How Replacements of the 12 Conserved Histidines of Subunit I Affect Assembly, Cofactor Binding, and Enzymatic Activity of the Bradyrhizobium japonicum cbb₃-type Oxidase*

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Alignments of the amino acid sequences of subunit I (FixN or CcoN) of the cbb₃-type oxidases show 12 conserved histidines. Six of them are diagnostic of heme-copper oxidases and are thought to bind the following cofactors: the low spin heme B and the binuclear high spin heme B-Cu₃ center. The other six are FixN(CcoN)-specific and their function is unknown. To analyze the contribution of these 12 invariant histidines of FixN in cofactor binding and function of the Bradyrhizobium japonicum cbb₃-type oxidase, they were substituted by valine or alanine by site-directed mutagenesis. The H131A mutant enzyme had already been reported previously to be defective in oxidative assembly and function (Zufferey, R., Thöny-Meyer, L., and Hennecke, H. (1996) FEBS Lett. 394, 349–352). Four of the remaining histidines were not essential for activity or assembly (positions 226, 246, 333, and 457); by contrast, histidines 331, 410, and 418 were required both for activity and stability of the enzyme. The last group of mutant enzymes, H420A, H280A, H330A, and H316V, were assembled but not functional. To purify the latter mutant proteins and the wild-type enzyme, a six-histidine tag was added to the C terminus of subunit I. The His₆-tagged cbb₃-oxidase complexes were purified 20-fold by a three-step purification protocol. With the exception of the H420A mutant oxidase, the mutant enzymes H280A, H316V, and H330A contained normal amounts of copper and heme B, and they displayed similar visible light spectroscopic characteristics like the wild-type His₆-tagged enzyme. The His₆-tagged H420A mutant oxidase differed from the His₆-tagged wild-type protein by showing altered visible light spectroscopic characteristics. No stable mutant oxidase lacking copper or heme B was obtained. This strongly suggests that copper and heme B incorporations in subunit I are prerequisites for assembly of the enzyme.

Terminal cytochrome c oxidases are multimeric membrane protein complexes that catalyze the 2-electron oxidation of cytochrome c and the 4-electron reduction of oxygen to water. This process is usually coupled with the extrusion of protons toward the periplasm. Cytochrome c oxidases belong to the so-called superfamily of heme-copper oxidases (for a review, see Ref. 2). They have in common a low spin heme moiety and a so-called binuclear center composed of a high spin heme and Cu₃B, where the oxygen reduction chemistry occurs. These cofactors are associated with subunit I and are bound by six strictly conserved histidines (3–5). Other typical features of cytochrome c oxidases are the presence of (i) a binuclear Cu₃ center in subunit II, which is the entry site for electrons derived from reduced cytochrome c, and (ii) a Mn²⁺ or Mg²⁺ redox-inactive center that lies between subunits I and II (6, 7). The cbb₃-type oxidase was found a few years ago to be a novel type of a cytochrome c oxidase with regard to subunit composition and content of prosthetic groups. It was first discovered in Bradyrhizobium japonicum and other rhizobial species and found to be essential for nitrogen fixation in symbiosis with the host plant (8–11). For this reason, the operon encoding the cbb₃-type oxidase subunits was named fixNOQP. In the meantime, similar operons were also found in non-symbiotic bacteria, for which the ecoNOQP nomenclature (mnemonic for cytochrome c oxidase) was the preferred designation (12, 13). The B. japonicum cbb₃-type oxidase is expressed under a low oxygen concentration (free-living and symbiotic) (14). This is in accordance with the high affinity for oxygen of the B. japonicum enzyme (Kₘ = 7 nM; see Ref. 14).

Subunit I (FixN in B. japonicum), the largest subunit of the cbb₃-type oxidase, is a 12-transmembrane-helix protein with a molecular mass of 61 kDa and contains two hemes of the B type and a Cu₃ (1, 14, 15). The high spin heme B forms a binuclear center with the Cu₃ metal (15, 16). The cbb₃-type oxidase lacks a Cu₃-containing subunit II. Instead, two subunits (FixO and FixP) are c-type cytochromes that are anchored in the cytoplasmic membrane by their N-terminal transmembrane helix. FixO corresponds to subunit II, as inferred from the redox potential of the homologous Rhodobacter capsulatus protein (16) and its role in assembly (17, 18). It is a monoheme cytochrome c of 28 kDa. FixP is a diheme cytochrome c of 32 kDa (18). The fixQ gene product has a molecular mass of 6 kDa and is thought to be inserted in the membrane by its hydrophobic N terminus. This small subunit is neither essential for the function nor for assembly of the enzyme (17). It is still unknown whether or not this gene product is part of the cbb₃-type oxidase complex. The purified enzyme of B. japonicum as well as that of three non-symbiotic bacteria were identified as three-subunit complexes, and the presence of FixQ in such preparations has never been reported (15–17, 19).

Subunit I of the cbb₃-type oxidase differs from the classical subunit I of terminal oxidases of the heme-copper oxidase family by two notable criteria: (i) it has a lower degree of similarity on the amino acid sequence level (20%, see Ref. 8), and (ii) it possesses in addition to the six canonical histidines conserved in all subunits I of heme-copper oxidases a further six histidines conserved in all FixN homologues (Fig. 1). The canonical histidines His-131, His-280, His-330, His-331, His-418, and His-420 of the B. japonicum FixN protein correspond to the
Recombinant DNA Work and Construction of the Mutants—Standard procedures were used for cloning, Southern blotting, and hybridization (25). Chromosomal DNA of *B. japonicum* was isolated according to Hahn and Hennecke (29). DNA hybridization probes were radioactively labeled using the nick-translation technique (25). DNA sequence analyses were performed using the chain termination method (30) and the equipment for automated DNA sequencing (Sequencer model 370A and fluorescent dye terminators from Applied Biosystems, Foster City, CA).

All mutagenic primers used in this work are listed in Table I. The following mutations in FixN, H227A, H246V, H330A, and H333V were point-mutated to a neutral, smaller localized on a periplasmic loop (20–22).

histidines His-94, His-276, His-325, His-411, and His-413 of the *Paracoccus denitrificans* aa3-type cytochrome *c* oxidase subunit I, which ligate the hemes and CuB cofactors, as shown unequivocally by the crystal structure of the bacterial and mitochondrial aa3-type cytochrome oxidases (3–5). All of them are located on the periplasmic face of the membrane. His-276, His-325, and His-326 (using the numbering of *P. denitrificans* cytochrome aa3) are involved in complexing CuB. His-411 serves as the high spin heme ligand. The two other histidines, His-94 and His-413, bind the low spin heme. One of the CuB ligands (His-325) was proposed to be implicated in both reduction of oxygen and proton pumping (3). The six non-conserved histidines His-94, His-276, His-325, His-326, His-411, and His-457 serve as the high spin heme ligand. The two other histidines His-227, His-316, and His-457 on the cytoplasmic side (using the numbering) are proposed to be on the periplasmic side and His-227, His-316, and His-457 on the cytoplasmic side of the membrane (1). His-410 corresponds to the histidine that ligates a Mg2+ or Mn2+ metal center in many cytochrome *c* oxidases but not in quinol oxidases (6, 7). It is predicted to be localized on a periplasmic loop (20–22).

In the present work, all of the conserved histidines of *B. japonicum* FixN were point-mutated to a neutral, smaller amino acid. These mutant variants were analyzed with respect to assembly in the membrane, cytochrome *c* oxidase activity, and nitrogen fixation in symbiosis with soybean. Among these mutations, only four, H316V (conserved FixN-specific residue), H420A (putative low spin heme B ligand), H280A, and H330A (putative CuB ligands) were found to cause inactive enzymes that were assembled in the membrane. Hexahistidine-tagged derivatives of these enzymes and of the wild-type enzyme were purified to homogeneity in a three-step purification procedure, including anion-exchange and Ni2+ affinity chromatographies and a gel filtration. We determined the copper and heme *B* contents and performed difference visible light spectroscopy with the purified mutant proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—All *B. japonicum* strains used in this work are listed in Table I. *B. japonicum* strains were grown at 28 °C either aerobically or anaerobically in PSY medium (8, 23) or in YEM medium supplemented with 10 mM KNO3, respectively (24). Antibiotics were added at the following concentrations (μg/ml): spectinomycin (100); tetracycline (60); chloramphenicol (10). *Escherichia coli* was grown in LB medium (25) to which antibiotics were added at the following concentrations (μg/ml): ampicillin (150); kanamycin (50); tetracycline (10). *E. coli* DH5α (26) was used for amplification of double strand plasmids and pM101 (27) for amplification of coliphage M13. *B. japonicum* was conjugated with the help of the *E. coli* donor strain S17-1 (28).

![FIG. 1. Topological model of subunit I (FixN) of *B. japonicum* cbb3-type oxidase. The black circles represent the invariant histidines present in subunit I of all heme-copper oxidases, whereas the gray circles symbolize the histidines conserved only in the FixN-homologous proteins. The histidines are numbered according to the amino acid sequence. The rectangles depict the hydrophobic segments. C, C terminus; N, N terminus; i, cytoplasm (inside); o, periplasm (outside).](image-url)
Role of Conserved Histidines in Subunit I of cbb₃-type Oxidase

TABLE I

| Strain     | Mutation (Wild-type) | Primer name | Sequence of the primer |
|------------|----------------------|-------------|------------------------|
| H227A      | ΔfxN                 | H15         | 5′-agtccggcagcaagatcgcgggtcc-3′ |
| H227A      | ΔfxNOQP             | H15         | 5′-agtccggcagcaagatcgcgggtcc-3′ |
| H246V      | ΔfxN                 | H16         | 5′-ctgtaacctggtctgctacgtctccgcgg-3′ |
| H316V      | ΔfxN                 | H11         | 5′-gtgtaacctggtctgctacgtctccgcgg-3′ |
| H330A      | ΔfxN                 | H3          | 5′-ctgtaacctggtctgctacgtctccgcgg-3′ |
| H330A      | ΔfxN                 | H4          | 5′-ctggggacagctcgcttgccttcg-3′ |
| H331A      | ΔfxN                 | H5          | 5′-gcaagcttcgcaagctgcctcgcctg-3′ |
| H333V      | ΔfxN                 | H7          | 5′-ctgggccagctgccacactgttgatcgcgg-3′ |
| H410A      | ΔfxN                 | H7          | 5′-ctgggccagctgccacactgttgatcgcgg-3′ |
| H418V      | ΔfxN                 | H6          | 5′-ctgggccagctgccacactgttgatcgcgg-3′ |
| H420A      | ΔfxN                 | H7          | 5′-ctgggccagctgccacactgttgatcgcgg-3′ |
| H457A      | ΔfxN                 | H6          | 5′-ctgggccagctgccacactgttgatcgcgg-3′ |
| H4621      | ΔfxN                 | H7          | 5′-ctgggccagctgccacactgttgatcgcgg-3′ |
| H4622      | ΔfxN                 | H18         | 5′-cttgccagctgccacactgttgatcgcgg-3′ |
| H4623      | ΔfxN                 | H11         | 5′-cttgccagctgccacactgttgatcgcgg-3′ |
| H4624      | ΔfxN                 | H3          | 5′-cttgccagctgccacactgttgatcgcgg-3′ |
| H4625      | ΔfxN                 | H8          | 5′-cttgccagctgccacactgttgatcgcgg-3′ |

by the manufacturer and equilibrated with buffer B (20 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.01% dodecyl maltoside, and 5 mM imidazole). After loading, the column was washed with 3 bed volumes of buffer B. Then, the contaminants were removed with 3 bed volumes of buffer B containing 20 mM imidazole in imidazole. The fractions containing the cbb₃-type oxidase were eluted with 250 mM imidazole in buffer B. The column was washed with 3 bed volumes of buffer B. The nitrocellulose membrane. Visible light spectroscopy of purified enzyme preparations in anaerobic conditions, because this terminal oxidase is expressed under such conditions (8). In this assay, TMPD is used as the artificial electron donor. The cbb₃-type oxidase contributes to at least 50–60% of the total cellular TMPOxidase activity under such conditions because the ΔfixN deletion mutant Bj4526 showed about 40% residual activity as compared with the positive control strain Bj4504 (fixNOQP⁺, Table II). This relatively high residual activity is caused by alternative cytochrome c oxidases (37) or by enzymes of the denitrification pathway (36, 39). The H227A, H246V, H333V, and H457A mutants oxidized TMPD to a similar extent as the positive control strain Bj4504 (70–100%). By contrast, histidine replacements at positions 280, 316, 330, 331, 410, 418, and 420 led to inactive enzymes because they all showed less than 50% TMPD oxidase activity like the ΔfixN in-frame deletion strain Bj4526.

The cbb₃-type terminal oxidase had been shown to be essential in supporting nitrogen fixation (Fix phenotype) in symbiosis with soybean (8). Therefore, we measured nitrogenase activity in root nodules infected with the histidine mutant strains (Table II). The following mutants H227A, H246V, H333V, and H457A fixed nitrogen to a similar extent as the positive control strain Bj4504 (87–135%). By contrast, mutations at positions 280, 316, 330, 331, 410, 418, and 420 led to inactive enzymes because they all showed less than 50% TMPD oxidase activity like the ΔfixN in-frame deletion strain Bj4526.

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Roles of Conserved Histidines in Subunit I of \(cbb_3\)-type Oxidase

Table II Phenotypes of the \(B. japonicum\) mutants

| \(B. japonicum\) \(\) strains | \(\) TMPD oxidase activity \(\) | \(\) Cytochrome \(c\) oxidase activity \(\) | \(\) Nitrogenase activity \(\) |
|-----------------------------|--------------------------|---------------------------|--------------------------|
| Bj4504                      | 100 ± 8                  | 100 ± 5                   | 100 ± 8                  |
| Bj4526                      | 38 ± 8                   | 8 ± 3                     | 6 ± 1                     |
| H420A                       | 45 ± 4                   | 9 ± 4                     | 10 ± 3                    |
| H418V                       | 26 ± 9                   | 17 ± 8                    | 6 ± 1                     |
| H280A                       | 30 ± 6                   | 17 ± 5                    | 11 ± 2                    |
| H330A                       | 52 ± 3                   | 13 ± 4                    | 10 ± 1                    |
| H331A                       | 41 ± 6                   | 9 ± 1                     | 14 ± 2                    |
| H410A                       | 27 ± 1                   | 9 ± 4                     | 12 ± 2                    |
| H227A                       | 70 ± 7                   | 69 ± 8                    | 87 ± 8                    |
| H246V                       | 72 ± 6                   | 74 ± 18                   | 101 ± 14                  |
| H316V                       | 40 ± 17                  | 14 ± 2                    | 18 ± 8                    |
| H333V                       | 98 ± 7                   | 60 ± 12                   | 87 ± 18                   |
| H457A                       | 68 ± 4                   | 12 ± 1                    | 135 ± 13                  |
| Bj4621                      | ND                       | 69 ± 10                   | ND                       |

a Bj4504 was used as the wild-type standard (100%). Values are given in percent and represent the means ± S.D.
b 100% of TMPD oxidase activity corresponds to 100 nmol of \(O_2\) × min \(^{-1}\) × mg of protein \(^{-1}\), and at least three measurements were made with two independent cultures of anaerobically grown cells.
c 100% of cytochrome \(c\) oxidase activity corresponds to 0.8 \(\mu\)mol of cytochrome \(c\) × min \(^{-1}\) × mg of protein \(^{-1}\), and at least two measurements were performed with solubilized membrane fractions of two independent anaerobically grown cultures.
d 100% of nitrogenase activity corresponds to 146 \(\mu\)mol of ethylene h \(^{-1}\) × mg of nodules \(^{-1}\) (dry weight); at least five plants per strain were used.

pared with the positive control strain.

Presence of Subunits in the Oxidase—We analyzed the mutant enzymes on the protein level to check whether the functionally defective mutant strains still contained \(cbb_3\)-type oxidase subunits. For this purpose, we performed Western blot analyses with antibodies specific to FixN, FixO, FixP, and CycM. The latter antibody was used as an internal control to ascertain that comparable levels of protein had been loaded in each lane. Membrane fractions of anaerobically grown cells were isolated. As expected, the positive control strain, Bj4504, contained the FixN, FixO, and FixP proteins, whereas the negative control strain, Bj4526, had an absent defect of the enzyme (17), lacking all three proteins (Fig. 2, lanes 2 and 1, respectively. The mutants H418V (lane 4), H331A (lane 7), H410A (lane 8), and H457A (lane 13) had strongly decreased amounts of \(cbb_3\)-type oxidase subunits in their membranes as compared with the positive control strain Bj4504. By contrast, H420A (lane 3), H280A (lane 5), H330A (lane 6), H227A (lane 9), H246V (lane 10), H316V (lane 11), and H333V (lane 12) displayed wild-type amounts of FixN, FixO, and FixP proteins in their membranes. Consequently, alanine replacement at positions 420, 280, 330, and 227 and valine substitution at positions 316 and 333 in FixN did not affect the assembly of the \(cbb_3\)-oxidase, whereas the H418V, H331A, H410A, and H457 mutations led to assembly defective or instable enzymes. Heme staining of the membrane-bound c-type cytochromes indicated that whenever FixO and FixP were present, they were synthesized as holoproteins (data not shown).

Considering the activities of the mutants and the detection of their subunits in the membrane, lack of functional activities correlates with the decreased amounts of \(cbb_3\)-type oxidase subunits present in the membrane for mutants H331A, H410A, and H418V. This was not the case for mutants H280A, H316V, H330A, and H420A, which are apparently assembled and stable in the membrane. Thus, these four enzymes were considered to be good candidates for purification and further analysis. Their functional defect might be explained by structural alterations that do not disturb the assembly process.

Purification and Characterization of the His\(_6\)-tagged \(cbb_3\) Oxidase—To purify the \(cbb_3\)-type oxidase, six histidines were fused to the C terminus of FixN by genetic means, and the respective DNA construct was integrated in the chromosome of the \(\Delta fixNOQP\) mutant Bj4503 (see “Experimental Procedures”). Preliminary tests showed that such a complex is properly assembled in the membrane, because FixN, FixO, and FixP subunits were detected by Western blot analyses with the respective immunoglobulins (data not shown), and because the cytochrome \(c\) oxidase activity was hardly affected (Table II). Hence, the addition of six histidines at the C terminus of FixN does not appear to affect the biogenesis and the activity of the enzyme.

The His\(_6\)-tagged \(cbb_3\)-type oxidase was purified from the solubilized membrane fraction by a three-step protocol as described under “Experimental Procedures” (Table III). A 20-fold enrichment of specific cytochrome \(c\) oxidase activity and a recovery of 70% of total membrane activity were obtained (Table III). From 40 mg of membrane protein, 1.3 mg of purified \(cbb_3\)-type oxidase was recovered, which represents approximately 3.3% of solubilized membrane proteins. Three major bands of apparent molecular masses of 47, 32, and 28 kDa were visible on a SDS-polyacrylamide gel (Fig. 3A, lanes 1–4). The 47-kDa band corresponded to the His\(_6\)-tagged FixN subunit because it was recognized by anti-FixN serum (Fig. 3B). The 32- and 28-kDa bands are cytochromes \(c\) because they are heme-stainable (Fig. 3A, lane 5) and correspond to FixO and FixP, respectively, as shown by their cross-reaction with immunoglobulins against FixP and FixO (Western blot not shown). The intensity of FixP heme staining was consistently stronger than that of FixO. This might reflect the fact that FixP is a diheme cytochrome \(c\) whereas FixO is a monoheme cytochrome \(c\) (14, 17, 18). The small \(fixQ\) gene product was not seen on a Schägger and von Jagow gel stained with Coomassie Blue and could be only detected with an antibody specific to a FixQ peptide. To test the specificity of the immunoglobulins, membrane proteins of wild-type \(cbb_3\)-type oxidase (Bj4504) and Bj4503 (\(\Delta fixNOQP\)) were loaded on a Schägger gel as positive and negative controls, respectively. The membrane proteins were blotted onto a nitrocellulose membrane laid behind a polyvinylidene difluoride membrane as described under “Experimental Procedures.” Only the nitrocellulose membrane was stained with immunoglobulins specific to the C terminus of FixQ. A 4-kDa band could be assigned to FixQ because it was detected in the purified \(cbb_3\)-type oxidase and in the membrane fraction of strain Bj4504 (\(\Delta fixNOQP\)) but was absent in the Bj4503 (\(\Delta fixNOQP\)) mutant (Fig. 3C, lanes 1–3). Thus, FixQ had a slightly aberrant migration behavior during SDS-PAGE (apparent molecular mass of 4 kDa compared with the theoretical molecular mass of 6,031 Da), which is not uncommon for membrane proteins. Hence, the \(cbb_3\)-type oxidase will be also...
Role of Conserved Histidines in Subunit I of cbb₃-type Oxidase

Protein determination was done with the bicinchoninic acid assay, and horse heart cytochrome c oxidase activity was measured at 30 °C as described under “Experimental Procedures.”

| Purification step                     | Cytochrome c oxidase activity | Purification factor | Protein |
|--------------------------------------|------------------------------|--------------------|---------|
| 1. Solubilized membrane fraction     | 0.58 μmol cytochrome c mg⁻¹×mg⁻¹ | 1                  | 40.9    |
| 2. Q-Sepharose fast flow             | 1.15 mg                      | 2                  | 19.8    |
| 3. His bond resin                    | 5.20 mg                      | 9                  | 1.1     |
| 4. Sephadex G-25                     | 11.60 mg                     | 19.8               | 1.3     |

Proteins from solubilized membranes and from the active fractions of the Q-Sepharose chromatography (15 and 8 min) were loaded in lanes 1 and 2, respectively. Lanes 3 and 4 contain 2-μg samples from the Ni²⁺ affinity chromatography and Sephadex G-25 gel filtration, respectively. B, Western blot with immunoglobulins against FixN with 0.5 μg of the purified fraction. C, Western blot (12% Schägger gel) with immunoglobulins against FixQ. One μg of the purified oxidase (lane 1) and 70 μg of membrane proteins of Bj4503 (fixNOQP, lane 2) and Bj4504 (fixNOQP⁺, lane 3) were loaded. The apparent molecular masses (kDa) of the marker proteins are indicated on the left.

The dithionite-reduced minus air-oxidized visible light spectra of the His₆-tagged purified oxidase showed features similar to the wild-type enzyme as follows: two peaks at 551 and 561 nm diagnostic of c- and b-type cytochromes, respectively (Fig. 4A). The ratio between heme C and B (peaks at 551 and 561 nm, respectively) was estimated to be 1.73 by using the respective molar extinction coefficients 19.1 and 22 mM⁻¹×nm⁻¹, respectively) was estimated to be 1.73 by using the respective molar extinction coefficients 19.1 and 22 mM⁻¹×nm⁻¹, respectively. 

Molecular masses (kDa) of the marker proteins are indicated on the left.

CycYHis⁺⁺ was measured, and a stoichiometry of 0.03 per mole (mass = 17,935 Da) was obtained. This value corresponded to 3.75% that of the purified wild-type His₆-tagged enzyme produced by Bj4621 (Table IV). From this result, we would like to exclude a role of the His₆-tag in copper binding.

Biochemical Characterization of Four His₆-tagged Histidine Mutant Enzymes—The H280A, H330A, H316V, and H420A mutants were chosen for purification and further examination because they produced inactive enzymes that were apparently assembled stably in the membrane. For this purpose, six histidines were added by genetic means at the C terminus of FixN as for the wild-type enzyme to allow a faster and more efficient purification procedure. It was not reasonable to attempt purification of the following mutant enzymes His₆-H331A, H410A, H418V, and H457A because they were present in very small amounts in the membrane or even completely absent. Moreover, it is quite possible that they are not suitable for purification because of their instability.

The H290A, H330A, H316V, and H420A His₆-tagged mutant enzymes (from strains Bj4623, Bj4622, Bj4624, and Bj4625, respectively) were purified by the same procedure as the His₆-tagged wild-type and mutant cbb₃-type oxidases (0.1 mg/ml) by room temperature difference spectroscopy. A, dithionite reduced minus air-oxidized difference spectra between 500 and 600 nm. The 551- and 561-nm peaks are diagnostic of c-type cytochromes and b-type cytochromes, respectively. B, second derivatives of dithionite reduced minus air-oxidized spectra between 510 and 590 nm. The 551- and 561-nm troughs are diagnostic of c-type cytochromes and b-type cytochromes, respectively.

| Mutant proteins (amino acid exchange) | Cytochrome c oxidase activity | Copper content | Heme B content |
|---------------------------------------|------------------------------|----------------|----------------|
| Bj4621                                | 100                          | 100.0 ± 0.1    | 100.0 ± 7.8    |
| Bj4622 (H330A)                        | 10.1                         | 150.8 ± 1.3    | ND             |
| Bj4623 (H280A)                        | <1                           | 80.9 ± 2.35    | ND             |
| Bj4624 (H316V)                        | <1                           | 120.9 ± 3.3    | ND             |
| Bj4625 (H420A)                        | 2.7                          | 111.5 ± 0.8    | 90.4 ± 4.5     |
| CycYHis⁺⁺                             | ND                           | 3.7 ± 0.6      | ND             |
tagged wild-type enzyme (from strain Bj4621). All of these mutant enzymes displayed less than 10% cytochrome c oxidase activity in vitro with reduced horse heart cyt c as the electron donor as compared with the His₆-tagged wild-type enzyme (Table IV). Visible light spectroscopy of dithionite-reduced minus air-oxidized forms of the purified enzymes from Bj4622, Bj4623, and Bj4624 revealed similar amounts of cytochromes b and c compared with the His₆-tagged wild-type enzyme, because the 551- and 561-nm peaks were present in a comparable stoichiometry (Fig. 4A). By contrast, the Bj4625 mutant apparently lacked the reducible heme B moiety because the specific peak at 561 nm was missing in the dithionite-reduced minus air-oxidized spectrum, which was even more obvious in its second derivative (Fig. 4B). To check whether the enzyme from the Bj4625 mutant still contained heme B or not, the non-covalently bound heme B was extracted and quantified by pyridine hemochrome spectroscopy according to Berry and Trumpower (36). The Bj4625 enzyme contained a similar amount (90%) of heme B compared with the wild-type His₆-tagged enzyme of Bj4621 (Table IV), indicating that its heme B moiety is present but probably cannot be reduced.

The copper content was measured by flame atomic absorption spectrometry, using purified enzyme preparations that had been treated with Chelex 100 as the wild-type enzyme. All mutants, Bj4622, Bj4623, Bj4624, and Bj4625, contained enzymes with similar stoichiometric amounts of copper as the His₆-tagged wild-type enzyme from Bj4621 (Table IV), indicating that its heme B moiety is present but probably cannot be reduced.

The corresponding purification protocol reported here, it was possible to obtain a 2–3-fold higher yield of the cbb₃-type oxidase with apparently fewer contaminating proteins. This allowed us to measure the contents of heme and copper in the enzyme. A relatively low value of 1.1 heme B molecule per oxidase complex was obtained, which might be due to some loss of heme during extraction. With respect to copper, a stoichiometry of 0.76 atoms per enzyme complex was calculated, supporting the previous assumption that the B. japonicum cbb₃-type oxidase contains one copper per enzyme complex. This copper atom is most likely part of the heme B-Cu₄ binuclear center present in subunit I, because a subclass subunit II with a binuclear Cu₄ center is missing in the cbb₃-type subclass of cytochrome c oxidases (8, 15, 16).

Subunit I of the cbb₃-type oxidases contains 12 conserved histidine residues, of which 6 can be predicted to serve as the cofactor ligands based on sequence similarities with the P. denitrificans aa₃-type oxidase whose structure is known (8). We created a complete set of fixN mutants by changing each of the conserved histidines to a smaller, neutral amino acid to test the importance of these residues in oxidase assembly and function.

All six histidines with predicted cofactor liganding functions turned out to be essential for cbb₃-type oxidase activity. We had shown previously that His-131 was essential for assembly of the oxidase subunits (18). This histidine resides on the periplasmic side of the second transmembrane helix of FixN (Fig. 1) and therefore serves most probably as a low spin heme ligand. However, since the H131A mutant oxidase is unstable, a role of this histidine in binding heme B could not be assessed directly.

Based on the amino acid sequence similarity between subunits I of heme copper oxidases, the second presumed low spin heme ligand of His-420. The H420A mutant contained a fully assembled but inactive cbb₃-type oxidase. The corresponding purified His₆-tagged mutant protein contained normal amounts of heme B and copper, suggesting that His-420 is not required for incorporation of the cofactors. However, the reduced minus oxidized difference spectrum lacked the peak at 561 nm that is indicative for reducible heme B. A similar phenotype was found in the equivalent h₃-type oxidase mutant of E. coli (44). We propose that His-420 is the second low spin heme ligand and is required for correctly positioning heme in the assembled oxidase so that electron flow can take place.

His-418 was predicted to be the high spin heme ligand. Mutation of this residue led to an assembly defect of the oxidase and, by consequence, to lack of enzymatic activity. Therefore, the role of His-418 as a cofactor ligand could not be investigated further.

The putative Cu₉ ligands His-280, His-330, and His-331 were changed to alanines, which resulted in inactive oxidases. It thus appeared as if all three ligands are necessary to hold Cu₉ in a reactive binuclear center to permit full oxidase activity. Although the H331A mutant oxidase had a defect in assembly, the H280A and H330A mutant oxidases could be purified as His₆-tagged variants and showed normal amounts of heme B and copper cofactors. This result does not contradict the assumption that these residues are Cu₉ ligands. If either His-280 or His-330 is altered, the other two putative ligands (His-330 plus His-331 or His-280 plus His-331, respectively) might be sufficient to bind copper to the oxidase during the assembly process. To ascertain the role of these two histidines

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nis, and M. Wikström, GenBank accession number U58092.
in complexing Cu_{II}, further refined spectroscopic analyses and the construction of various double mutants would be required.

Three of the six histidines that are conserved in cbb_{3}-type oxidases, but not in other classical heme copper oxidases, can be altered without any effect on oxidase formation or activity as follows: His-227 and His-246 on either side of transmembrane helix V and His-333 on the periplasmic side of transmembrane helix VII (Fig. 1).

The mutant H457A, in which the conserved histidine on the cytoplasmic side of transmembrane helix XI was altered, displayed wild-type levels of TMPD oxidase and nitrogenase activities in vivo. However, a slight defect in cytochrome c oxidase activity was observed in isolated membranes that also contained diminished amounts of cbb_{3} oxidase subunits. This suggests that the H457A exchange per se, rather than affecting the formation and activity of the oxidase, renders the FixN protein more fragile during cell fractionation and, therefore, causes some loss of the complex in membrane preparations.

His-410 resides in a periplasmic domain between the transmembrane helices IX and X and appears to be required for the formation of a stable oxidase, because the H410A mutant enzyme does not assemble in the membrane. In the P. denitrificans aa_{3}-type oxidase a histidine (His-403) in the corresponding periplasmic loop has been proposed to be part of a binding site for a redox-inactive Mg^{2+} or Mn^{2+} center (6, 7), and mutation of the corresponding His-411 in the Rhodobacter sphaeroides aa_{3}-type oxidase led to lower activity and loss of Mn^{2+} binding (21). Therefore, it is possible that the cbb_{3}-type oxidase also possesses such an additional metal center involving His-410 and that the metal contributes to assembly of the subunits.

Beyond the essential histidines discussed so far, whose function could be backed up by comparison with the cytochrome aa_{3} structure, His-316 was the sole FixN-specific, conserved histidine found to be essential for oxidase function. Cu_{II} content and visible spectroscopic features of the His_{316}-tagged H316V mutant oxidase resembled those of the corresponding His_{316}-tagged wild-type enzyme. This histidine is predicted to be localized on the cytoplasmic side of the membrane of transmembrane helix V (Fig. 1) and might be a candidate for binding and releasing protons. Two processes involving proton translocation can be expected for the cbb_{3}-type oxidase: (i) scalar proton translocation to the bimolecular center for H_{2}O formation, and (ii) vectorial proton pumping across the membrane to establish an electrochemical proton gradient, a process demonstrated for the P. denitrificans cbb_{3}-type oxidase (13). Since several amino acids that have been proposed to be involved in these translocation processes in the aa_{3}-type oxidase are not conserved in the cbb_{3}-type oxidase, the mechanisms how protons move inside and through the latter enzyme are completely unknown. Moreover, proton translocation has not been demonstrated yet for the B. japonicum FixNOQP oxidase. At present it is therefore too early to assign a role in proton translocation to His-316. Since the H316V mutant enzyme is not affected in membrane insertion, stability, and cofactor binding, it might serve as a valuable tool to analyze the coupling of proton translocation and electron transfer reactions in this type of oxidase.

By and large, the replacement of putative cofactor ligands by other amino acids provided valuable insight into the assembly process of the cbb_{3}-type oxidase. Assembled but inactive histidine mutant proteins lacking the heme B or Cu_{II} moieties have never been found. This strongly suggests that low spin and high spin heme B as well as Cu_{II} incorporations are essential for the assembly and stability of the B. japonicum cbb_{3}-type oxidase, and these processes seem to represent an early step in the biogenesis of the enzyme. At this stage of the analysis, it is not possible to predict whether heme B and copper insertions occur as sequential processes.

The results presented in this paper are fully consistent with the assumption that those six invariant histidines of the cbb_{3}-type oxidase that are common to all heme-copper oxidases are involved in cofactor binding in a similar way as it is known for the aa_{3}-type oxidase. Also, a similar organization of the transmembrane helices around the heme B and Cu_{II} cofactors can be postulated. Our mutational analysis of subunit I (FixN) reinforces the idea that the cbb_{3}-type oxidases share structural similarities with the classical heme-copper oxidases. By contrast, the role of the additional six histidines and the reason why they were conserved during evolution of almost all of the cbb_{3}-type oxidases remain obscure. Notably, after this manuscript was completed, we learned from the complete nucleotide sequence of the Helicobacter pylori genome that the sole cytochrome oxidase this bacterium has is of the cbb_{3}-type and that its subunit I lacks five of the FixN-specific conserved histidines (corresponding to His-227, His-246, His-316, His-333, and His-457 of B. japonicum FixN; see Ref. 42, GenBank accession number AE000536). It is remarkable and fully consistent with our data that among the seven remaining histidines of the H. pylori subunit I are the six canonical ones and the one that has been implicated in Mg^{2+} or Mn^{2+} binding (see above).

For future studies with the B. japonicum cbb_{3}-type oxidase, we are in need of larger quantities of the enzyme. The His_{316}-tagged subunit I, as shown in this work, has aided in the purification, and we hope that this tool will be useful in scaling up the purification procedure.

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