (43.5 nM) of oxygenated leghemoglobin (Lh), the natural O₂ delivery system for bacteroids (1). The formerly enigmatic bacteroid oxidase has been identified and characterized in our laboratory, first genetically (30) and now biochemically, as reported here. The work was done with Bradyrhizobium japonicum, the root nodule bacterium of soybean.

Under aerobic conditions B. japonicum uses a respiratory chain whose composition is quite similar to that known from mitochondria and which terminates with an aa₃-type cytochrome c oxidase (Fig. 1) (5, 13, 28). Mutations in the genes for cytochrome c (cytc) (7) and for subunit I of cytochrome aa₃ (cox) (5, 28) did not disturb bacteroid development and symbiotic nitrogen fixation (Fix⁺); hence, this respiratory branch is not essential for symbiosis. Likewise, mutations in the cox MNOP operon encoding an alternative cytochrome c oxidase led to a Fix⁺ phenotype (Fig. 1) (6). Recently, a B. japonicum gene (coxX) for yet another oxidase, most probably a ubiquinol oxidase (Fig. 1), has been discovered (35), but the coxX mutant phenotype is still unknown.

A first genetic indication regarding the composition of a bacteroid-specific respiratory branch in B. japonicum came from an analysis of mutations in the fbcFH genes for the cytochrome bc₁ complex (Fig. 1) (38) which caused a defect in symbiotic nitrogen fixation (Fix⁺). This suggested an electron transfer from the bc₁ complex to a new, symbiosis-specific cytochrome oxidase. In subsequent work it was proposed that this new oxidase is encoded by the fixNOQP operon (30) for the following reasons: (i) B. japonicum mutants with insertions in fixNOQP are Fix⁻ and show an 80% decreased TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) oxidase activity in cells grown microaerobically; (ii) the operon is induced under microaerobic and anaerobic conditions (the latter with NO₃⁻ as the electron acceptor), which is consistent with the proposed role of the gene products under O₂-limiting conditions; and (iii) the amino acid sequences derived from the DNA sequence suggested an oxidase function for the fixNOQP gene products. The FixN protein has the characteristics typical of a subunit I of the heme-copper oxidases containing six conserved histidines whose imidazole side chains function as the ligands to a low-spin heme and the high-spin heme-CuB binuclear center (15, 19, 39). The FixO and FixP proteins are thought to be membrane-anchored mono- and diheme c-type cytochromes. The small FixQ protein (6 kDa) does not appear to play an essential role because we recently associated a Fix⁺ phenotype with a mutant suffering an in-frame deletion in the fixQ gene (40).
FIG. 1. Current model of branched respiratory chain of B. japonicum. Under aerobic conditions the electrons are transferred from the ubiquinol pool (Q) via the Rieske FxP protein-cytochrome bc complex (fcFH gene products) and a 20-kDa membrane-bound cytochrome c (ccyM gene product) to the O2-reducing aa3-type terminal oxidase encoded by ccoBA (see the text for references; for ccoB, see reference 24). The function of the CoxMNP oxidase is still unknown; its protein sequence shows similarity to that of a heme-copper cytochrome c oxidase (6). The presence of a quinol oxidase has been postulated because few comparative studies on O2 affinities of bacterial terminal oxidases (10). Most of them were done with the insensitive hemochrome assay (3). The standard blotting protocol in the latter technique was used for FixO and FixP, whereas it did not work with the FixN protein. This protein band was excised directly from the SDS-polyacrylamide gel. The protein was then electroeluted and centrifuged onto a polycrylamide diode membrane by using a ProSpin cartridge (Applied Biosystems, Foster City, Calif.). Cytochrome c oxidase activity was determined spectrophotometrically in a total volume of 1.5 ml of 50 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [pH 7.4]) containing 0.1 mg of dodecyl maltoside per ml and 40 μM reduced horse heart cytochrome c at 30°C (32, 33). Here, enzyme activity was determined by the diffusion-absorptiometric high-affinity bacteroid oxidase (HPLC) (36).

Genes homologous to fixNOQP are present not only in rhizobial species (21, 25) but also in nonsymbiotic bacterial species (34, 36). In Rhodobacter capsulatus (16, 17, 36). Because of the presence of only c- and b-type cytochromes and the absence of subunit II carrying the CuA center, this novel oxidase was called the cbb3-type cytochrome oxidase. This type is now generally realized as a new subfamily of the heme-copper oxidase superfamily (15-17, 39). A cytochrome c oxidase complex consisting of seven or eight subunits was previously isolated from B. japonicum bacteroids (22). Interestingly, this oxidase preparation contained only b- and c-type hemoproteins, but its identification as a bona fide cbb3- or FixNOQP-type oxidase was not possible at that time. Also, attempts to determine the oxidase’s affinity for O2 were unsuccessful.

The determination and comparison of O2 affinities of terminal oxidases is important to understand the physiological role of branched respiratory systems. Unfortunately, there are only a few comparative studies on O2 affinities of bacterial terminal oxidases (10). Most of them were done with the insensitive polarographic method with the use of an O2 electrode. A more sensitive way to determine the O2 affinity is the spectrophotometric method with oxygenated myoglobin or Lb, the use of either of which as an O2 delivery system has improved Km determinations below 2 μM O2 or even below 200 nM O2, respectively (3, 10-12, 22, 26). In such experiments the free O2 concentration in a closed system is determined indirectly by measuring the fractional degree of oxygenation of added globin. Here, we report the biochemical characterization of a 27-fold-purified B. japonicum cbb3-type terminal oxidase and provide evidence for its presence in bacteroids. Furthermore, its affinity for O2 was determined and found to be ideally suited for a function as a bacteroid oxidase.

Materials and Methods

Strains and growth. B. japonicum 110tip4-31 (31) is called the wild type throughout this paper. Mutant Cox132 has a Trn insertion in the gene ccx that encodes subunit I of the aa3-type terminal oxidase (5). The fixNOQP operon mutant was constructed by deleting a 3-kb DNA segment between the ProII site in fixN and the BgIII site infixP and replacing it with a 1.3-kb SmaI-BamHI fragment containing the aphII cassette (kanamycin resistance). B. japonicum cells were grown microaerobically or aerobically in peptone-salts-yeast extract medium at 30°C (30, 31) and harvested in the exponential growth phase for biochemical experiments. Anaerobic growth was achieved in yeast extract mannitol medium plus 10 mM KNO3 at 30°C (30). For protein purification, cells were grown anaerobically in a 20-liter batch culture and harvested in the early stationary phase of growth. Bacteroids isolated from root nodules were enriched by a Percoll gradient (9).

Standard biochemical techniques. The following techniques were described previously—protein determination and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without heating the loaded sample (38), heme staining (7), Western blot (immunoblot) analysis (38) with rabbit anti-CoxM (32) or anti-MalE-FixP serum (29), and N-terminal amino acid sequence determination (37). The standard blotting protocol in the latter technique was used for FixO and FixP, whereas it did not work with the FixN protein. This protein band was excised directly from the SDS-polyacrylamide gel. The protein was then electroeluted and centrifuged onto a polycrylamide diode membrane by using a ProSpin cartridge (Applied Biosystems, Foster City, Calif.). Cytochrome c oxidase activity was determined spectrophotometrically in a total volume of 1.5 ml of 50 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [pH 7.4]) containing 0.1 mg of dodecyl maltoside per ml and 40 μM reduced horse heart cytochrome c at 30°C (32, 33). Heme activity was determined by the diffusion-absorptiometric HPLC (36).

Protein purification. Frozen B. japonicum wild-type cells from 10 to 20 liters of anaerobic cultures were resuspended in 40 to 60 ml of 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg of DNase I per ml, and 10 μg of RNase A per ml. The bacteria were broken by three passages through a French pressure cell (18,000 lb/in2). The extract was centrifuged at 26,000 × g for 20 min. Membranes were collected by a second centrifugation of the supernatant at 130,000 × g for 2 h and then stirred in 20 ml of 50 mM HEPES (pH 7.4) containing 2% dodecyl maltoside and 1 mM DTT for 1 to 2 h at 4°C. Precipitates were removed from the solubilized membranes by centrifugation at 130,000 × g for 2 h. The supernatant was loaded onto a Sephacryl S-300 gel filtration column (80 by 3 cm; Pharmacia, Uppsala, Sweden) equilibrated with the standard buffer of 50 mM HEPES (pH 7.4) containing 0.1 mg of dodecyl maltoside per ml. Protein was eluted with a flow rate of 0.8 ml min−1 at 4°C. Fractions were tested for TMPD oxidase activity. Active fractions (50 ml) were pooled and concentrated to 8 to 10 ml by ultrafiltration with a YM-10 filter (Amicon, Danvers, Mass.) containing a molecular mass cutoff of 100 kDa. Further purification steps were carried out by a Hewlett Packard HPLC system (model 1050) at room temperature and a flow rate of 1 ml min−1. The concentrated solution was applied to a hyper-Sepharose column (15 by 1 cm; Pharmacia) with a similar void volume. The column was then washed with a 100-ml buffer of 50 mM HEPES (pH 7.4) containing 1 mM DTT, 0.1 M KCl, and 5 nM bovine serum albumin (BSA) and eluted with a linear gradient of 0 to 500 mM KCl in the same buffer. Fractions containing oxidase activity eluted as a single peak (the range of 200 to 240 mM KCl); they were pooled by a second ultrafiltration to a volume of less than 2 ml. The sample was then loaded onto a Sephacryl S-300 gel filtration column (30 by 1 cm), and the eluate was pooled.

Glycerol (10% [vol/vol]) was added to the desalted, enriched oxidase preparation. The oxidase was stored at -20°C.

Spectroscopy. Dithionite-reduced-minus-air-oxidized spectra and CO plus dithionite-reduced-minus-dithionite-reduced spectra were recorded by a Hitachi model U-3300 spectrophotometer equipped with a head-on photomultiplier (5).

Determination of Km for O2. Crude Lb extract from soybean nodules was further purified by DEAE Fract乔治 EM anion-exchange chromatography and concentrated by ultrafiltration through a YM-10 membrane (Amicon). Enriched Lb was reduced by a pinch of sodium dithionite, which was subsequently separated from the protein on a PD-10 desalting column (Pharmacia). Exposure of reduced Lb to air in the absence of dithionite is sufficient for its oxygenation. The Lb concentration was measured spectrophotometrically by the alkaline pyridine method (3). The deoxygenation experiment was carried out in a stopped cuvette filled in an anerobic glove box with 3 ml of degassed buffer.

Thus, the gas phase in the cuvette was small. Two different approaches were used to determine the Km values of membranes and the partially purified oxidase. In the first case, fresh membranes (0.1 to 1.2 mg of protein per sample) were resuspended in 20 ml of 10 mM Tris-HCl (pH 7.0) with 1.3 mM NADH and 25 mM KNO3 at 30°C. Spectrophotometric measurements were made in a Cary 118 spectrophotometer at 574 nm measured immediately after Lb addition (absorption coefficient: 14 m−1 cm−1 [31]). Scans from 650 to 500 nm were recorded every minute. The
sequence of the 42-kDa protein turned out to be a mixture of amino acid sequences from two protein species, one being identical to the amino terminus of the fixN gene product (predicted \( M_r \) of 61 kDa). The significantly smaller apparent \( M_r \) of FixN in SDS-polyacrylamide gels is probably due to the extremely hydrophobic nature of the protein with up to 14 potential membrane-spanning helices (30, 36). A similar discrepancy was observed previously with cytochrome \( b \) of the \( bc_1 \) complex (37, 38). The amino acid sequences derived from the 31- and 29-kDa proteins were identical to the \( N \) termini of FixP and FixO (predicted \( M_r \) of 31 and 27 kDa for the respective apoproteins). The small \( fixO \) gene product (calculated \( M_r \) of 6 kDa) was not found. Molecular mass determination of the entire oxidase complex was done by calibrated gel filtration whereby an apparent \( M_r \) of 180 kDa was obtained.

The oxidase complex contains cytochromes \( b \) and \( c \). Subunit I of heme-copper oxidases always possesses two noncovalently bound hemes, a low-spin heme and a high-spin heme. We analyzed the heme composition of the oxidase preparation by reversed-phase HPLC. Acid-acetone extracts from membranes of aerobically grown cells of *Escherichia coli* and *Bacillus subtilis* were used as standards for heme \( B \) plus \( O \) and heme \( B \) plus heme \( A \), respectively. Only heme \( B \) was detectable in the heme extract from the purified FixNOQP oxidase (data not shown). The amounts of oxidase isolated were too small to permit a reliable determination of the copper content.

The presence of covalently bound heme in the oxidase preparation was shown by the peroxidase activity of cytochrome \( c \) in SDS-polyacrylamide gels (Fig. 3, lane 6). The FixO and FixP bands were clearly visualized by heme staining. The intensity of FixP staining was consistently better than that of FixO. This may reflect the prediction that FixP is a diheme cytochrome \( c \) whereas FixO is a monoheme cytochrome \( c \) (30).

The dithionite-reduced-minus-air-oxidized difference spectrum of the oxidase confirmed that it contains both \( b \)-type and \( c \)-type cytochromes (Fig. 4A) but no \( a \)-type cytochrome that would give a characteristic peak at 603 nm. The ratio between cytochrome \( c \) (peak at 551 nm) and cytochrome \( b \) (shoulder at 560 nm) was estimated to be 1.7 by using the respective molar extinction coefficients 19.1 and 22 mM \(^{-1} \) cm \(^{-1} \) (20). Troughs at 552 and 560 nm were obtained in CO plus dithionite-reduced-minus-dithionite-reduced spectra of the FixNOQP oxidase, as shown in Fig. 4B. The trough at 552 nm may originate from a CO-binding cytochrome \( c \) or \( b \), whereas the inverted shoulder at 560 nm appears to be due to a cytochrome \( b \) (20). Taken together, all of the described properties show that the FixNOQP oxidase is a member of the \( cbb_3 \)-type subclass of heme-copper oxidases.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Cytochrome c oxidase activity (( \mu )mol of cytochrome c min (^{-1} ) mg (^{-1} ))</th>
<th>Purification factor</th>
<th>Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Solubilization of membranes</td>
<td>1.9</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2. Sephacryl S-300 gel filtration</td>
<td>5.6</td>
<td>2.9</td>
<td>35</td>
</tr>
<tr>
<td>3. Lysine-Sepharose chromatography</td>
<td>29.7</td>
<td>15.6</td>
<td>20</td>
</tr>
<tr>
<td>4. DEAE Fractogel EMD anion-exchange chromatography</td>
<td>47.3</td>
<td>24.9</td>
<td>2.6</td>
</tr>
<tr>
<td>5. Sephacryl S-300 gel filtration</td>
<td>51.5</td>
<td>27.1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Protein determinations and measurements of horse heart cytochrome \( c \) oxidase activity at 30°C were done as described in Materials and Methods.

FIG. 2. Western blot analysis of cytochromes. The FixP and cycM gene products were analyzed in membranes isolated from bacteroids of the wild type (wt) and the fixNOQP mutant (Bj4503). Each lane was loaded with 20 \( \mu \)g of membrane proteins. The upper blot shows a Western blot developed with antibodies directed against MalE-FixP, whereas in the lower blot the membranes were incubated with anti-CycM serum. The apparent \( M_r \) (in thousands) of molecular mass markers are indicated on the left.
Determination of $O_2$ affinities. Lb is known to function as an $O_2$ buffer to maintain very low concentrations of free $O_2$ in the nodule interior (1). The fractional degree of Lb oxygenation is dependent on the concentration of free $O_2$ and can be determined spectrophotometrically: the value is 0.5 at 43.5 nM free $O_2$ regardless of the Lb concentration. This property is very useful for a determination of the apparent $K_m$ values for $O_2$ of oxidases in a closed system with Lb as the sole $O_2$ delivery system (3). The respiratory activity was studied with intact membranes from different strains. The conditions were 30°C with 8 mM succinate as the electron donor and 85 μM EDTA to protect Lb against oxidation by polyvalent metal ions. The results of such a deoxygenation experiment is shown in Fig. 5. Membranes from aerobically and anaerobically grown cells of the B. japonicum wild type, mutant COX132 (coxA, no cytochrome $aa_3$), and mutant Bj4503 (fixNOQ, no cytochrome $cbb_3$) were used. Each deoxygenation experiment led to a set of $\Delta A_{574-560}$ values measured every minute. These values were used to plot the rate of $O_2$ consumption versus the concentration of free $O_2$ (Fig. 6). The consumption followed a hyperbolic curve characteristic of Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values were calculated by linear regression from
an Eadie-Hofstee plot. $K_m$ values were independent of the amount of membranes added, whereas $V_{max}$ values showed such a dependence. As a further control, the $V_{max}$ values were compared with the values for O$_2$ consumption obtained from experiments using an O$_2$ electrode and air-saturated buffer without Lb. The measured values at a high concentration of free O$_2$ were in a range similar to that of the $V_{max}$ values determined from deoxygenation experiments. Oxidation of Lb did not occur to significant extents, as shown by the spectra (Fig. 5).

Table 2 summarizes the obtained values. The $K_m$ values for membranes from aerobically grown cells of the three strains were roughly in the same range, although COX132 mutant membranes always showed a somewhat lower $K_m$ of 32 nM O$_2$ compared with 55 to 62 nM for wild-type and mutant Bj4503 membranes. Interestingly, the situation was much different for membranes from anaerobically grown cells. Clearly, COX132 membranes respiring with a low $K_m$ of 7 nM O$_2$, whereas Bj4503 membranes possessed an eightfold-higher $K_m$ of 56 nM. Wild-type membranes consumed O$_2$ with a characteristic biphasic curve, revealing two $K_m$ values of 19 and 4 nM. A comparison between the apparent $V_{max}$ values showed that membrane preparations of aerobically grown COX132 cells possessed a substrate turnover rate significantly lower than those for the wild-type and Bj4503 mutant membranes. In the case of anaerobically grown COX132 mutant cells, membranes still respired with the lowest $V_{max}$ resulting in 57 and 41% of wild-type and Bj4503 activities, respectively (see Discussion).

The determination of the $K_m$ value of the partially purified oxidase was more difficult because of two problems. First, a relatively high concentration of reduced horse heart cytochrome c (40 μM) had to be used as the electron donor, whose redox changes had a small but significant effect on the Lb spectra at 560 nm. However, this amount of electron donor was necessary to obtain acceptable respiration rates. Second, partial oxidation to Lb(III$^+$) occurred during the experiment and disturbed the equilibrium of Lb(II$^+$) with O$_2$. The oxidation seems to be due to an interaction of deoxygenated Lb either with oxidized cytochrome $c$ or with ascorbate. While all three substrates, oxygenated Lb, cytochrome $c$, and ascorbate, were stable in the premix, an uncontrolled oxidation of Lb was observed after the start of the enzymatic reaction. An apparent $K_m$ value of 12.0 ± 2.5 nM O$_2$ for the $cbb_3$-type oxidase was obtained under these conditions.

**DISCUSSION**

A $K_m$ for O$_2$ of 7 nM is the lowest $K_m$ value reported so far for a heme-copper terminal oxidase, a unique property that makes the $cbb_3$-type oxidase encoded by the $fixNOQP$ operon ideally suited to support bacteroid respiration in the nearly anoxic root nodule interior. This conclusion is corroborated by other observations such as the induction of the $fixNOQP$ operon under microaerobiosis, the immunological detection of the oxidase subunit FixP in bacteroid membranes, and the Fix$^-$ phenotype of $fixNOQP$ mutants. Therefore, it seems safe to say that the $cbb_3$-type heme-copper oxidase terminates a symbiosis-specific respiratory chain which branches off at the cytochrome $bc_1$ complex as illustrated in Fig. 1 (see also below). While it is clear that *B. japonicum* does possess at least three alternative terminal oxidases (Fig. 1), none of them is obviously capable of fully compensating for the strongly impaired oxidase activity in $fixNOQP$ mutants. In such mutants, we consistently measured 20% residual oxidase activity with bacteroids or with cells grown microaerobically or anaerobically (30), but we do not know the relative contribution of each of the three alternative oxidases to this activity. It has been suggested that the level of cytochrome $aa_3$ is down-regulated in symbiosis (14, 23), and the physiological conditions for maximal expression of the CoxMNOP oxidase and the CoxX oxidase (Fig. 1) are not known at all. At any rate, microaerobically expressed oxidases other than that encoded by $fixNOQP$ must have a comparatively low affinity for O$_2$. This becomes evident by an inspection of the kinetics obtained with membranes from the $fixNOQP$ mutant (Fig. 6), in which the rate of O$_2$ consumption is much lower at low O$_2$ concentrations compared with that for membranes from $fixNOQP^+$ strains (wild type and COX132). A $K_m$ of 56 nM was determined for such an alternative oxidase (or oxidase mixture). This value is about eightfold higher than that of the $cbb_3$-type oxidase and falls within

![FIG. 6. Respiration by membranes isolated from anaerobically grown *B. japonicum* strains at low concentrations of free O$_2$ (<200 nM). The amount of membranes was adjusted to obtain similar $V_{max}$ values in each of the three experiments. The use of 0.36 mg of wild-type ( ), 0.36 mg of mutant COX132 (coxA $fixNOQP^+$, □), and 0.3 mg of mutant Bj4503 (coxA$^+$ $fixNOQP^+$, ▲) membrane proteins revealed $V_{max}$ values of 6.4, 5.6, and 6.5 nmol of O$_2$ per min, respectively, in this experiment.](http://jb.asm.org/)

### TABLE 2. O$_2$ affinities ($K_m$) and apparent maximal velocities ($V_{max}$) calculated from respiration experiments using membranes from aerobically and anaerobically grown cells

<table>
<thead>
<tr>
<th>B. japonicum strain and culture conditions</th>
<th>Relevant genotype</th>
<th>O$_2$ affinity ($K_m$ [nM O$_2$])$^a$</th>
<th>Specific O$<em>2$ consumption ($V</em>{max}$ [nmol O$_2$ min$^{-1}$ mg$^{-1}$])$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td>55.7 ± 24.2</td>
<td>37.4 ± 9.2</td>
</tr>
<tr>
<td>COX132</td>
<td>coxA $fixNOQP^+$</td>
<td>31.7 ± 5.2</td>
<td>8.6 ± 1.7</td>
</tr>
<tr>
<td>Bj4503</td>
<td>coxA$^+$ $fixNOQP$</td>
<td>62.2 ± 14.7</td>
<td>28.8 ± 3.9</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td>19.3 ± 7.0</td>
<td>15.3 ± 2.6</td>
</tr>
<tr>
<td>COX132</td>
<td>coxA $fixNOQP^+$</td>
<td>7.0 ± 2.5</td>
<td>8.7 ± 1.8</td>
</tr>
<tr>
<td>Bj4503</td>
<td>coxA$^+$ $fixNOQP$</td>
<td>56.2 ± 10.6</td>
<td>21.2 ± 3.8</td>
</tr>
</tbody>
</table>

$^a$Values are the means (± standard deviations) based on at least five measurements from two or three independent cultures.
the 30 to 60 nM range measured for membranes from aerobically grown cells. Since cytochrome aa₃ appears to be the predominant oxidase under aerobicosis (5, 14), the values of 55 to 62 nM measured with aerobically grown coxA⁺ strains probably reflect its Keₐ for O₂, even more so as the Vₜₐₘₚ for the coxA mutant drops by almost 80% compared with that for the wild type (Table 2). In this context it should be noted that the derived Keₐ for O₂ of the aerobically expressed B. japonicum oxidases accords well with the recently reported Keₐ values of the low-affinity cytochrome bo⁻ from E. coli (46 nM) (11) and the mitochondrial cytochrome aa₃ from soybean (50 to 147 nM) (26, 27).

The presence of at least two oxidases with different affinities in anaerobically grown wild-type cells can also be deduced from the biphasic kinetics. While an interpretation of this result is not straightforward, we suspect that it may have been caused by the superimposition of two kinetics, one of the cbb₃-type oxidase (Keₐ of 4 nM) (Table 2) and another of an alternative oxidase (Keₐ of 19 nM) (Table 2). The latter value comes close to the single Keₐ of 25.5 nM O₂ measured for intact, succinate-respiring B. japonicum bacteroids by Bergersen and Turner (4). Since the lowest free O₂ concentration in their experiments was 8 nM, a Keₐ value as low as 4 nM could have escaped their attention. We are aware that the use of complex membrane preparations or even whole cells can be delicate, but there is no other choice because cytochrome c-dependent oxy-Lb deoxygenation experiments with purified oxidases must be met with caution because of the Lb oxidation problem (see Results). Similar pitfalls have been encountered by Keefe and Maier (22).

Having described the fixNOQP-encoded cytochrome cbb₃ complex as a high-affinity oxidase in B. japonicum, we must emphasize that this may not necessarily be an inherent property of all cbb₃-type oxidases found in other bacteria. The corresponding R. capsulatus oxidase, for example, is one of the major oxidases supporting aerobic growth (17, 36). It will be of interest to see whether its Keₐ value equals that of the low-affinity oxidase.

The partially purified cytochrome oxidase complex described here has other properties that are fully compatible with the recent definition of the novel, third subclass (cbb₃ type) of the heme-copper oxidase superfamily (8, 15, 39). This concerns primarily the presence of the three major subunits, one subunit (FixN) binding two B hemes and Cu and two subunits (FixO and FixP) that are mono- and diheme c-type cytochromes. We determined a cytochrome c-to-b ratio of 1.7, which is in good agreement with that assignment. Whether the small, nonessential FixO protein is also associated with the complex might be clarified when larger amounts of pure enzyme become available. The eight-subunit cytochrome oxidase preparation from B. japonicum bacteroids that has been enriched fivefold by Keefe and Maier (22) not only shares similar spectral properties with our preparation but also contains two c-type heme-proteins with molecular masses similar to those of FixO and FixP. Although we cannot exclude the possibility that they have purified a completely different cytochrome oxidase, it is intriguing to learn that their preparation also includes subunits of the cytochrome bc₁ complex. A tight association of the bc₁ complex with a cbb₃-type oxidase would in fact be consistent with our postulate that the former serves as the electron donor for the latter in the symbiosis-specific respiratory branch of B. japonicum (Fig. 1).

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