Assembly and Function of the Cytochrome cbb₃ Oxidase Subunits in Bradyrhizobium japonicum*

(Received for publication, November 27, 1995, and in revised form, January 30, 1996)

Rachel Zufferey, Oliver Preisig†, Hauke Hennecke, and Linda Thöny-Meyer‡

From the Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

The Bradyrhizobium japonicum cbb₃-type cytochrome oxidase, which supports microaerobic respiration, is a multisubunit enzyme encoded by the genes of the fixNOQP operon. We investigated the contribution of the individual subunits to function and assembly of the membrane-bound complex. In-frame deletion mutants of fixN, fixO, and fixQ, and an insertion mutant of fixP were constructed. All mutants, except the fixQ mutant, showed clearly altered absorption difference spectra of their membranes and decreased oxidase activities, and they were unable to fix nitrogen symbiotically. The presence of the individual subunits was assayed by Western blot analysis, using subunit-specific antibodies, and by heme staining of the c-type cytochromes FixO and FixP. These analyses led to the following conclusions: (i) FixN and FixO are necessary for assembly of the multimeric oxidase, (ii) FixN and FixO assemble independently of FixP, and (iii) FixO is not required for complex formation and, therefore, does not seem to be an essential subunit. The possible oxidase biogenesis pathway involves the formation of a primary core complex consisting of FixN and FixO, which allows the subsequent association with FixP to form the complete enzyme.

Terminal oxidases are the ultimate components of respiratory chains catalyzing the four-electron reduction of molecular oxygen to water. The bacterial respiratory system is usually branched and comprises several terminal oxidases, allowing bacteria to grow at different oxygen tensions. Most of the bacterial oxidases belong to the so-called superfamily of heme-copper oxidases and the cytochrome c oxidases which is divided into two groups, the quinol oxidases and the cytochrome c oxidases (8, 9).

In Bradyrhizobium japonicum, the fixNOQP-encoded cbb₃-type oxidase is expressed only under microaerobic and anaerobic conditions (2). The 61-kDa fixN gene product has an up to 14 putative transmembrane helices and binds heme B (2, 11). The fixN and fixP genes code for membrane-anchored c-type cytochromes of 28 and 32 kDa, respectively. FixO is a 54-amino-acid polypeptide that is thought to be membrane-bound by its hydrophobic N-terminal half. Having a Kₘ as low as 7 nM, the B. japonicum cbb₃-type oxidase supports respiration under oxygen-limiting conditions and is, therefore, required for nitrogen fixation in symbiosis with soybean (Glycine max L. Merr.) (2, 11).

Correct folding and assembly of the individual subunits of a multimeric membrane protein complex may involve a specific nucleation and a precise pathway. Little is known about the assembly of oxidase complexes in bacteria. Minagawa et al. (13) reported the absence of the Escherichia coli cyoABCDE gene products when only one of the subunit genes was deleted. Studies on assembly of the Paracoccus denitrificans aa₃-type oxidase revealed that subunit I was not present in a subunit I₁II₁ gene deletion mutant, whereas subunit I₁I₁ was not absolutely essential for the formation of a subunit I₁I₁I₁ subcomplex (14, 15).

Using the B. japonicum cbb₃-type oxidase as an example, we attempted to elucidate the role of its individual subunits in assembly and biological function.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—B. japonicum 110spd is called the wild type throughout this work. All strains are listed in Table I. B. japonicum strains were grown aerobically or microaerobically at 28 °C in PSY medium (2, 16) or anaerobically in YEM medium supplemented with 10 mM KNO₃ (17). Antibiotics were added at the following concentrations (µg/ml): spectinomycin (100); streptomycin (100); kanamycin (100); tetracycline (60); chloramphenicol (10). E. coli was grown in LB medium (18) to which antibiotics were added at the following concentrations (µg/ml): ampicillin (150); kanamycin (100); tetracycline (10).

**Recombinant DNA Work**—Standard procedures were used for cloning, Southern blotting and hybridization (18). Chromosomal DNA of B. japonicum was isolated as described previously (19). DNA hybridization probes were radioactively labeled using the nick-translation technique (18). DNA sequence analyses were performed using the chain-termination method (20) and the equipment for automated DNA sequencing (Sequence model 370A and fluorescent dye terminators from Applied Biosystems, Foster City, CA).

**Marker Replacement Mutagenesis and Genetic Complementation**—Relevant plasmids used or constructed throughout this work are listed in Table I. For complementation of the fixNOQP operon deletion mutant Bj4503 (11), we constructed plasmid pRJ4504 that contained the entire fixNOQP operon, orf141, and most of orf177 gene (see Fig. 1). The fixN in-frame deletion construct pRJ4526 lacks a 1.2-kilobase pair.

*This work was supported by grants from the Swiss National Foundation for Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C., Section 1734 solely to indicate this fact.

† Present address: Dept. of Microbiology and Biochemistry, University of the Orange Free State, P. O. Box 339, 9300 Bloemfontein, South Africa.

‡ To whom correspondence should be addressed. Tel.: 41-1-632-4419; Fax: 41-1-632-1148; E-mail: lthoeny@micro.biol.ethz.ch.
Assembly of the B. japonicum cbb₃ Oxidase

**Table I**

| Bacterial strains and plasmids | | Source or reference |
|---------------------------------|---|-------------------|
| E. coli strains | Relevant characteristics | Source or reference |
| DH5α supE44 ΔlacU169 (Δ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | 38 |
| S17-1 hsdRΔRP4-2 kan-2 Tn7 thi-1 Mu, integrated in the chromosome | 39 |
| B. japonicum strains | | |
| 110spc4 Sp-reactive (wild type) | 16 |
| B) 3067 Smr, Sp², 1 I cassette in Smal site of fbcF | 30 |
| B) 110spc4, prJ 3603 cointegrated, fixP-1-lacZ, Sp³, Tc | This work |
| B) 3618 fixP-apnH, Sp, Km² | 2 |
| B) 4503 ΔfixNOQ::aphH1, Sp, Km² | This work |
| B) 4504 B) 4503, prJ 4504 cointegrated, fixNOQ-1, Sp², Km², Tc | This work |
| B) 4518 B) 4503, prJ 4518 cointegrated, ΔfixO, Sp², Km², Tc | This work |
| B) 4526 B) 4503, prJ 4526 cointegrated, ΔfixN, Sp², Km², Tc | This work |
| B) 4528 B) 4503, prJ 4528 cointegrated, ΔfixQ, Sp², Km², Tc | This work |
| B) 4532 B) 4503, prJ 4532 cointegrated, ΔfixP, Sp², Km², Tc | This work |
| B) 4537 B) 4503, prJ 4537 cointegrated, ΔfixQ, Sp², Km², Tc | This work |
| B) 4539 B) 4503, prJ 4539 cointegrated, ΔfixN, Sp², Km², Tc | This work |
| B) 4540 B) 4503, prJ 4540 cointegrated, ΔfixO, Sp², Km², Tc | This work |
| B) 4542 B) 4503, prJ 4542 cointegrated, ΔfixQ, Sp², Km², Tc | This work |
| B) 4543 B) 4503, prJ 4543 cointegrated, ΔfixQ, Sp², Km², Tc | This work |
| B) 4545 B) 4503, prJ 4545 cointegrated, ΔfixQ, Sp², Km², Tc | This work |
| B) 4546 B) 4503, prJ 4546 cointegrated, ΔfixQ, Sp², Km², Tc | This work |
| Plasmids | | |
| pMal-cRI | Ap' , lacI, P lac, MalE-1-lacZα | New England Biolabs |
| pNM481X/482 | Ap', lacI | 40 |
| pNM481X | Ap', lacI, Xba linker at Stul site | 22 |
| pNM481Xb | Ap', lacI, Xba linker at Stul site | 21 |
| pNM481Xa | Ap', lacI, Xba linker at Stul site | 21 |
| pSUP202pol2 | Tc', oriT from RP4, pBLS I-II polylinker | 21 |
| pSUP202pol6K | Tc', derived from pSUP202pol2; Sall to EcoRI of pBLS I-II polylinker, Km polylinker ligated into Smal site | P. Künzler, unpublished |

**Puvel-NsiI fragment.** To construct the fixO in-frame deletion, a 0.34-kilobase pair EcoT71I fragment was excised, resulting in prJ 4518. The fixO in-frame deletion was obtained in plasmid prJ 4528 by eliminating a 0.12-kilobase pair BsaI fragment whose ends were made blunt, and adding a XbaI linker (octamer) to regenerate the ribosome binding site of the downstream gene fixP. The plasmids prJ 4504, prJ 4526, prJ 4518, and prJ 4528 are pSUP202pol6K derivatives.

All of the newly fused sites at which remote DNA was joined after in-frame deletion were confirmed by sequencing. The plasmids prJ 4526, prJ 4518, and prJ 4528 were cointegrated into the Bj4503 chromosome by homologous recombination. Tetracycline-resistant clones were selected (single crossing over) and their DNA examined by Southern blot hybridization for the correct genomic structure. In the case of Bj4526, plasmid cointegration occurred at the fixG locus, i.e. downstream of the operon deletion, whereas the plasmids prJ 4518 and prJ 4528 cointegrated within the orf277orf141 locus, i.e. upstream of the operon deletion.

**Construction of Translational lacZ Fusions—**The lacZ gene of pNM481Xb was fused to the 7th codon of fixO at the BsaI site (made blunt) that was preceded by either an intact fixN gene or a fixN deletion (described above), resulting in prJ 4569 and prJ 4545, respectively.

The fixO-1-lacZ fusion (to the 15th codon of fixO) was constructed by ligating at the BsaI site (made blunt) the lacZ gene of pNM481Xb (21). This fusion was constructed either downstream of intact DNA fixN genes, or downstream of the ΔfixN or ΔfixO deletions (described above), yielding prJ 4542, prJ 4543, and prJ 4550, respectively. A translational fusion of the lacZ gene of pNM481X (22) to the 7th codon of fixP was generated at the EcoRI site, yielding prJ 3603. To position the fixP-1-lacZ fusion into the mutated ΔfixN, ΔfixO, and ΔfixQ operators (described above), the lacZ gene of pNM481Xb (21) was cloned into the EcoRI site of fixP, resulting in prJ 4539, prJ 4538, and prJ 4540, respectively. Plasmids prJ 4569, prJ 4545, prJ 4542, prJ 4543, prJ 4550, prJ 4539, prJ 4538, and prJ 4540 are the respective pSUP202pol6K derivatives.

All fusion sites were confirmed by sequencing. While prJ 3603 was cointegrated into the chromosome of the wild type B. japonicum 110spc4 at the fixNOQ locus, the plasmids prJ 4538, prJ 4539, prJ 4540, prJ 4542, prJ 4543, prJ 4545, prJ 4550, and prJ 4569 were cointegrated into the operon deletion mutant B) 4503 by conjugation. Tetracycline-resistant clones were selected and confirmed by Southern blot hybridization. Thus, strains B) 4538, B) 4539, B) 4540, B) 4542, B) 4543, B) 4545, B) 4546, and B) 4569 were obtained that contain the corresponding plasmids integrated at the homologous position in the chromosome.

**Antibodies—**MalE-1-FixO and MalE-1-FixP hybrid proteins were used as suitable antigens. The malE-1-FixO and the malE-1-FixP gene fusions (to the 7th codon of fixO and the 6th codon of fixP) were constructed at the BsaI site (made blunt) in fixO or at the EcoRI site in fixP with the malE gene of pMal-cRI plasmid, resulting in prJ 4532 and prJ 3620, respectively. The correct fusion sites were confirmed by sequencing. The MalE-1-FixO and MalE-1-FixP proteins were expressed and purified according to the instructions of the manufacturer (New England Biolabs, Schwalbach/Taunus, Germany). The purified fractions were separated by 5% polyacrylamide gel electrophoresis (PAGE), and the proteins were stained with 0.1% Comassie Brilliant Blue R250. Gel pieces with the visualized hybrid proteins were excised and used to immunize a New Zealand White rabbit. The MalE-1-FixP...
antiserum was without purification. The MaLe'::FixO antiserum was purified following the method of Smith and Fischer (23). Antiserum directed against three FixN-specific peptides (PEP1 from position 61 to 73: RYFERPAALPPAE; PEP2 from position 366 to 381: TLSGAWD andantiserum was without purification. The MaLe'::FixO antiserum was purified following the method of Smith and Fischer (23). Antiserum directed against three FixN-specific peptides (PEP1 from position 61 to 73: RYFERPAALPPAE; PEP2 from position 366 to 381: TLSGAWD and

Enzymatic Assays—β-Galactosidase activity was measured from 100 μl samples of three independent cultures as described by Miller (25). N,N,N',N'-Tetramethyl-p-phenylenediamine (TMPD)2 oxidase activity was measured with whole cells as described previously (26). Cytochrome c oxidase activity with horse heart cytochrome c as an electron donor was measured as described by Gerhus et al. (27).

Cell Fractionation—B. japonicum cells were harvested in the late exponential growth phase. Membrane fractions were isolated as described elsewhere (26) with the following modification: the membranes were solubilized in 50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride and 1% dodecyl maltoside (Sigma), and the ultracentrifugation step was repeated. The solubilized oxidase complex remained in the supernatant. Protein concentration, SDS-PAGE (according to Laemmli (28) or Schägger and von Jagow (29), and heme stains were performed as described previously (30, 31). Spectroscopy of membrane fractions was performed as described elsewhere (32).

Western Blotting—Proteins were separated by SDS-PAGE and electroblotted onto Hybond-C nitrocellulose (Amersham Corp., Bucking-

Physical map of the B. japonicum fixNOQP region and of mutant constructs. A, the top line shows a restriction map of the DNA region harboring the 3' part of orf277, orf141, fixNOQP and the 5' part of fixG. Restriction sites: Bb, BstBI; Bg, BglII; Bs, BsaI; E, EcoRV; Ec, Eco471I; Nc, NcoI; Ns, NsiI; P, PvuII; Ps, PstI; R, EcoRI; S, SacI.

RESULTS

Mutagenesis of the Individual Genes in the fixNOQP Operon—To investigate the role of each of the four genes in function and biogenesis of the multisubunit enzyme complex, we constructed in-frame deletion mutations in fixN, fixO, and fixQ and used a fixP insertion that had been generated previously (24). The idea was to create nonpolar mutations in the first three genes that would not affect transcription and translation of the downstream genes in the operon. In the case of fixN, 394 codons (corresponding to amino acid positions 110–504) were deleted. From fixO, 114 internal codons (corresponding to amino acid positions 85–199) were removed. In the fixQ deletion the last 39 codons of the gene (amino acid positions 16–54) were removed. To restore the Shine-Dalgarno sequence of the downstream fixX gene, and to bring the fixQ stop codon in-frame, an Xhol-linker had to be inserted (see "Experimental Procedures"). All of the constructs contained the fixN promoter region (between orf141 and fixN)2 that drives transcription of the mutated operon. The ΔfixN, ΔfixO and ΔfixQ deletion plasmids were co-integrated into the chromosome of the mutant strain BJ4503 (11) in which the entire fixNOQP operon was deleted (Fig. 1). To make sure that a functional oxidase can be expressed from such co-integrates, a plasmid containing the entire wild type fixNOQP operon was also co-integrated into BJ4503. The resulting strain BJ4504 served as a positive control. Since fixN is the last gene of the operon, an aphII cassette was inserted into it, and the insertion was transferred into the wild type chromosome by marker replacement (strain BJ3618; 1 The abbreviations used are: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; PAGE, polyacrylamide gel electrophoresis.

Western Blotting—Proteins were separated by SDS-PAGE and electroblotted onto Hybond-C nitrocellulose (Amersham Corp., Bucking-

Plant Infection Test—Plant infection tests with soybean (G. max L. Merr.) were performed as described previously (19). Nitrogen fixation activity was determined by the acetylene reduction assay (33).

Control for Nonpolarity of the In-frame Deletions—Expression of the genes downstream of the in-frame deletions was tested. The fixNOQP-"fixP::lacZ fusion plasmid (pR) 3603 was co-integrated at its homologous site into the chromosome of the wild type, whereas all other fusion plasmids (Table II) were co-integrated at their homologous sites into the chromosome of the operon deletion strain BJ4503. Thus, expression of the fixO-"lacZ fusion was tested cotranscriptionally with either an intact fixN gene or the ΔfixN deletion. Expression of the fixQ-"lacZ fusion was tested cotranscriptionally with fixN-"ΔfixN, ΔfixN-"fixO, or ΔfixN-"ΔfixO. Expression of the fixQ-"lacZ fusion was tested cotranscriptionally with fixNO-"ΔfixN, ΔfixN-"fixO, ΔfixQ-"ΔfixO, or ΔfixNO-"ΔfixO. β-Galactosidase activity of microaerobically grown cells was measured because the fixNOQP operon was found to be maximally expressed under microaerobic growth conditions (data not shown). Table II shows that the fixO-"lacZ, fixQ-"lacZ, and fixP-"lacZ fusions were expressed to a similar extent. Thus, the in-frame gene deletion mutations ΔfixN, ΔfixO, and ΔfixQ neither disturbed transcription nor translation of the downstream genes. Notably, the fixP-"lacZ fusion in the ΔfixQ mutant led to a similar β-galactosidase activity as that in the wild type, proving that the reconstructed fixP ribosome binding site was functional. The results from these experiments allowed us to conclude that the single-gene mutants were suitable tools to study the role of the individual gene products in respiration and complex assembly.

Presence of the Subunits and Cofactors in the Oxidase—We first looked for the presence or absence of cbb3-type oxidase subunits in the ΔfixN, ΔfixO, ΔfixQ, and fixX::aphII mutants by Western blot analysis (Fig. 2), using antibodies specific for FixN, FixO, and FixP (see "Experimental Procedures"). B. japonicum cells were grown microaerobically, and membrane fractions were isolated. The cross-reaction of blotted membrane proteins with immunoglobulins specific for FixN or FixO gave no signal. The antiserum was used without purification. The MaLe'::FixO antiserum was purified following the method of Smith and Fischer (23). Antiserum directed against three FixN-specific peptides (PEP1 from position 61 to 73: RYFERPAALPPAE; PEP2 from position 366 to 381: TLSGAWD and

Control for Nonpolarity of the In-Frame Deletions—Expression of the genes downstream of the in-frame deletions was tested. The fixNOQP-"fixP::lacZ fusion plasmid (pR) 3603 was co-integrated at its homologous site into the chromosome of the wild type, whereas all other fusion plasmids (Table II) were co-integrated at their homologous sites into the chromosome of the operon deletion strain BJ4503. Thus, expression of the fixO-"lacZ fusion was tested cotranscriptionally with either an intact fixN gene or the ΔfixN deletion. Expression of the fixQ-"lacZ fusion was tested cotranscriptionally with fixN-"ΔfixN, ΔfixN-"fixO, or ΔfixN-"ΔfixO. Expression of the fixQ-"lacZ fusion was tested cotranscriptionally with fixNO-"ΔfixN, ΔfixN-"fixO, ΔfixQ-"ΔfixO, or ΔfixNO-"ΔfixO. β-Galactosidase activity of microaerobically grown cells was measured because the fixNOQP operon was found to be maximally expressed under microaerobic growth conditions (data not shown). Table II shows that the fixO-"lacZ, fixQ-"lacZ, and fixP-"lacZ fusions were expressed to a similar extent. Thus, the in-frame gene deletion mutations ΔfixN, ΔfixO, and ΔfixQ neither disturbed transcription nor translation of the downstream genes. Notably, the fixP-"lacZ fusion in the ΔfixQ mutant led to a similar β-galactosidase activity as that in the wild type, proving that the reconstructed fixP ribosome binding site was functional. The results from these experiments allowed us to conclude that the single-gene mutants were suitable tools to study the role of the individual gene products in respiration and complex assembly.

Presence of the Subunits and Cofactors in the Oxidase—We first looked for the presence or absence of cbb3-type oxidase subunits in the ΔfixN, ΔfixO, ΔfixQ, and fixX::aphII mutants by Western blot analysis (Fig. 2), using antibodies specific for FixN, FixO, and FixP (see "Experimental Procedures"). B. japonicum cells were grown microaerobically, and membrane fractions were isolated. The cross-reaction of blotted membrane proteins with immunoglobulins specific for FixN or FixO gave no signal. The antiserum was used without purification. The MaLe'::FixO antiserum was purified following the method of Smith and Fischer (23). Antiserum directed against three FixN-specific peptides (PEP1 from position 61 to 73: RYFERPAALPPAE; PEP2 from position 366 to 381: TLSGAWD and

1 The abbreviations used are: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; PAGE, polyacrylamide gel electrophoresis.

2 O. Preisig and J. Pleschke, unpublished results.
The B. japonicum mutant strains were grown microaerobically. Values are the means ± standard deviation of at least three measurements with two independent cultures of an A₆₀₀ of 0.2–0.3. The activity of the fixP⁻·IacZ fusion in the wild type background (BJ3604) was used as the standard (100%) and corresponded to 858 Miller units.

In summary, the results from Western blots, heme staining, dithionite-reduced minus air-oxidized difference spectra of membranes, and visible light spectroscopy implied that (i) complementation of an operon deletion with the wild type fixNOQP operon was successful; (ii) FixN, FixO and FixP were absent in the ΔfixN and ΔfixO mutants; (iii) the ΔfixQ mutation had no effect on the presence of the FixN, FixO and FixP subunits except perhaps a slight reduction in the amount of FixP; and (iv) FixN and FixO, but not FixP, were present in the fixP::aphII mutant.

Role of the fixNOQP-encoded Proteins in Microaerobic Respiration and Symbiotic Nitrogen Fixation—The functional consequences of the different mutations on respiration was tested.
by using TMPD as an artificial electron donor to measure cytochrome c-dependent oxidase activity from microaerobically grown cells with an oxygen electrode (Table III). The ΔfixQ strain had a similar TMPD oxidase activity as the wild type. The operon deletion mutant (Bj4503) showed a 25% residual oxidase activity, indicating that the cbb3 oxidase is responsible for about 75% of the total oxidase activity present in cells grown under microaerobic conditions. The ΔfixN, ΔfixO and the fixP::aphII mutants also showed decreased oxidase activities (18, 24.5, and 38%, respectively, as compared with the wild type). The fixP::aphII insertion mutant consistently had a slightly higher oxidase activity than the ΔfixN and ΔfixO deletion mutants. It thus appeared as if the FixN and FixO subunits alone are responsible for a small amount of TMPD oxidase activity. Interestingly, the cytochrome bc3 mutant showed almost wild type activity, suggesting that in the absence of the bc3 complex, the cbb3-type oxidase can still oxidize TMPD as a substrate. In addition to the tests shown in Table III we measured cytochrome c oxidase activity with reduced horse heart cytochrome c as electron donor, and similar results as with TMPD were obtained (not shown).

As shown previously (2), the cbb3-type oxidase is essential for symbiotic nitrogen fixation because it supports energy conservation under the extremely microaerobic conditions that prevail in soybean root nodules. Thus, we analyzed the ability of the single-gene deletion mutants to fix nitrogen in symbiosis (Fix phenotype; Table III). Soybean seedlings were inoculated with the B. japonicum mutant strains, and nitrogenase activity was measured as acetylene reduction activity 23 days after infection. While the fixNOQP+ strain and the ΔfixQ mutant were Fix+, the ΔfixN, ΔfixO and fixP::aphII mutants showed between 0 and 5% residual Fix activity. Nodules infected with the latter mutant strains were greenish inside, indicating the absence of functional leghemoglobin. Our results suggest that the FixN, FixO and FixP proteins, but not the FixQ protein, are essential to energetically support nitrogen fixation in root nodules.

**DISCUSSION**

To study the pathway for assembly of multisubunit membrane complexes is inherently difficult. Lack of a single subunit often causes complete absence of the entire complex because membrane proteins may be rapidly degraded unless they find their correct partner subunit(s) to become stabilized by protein-protein interaction. For example, the assembly of the F$_{1}$F$_{0}$-ATPase of E. coli relies on the presence of both α and β subunits (35), and cytochrome c$_{551}$ of the Rhodobacter capsulatus and P. denitrificans ubiquinol-cytochrome c oxidoreductase is required to maintain the stability of the other two subunits (cytochrome b, Rieske iron sulfur protein) (27, 36).

Here we report on studies to elucidate how assembly of the B. japonicum cbb3-type cytochrome oxidase takes place. The approach was to knock out separately each of the four genes in the fixNOQP operon, and then we assessed the presence or absence of the other gene products in the cytoplasmic membrane. It was mandatory to construct in-frame deletions and ensure that genes located downstream of the deleted gene were expressed at normal levels. In fact, none of the deletions showed any polarity effects on the downstream genes.

The mutations analyzed here affected cytochrome cbb3 formation to different extents, allowing us to draw conclusions with respect to subunit composition, function, and assembly. Surprisingly, the ΔfixQ mutation did not cause any severe deficiencies even though (i) a fixQ-like gene is conserved in all organisms examined for the presence of fixNOQP-like operons (2-6) and (ii) fixQ was shown here to be transcribed and translated. Hence, it is clear that the 54-amino acid FixQ protein is not essential for the formation and function of the cbb3 oxidase complex. However, this does not exclude the possibility that FixQ is bound to the oxidase, perhaps even in substoichiometric amounts. The question of whether or not FixQ is part of the complex will be answered only when substantial quantities of highly purified cytochrome cbb3 have become available. The partially purified oxidase preparation reported recently (11) was of sufficient quantity to identify a protein of the expected molecular mass of FixQ, i.e. approximately 6 kDa. In this context, we find it intriguing that the recent three-dimensional structure determination of P. denitrificans cytochrome aa$_{3}$ has uncovered the presence of an unprecedented 60-amino acid peptide whose functional role is also not known (37).

The FixN and FixO proteins, as opposed to FixQ, are absolutely essential for the function and assembly of cytochrome cbb3. Neither FixN and FixO nor the FixP protein were detectable in membranes isolated from ΔfixN and ΔfixO mutants. This supports previous biochemical data obtained with purified

![Fig. 4. Visible absorption difference spectra (dithionite-reduced minus air-oxidized). Membrane proteins (0.5 mg ml$^{-1}$) solubilized with dodecyl maltoside from the following B. japonicum strains were tested: Bj4504 (fixNOQP+ by complementation), Bj4503 (ΔfixNOQP), Bj4526 (ΔfixN), Bj4518 (ΔfixO), Bj4528 (ΔfixQ), and Bj3618 (fixP::aphII).](image-url)

**Table III**

Phenotypes of B. japonicum wild type and mutant strains

| Phenotypic marker | 110p04 wild type | Bj3067 | Bj4503 | ΔfixNOQP | Bj4504 fixNOQP+ | Bj4504 ΔfixN | Bj4504 ΔfixO | Bj4504 ΔfixQ | Bj4504 ΔfixP::aphII |
|-------------------|------------------|--------|--------|----------|----------------|-------------|-------------|-------------|-------------------|
| TMPD oxidase activity | 63.4 ± 7.5 | 57.6 ± 11.8 | 17.3 ± 3.3 | 67.1 ± 18.9 | 12 ± 0.8 | 15.5 ± 1.1 | 56.6 ± 14.6 | 24.0 ± 2.6 |
| Nitrogenase activity | 145 ± 46 | 0 | 10 ± 2 | 199 ± 41 | 3 ± 2 | 0 | 172 ± 39 | 2 ± 3 |

* Nanomoles of O$_{2}$ reduced min$^{-1}$ mg$^{-1}$; at least three measurements were made with cultures grown microaerobically to an OD$$_{550}$ of 0.3.
* Micromoles of ethylene h$^{-1}$ mg nodules (dry weight)$^{-1}$; at least five plants per strain were used.
* All values are the means ± standard deviation.
Assembly of the B. japonicum cbb\(_3\) Oxidase

**cbb\(_3\) oxidase from B. japonicum and two Rhodobacter species (8, 9, 11)** which showed that it is a three-subunit enzyme complex consisting of FixN+, FixO+, and FixP-like proteins. Interestingly, in the B. japonicum fixP insertion mutant we detected almost wild type levels of the FixN and FixO proteins, whereas only FixP itself was absent. Moreover, the mutant had wild type levels of cytochrome b, but a decreased level of cytochrome c probably due to the absence of FixP. Notably, FixO was shown by heme-staining after SDS-PAGE to be present as mature holocytochrome c in the fixP mutant. The results are reminiscent of studies with the cytochrome bc\(_1\) complex, in which cytochromes b and c\(_1\) were still detectable in a mutant defective in the gene for the Rieske iron-sulfur protein (36).

Taken together, our results led us to propose an ordered biogenesis pathway for the cbb\(_3\)-type cytochrome oxidase. After translation of the individual subunit polypeptides from the fixNOQ mRNA, FixN and FixO are probably the first to be inserted into the membrane to form an apparently stable FixNO core complex. This primary nucleation step occurs also in the absence of FixP and, therefore, precedes assembly of the FixNO core with the FixP protein. The fact that not even traces of FixN plus FixP and FixO plus FixP are detectable in ΔfixO and ΔfixN mutants, respectively, suggests that the subunits are degraded extremely rapidly when assembly in the membrane is not possible for lack of cognate, stabilizing protein partners.

In the aerobic respiratory chain of B. japonicum the bc\(_1\) complex appears to be a nucleation center for the formation and stable maintenance of the subsequent electron transport cytochromes CymM and aa\(_3\), which are thought to be organized in a bc\(_1\)-CymM-aa\(_3\) supercomplex (24, 30, 32). By contrast, the FixNOQ proteins do assemble also in the absence of, and therefore independently from, the bc\(_1\) complex.

The phenotypical analyses of the mutants also help toward understanding the organization of the microaerobic electron transport chain in B. japonicum. It is now well established that under microaerobic conditions electrons are transferred from ubiquinol to the bc\(_1\)-CytM-aa\(_3\) complex and then to FixP and from FixP to FixO and FixN, where they are finally used for the reduction of molecular oxygen to water.

Acknowledgments—We are grateful to R. Fischer for his help with antibody production, and to P. Künzler for constructing pSUP202pOl6K.

**REFERENCES**

1. García-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J., and Gennis, R. B. (1993) J. Bacteriol. 176, 5387–5600
2. Preissig, O., Anthamatten, D., and Hennecke, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3309–3313
3. Mondon, K., Kaninsky, P. A., and Elmerich, C. (1994) J. Bacteriol. 176, 6444–6454
4. Kahn, D., Batut, J., Davenant, M.-L., and Fourment, J. (1993) in New Horizons in Nitrogen Fixation (Palacios, R., Mora, J., and Newton, W. E., eds.) p. 474, Kluwer Academic Publishers, Dordrecht
5. Schütler, A., Patschkowski, T., Weidner, S., Under, G., Hynes, M. F., and Priefert, U. B. (1993) in New Horizons in Nitrogen Fixation (Palacios, R., Mora, J., and Newton, W. E., eds.) p. 483, Kluwer Academic Publishers, Dordrecht
6. Thöny-Meyer, L., Beck, C., Preissig, O., and Hennecke, H. (1994) Mol. Microbiol. 17, 5001–5005
7. Schütler, A., Rüger, S., Krämer, M., Weidner, S., and Priefert, U. B. (1995) Mol. Gen. Genet. 247, 206–215
8. Gray, K. A., Groons, M., Myiliakkal, H., Moosw, C., Slaughter, C., and Daldal, F. (1994) Biochim. Biophys. Acta 1233, 3120–3127
9. García-Horsman, J. A., Berry, E., Shapleigh, J. P., Alben, J. O., and Gennis, R. B. (1994) Biochemistry 33, 3113–3119
10. Keefe, R. G., and Maier, R. J. (1993) Biochim. Biophys. Acta 1183, 91–104
11. Preisig, O., Zufferey, R., Thöny-Meyer, L., Appleby, C. A., and Hennecke, H. (1996) J. Bacteriol., in press
12. de Gier, J. W. L. (1995) The Terminal Oxidases of Paracoccus denitrificans. Ph.D. thesis, Vrije Universiteit, Amsterdam
13. Minagawa, J., Mogi, T., Gennis, R. B., and Anraku, Y. (1992) J. Biol. Chem. 267, 2096–2104
14. Steinrücker, P., Gerhus, E., and Ludwig, B. (1993) J. Biol. Chem. 268, 7676–7681
15. Haltia, T., Finel, M., Harms, N., Nakari, T., Raatto, M., Wilkström, M., and Sars, M. (1989) EMBO J. 8, 3571–3579
16. Regensburger, B., and Hennecke, H. (1983) Arch. Microbiol. 135, 103–109
17. Daniel, R. M., and Appleby, C. A. (1972) Biochim. Biophys. Acta 275, 347–354
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Hahn, M., and Hennecke, H. (1984) Mol. Gen. Genet. 193, 46–52
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
21. Göttler, M., Holzhäuser, D., Bäni, D., and Hennecke, H. (1992) Mol. Plant-Microbe Interact. 5, 305–316
22. Ritz, D., Bott, M., and Hennecke, H. (1993) Mol. Microbiol. 9, 729–740
23. Smith, D. E., and Fischer, P. A. (1983) J. Cell Biol. 99, 20
24. Thöny-Meyer, L., James, P., and Hennecke, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5001–5005
25. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Letters, H., Bott, M., and Hennecke, H. (1993) EMBO J. 12, 3373–3383
27. Gerhus, E., Steinrücker, P., and Ludwig, G. (1990) J. Bacteriol. 172, 2392–2400
28. Laemmli, U. K. (1970) Nature 227, 680–685
29. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
30. Thöny-Meyer, L., Stax, D., and Hennecke, H. (1989) Cell 57, 683–697
31. Francis, R. T., and Becker, R. R. (1984) Anal. Biochem. 136, 509–514
32. Bott, M., Baltzer, M., and Hennecke, H. (1990) Mol. Microbiol. 4, 2147–2157
33. Turner, G. L., and Gibson, A. H. (1980) in Molecular Biology of the Bacteria-Plant Interaction (Bergersen, F. J. ed) pp. 111–138, John Wiley & Sons, Chichester
34. Bott, M., Ritz, D., and Hennecke, H. (1991) J. Bacteriol. 173, 6766–6772
35. Cox, G. B., and Gibson, F. (1987) Curr. Top. Bioenerg. 15, 163–175
36. Davidson, E., Ohnishi, T., Tokito, M., and Daldal, F. (1992) Biochemistry 31, 3351–3358
37. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature 376, 660–669
38. Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
39. Simon, R., Priefert, U., and Pühler, A. (1983) in Molecular Genetics of the Bacteria-Plant Interaction (Pühler, A., ed) pp. 98–106, Springer Verlag, Heidelberg
40. Minton, N. P. (1984) Gene (Amst.) 31, 269–273