Histidine 131, not histidine 43, of the *Bradyrhizobium japonicum* FixN protein is exposed towards the periplasm and essential for the function of the cbb_3-type cytochrome oxidase

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Abstract In subunit I (FixN) of the *Bradyrhizobium japonicum* cbb_3-type oxidase, only five instead of the normal six strictly conserved histidines (H) could be unambiguously assigned as the putative heme or copper ligands. The ambiguity concerned H43 or H131 as the presumptive N-terminal ligands of the low-spin heme B. We report here that a H43A replacement had a wild-type phenotype, whereas the H131A mutant was defective in oxidase function and subunit assembly or stability, suggesting that H131 serves as the N-terminal low-spin heme ligand. Topological studies revealed that H131 resides on the periplasmic side of helix 2, where one of the low-spin heme ligands is normally found in conventional heme-copper oxidases.

Key words: Subunit assembly; cbb_3-type cytochrome *c* oxidase; Low-spin heme B ligand; Histidine; *Bradyrhizobium japonicum*

1. Introduction

In *Bradyrhizobium japonicum* the cbb_3-type oxidase functions as a cytochrome *c* oxidase and reduces oxygen to water under microaerobic and symbiotic conditions [1,2]. It belongs to the so-called super-family of heme-copper oxidases [3] and is encoded by the *fixNOQP* operon [1]. The purified oxidase contains at least three subunits [4]. The special structural feature of the cbb_3-type oxidase is the association of a subunit I (FixN protein) with two *c*-type cytochromes, FixO and FixP, representing subunit II and subunit III, respectively, which are anchored in the cytoplasmic membrane by their hydrophobic N-termini and which are exposed to the periplasm [1,2,4,5]. FixN is an integral membrane protein with up to 14 putative transmembrane α-helices [1,2]. It contains a low- and a high-spin heme B cofactor, the latter being associated with copper (Cu_B) in a binuclear center that is the catalytic site of oxygen reduction [3,4,6,7].

Subunit I of most of the conventional heme-copper oxidases has 12, not 14, transmembrane α-helices which are organized in a threefold symmetry [8,9]. Six strictly conserved histidines (H) are involved in liganding the heme and copper cofactors (for reviews, see [3,10,11]). All of them are located at the periplasmic face of the membrane [8]. H276, H325 and H326 (using the numbering system for the *Paracoccus denitrificans* aa_3-type cytochrome oxidase) are involved in complexing Cu_B. H411 serves as the high-spin heme ligand. The two other histidines, H94 near the N-terminus and H413 near the C-terminus bind the low-spin heme [8,9]. One of the Cu_B ligands (H325) was proposed to be involved in the reduction of oxygen and in proton pumping [8]. Multiple sequence alignments revealed that the overall amino acid sequence similarity between subunit I of the cbb_3-type oxidase and subunit I of the other heme-copper terminal oxidases is low, albeit significant (approx. 20% [1]). In the *B. japonicum* FixN protein, only five of the six canonical histidines could be readily identified as being conserved (Fig. 1A,B [1]). Three of these five histidines are thought to be the Cu_B ligands, one of them the high-spin heme B ligand and another one the C-terminal low-spin heme B ligand. Based on sequence alignments of FixN homologues from species of the *Rhizobiaceae* [1,12–14], the position of the histidine serving as the N-terminal low-spin heme ligand remained ambiguous [15]. There are two histidines at positions 43 and 131 which might fulfil this role, even more so as they both come to lie near the outer side of the membrane according to the topological model shown in Fig. 1B. Therefore, the purpose of this study was to test by site-directed mutagenesis which of these two histidines in FixN is essential for the function and assembly of the cbb_3-type oxidase. In the same context it was necessary to investigate the membrane topology especially of the N-terminal region of FixN as it differed from that of subunit I from conventional heme-copper oxidases (see Fig. 1).

2. Materials and methods

2.1. Recombinant DNA work and construction of mutants

Standard procedures were used for cloning, Southern blotting and hybridization [16]. Chromosomal DNA of *B. japonicum* was isolated as described previously [17]. DNA hybridization probes were radioactively labeled using the nick-translation technique [16]. DNA sequence analyses were performed using the chain-termination method [18] and the equipment for automated DNA sequencing (Sequencer model 370A and fluorescent dye terminators from Applied Biosystems, Foster City, CA).

The mutation H43A (in strain Bj4593) was constructed by a single-step polymerase chain reaction (PCR), using pRJ4561 (a pUCBM21-derivative carrying the 0.36-kb *Sac*II-*Nru*I fragment of *fixN*) as a template. Mutation H131A (in strain Bj4568) was obtained using a two-step PCR method, described by Landt et al. [19], with pRJ4557 (a pUCBM21-derivative carrying the 1.1-kb *Sac*II-*Sal*I fragment of *fixN*) as template. The following mutagenic primers were used: primer H13 for mutation H43A, 5′-TCGACGCGCCCTTCGCCTTCGCCGC-CGC-3′; primer H12 for mutation H131A, 5′-GCGTCCGCT-GGCCACCTCTGCCGTG-3′. The mutations were verified by sequencing. The corresponding *B. japonicum* mutants were generated by cointegration of pSUP202 derivatives into the chromosome of a Δ*fixNOQP* strain (Bj4503) as described previously [5].

The *fixN′-′lacZ* fusion was constructed by cloning the *lacZ* gene of pNM480X [20] into the *Sty*I site of *fixN*, leading to pRJ4598. For the

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Abbreviations: PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine

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fixN'-'phoA fusion, the *phoA* gene of pCH39X (pCH39 [21], with a *Xba*I linker in the *Xho*I site) was cloned into the *Pst*I site of pRJ4598, resulting in pRJ4604. The plasmids pRJ4598 and pRJ4604 were co-integrated into the chromosome of *B. japonicum* Bj4503 (Δ*fixNOQP*) as described in [5]. The correct cointegrates were confirmed by Southern blot hybridizations.

2.2. Enzymatic assays

β-Galactosidase and alkaline phosphatase activities were measured in duplicate from 100-ml samples of three independent cultures as described [22]. Tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity, cytochrome *c* oxidase activity with reduced horse heart cytochrome *c* as an electron donor and the nitrogen fixation activity were measured as described before [5].

2.3. Membrane preparation and Western blotting

B. japonicum cells were grown anaerobically in YEM medium supplemented with 10 mM KNO_3 [23] and harvested in the late exponential growth phase. Membrane fractions were isolated as described elsewhere [5]. Determination of protein concentrations, SDS-PAGE according to Laemmli [24], heme stains and Western blot analyses with antibodies against FixN, FixO, FixP and cytochrome c_1 were performed as described previously [5].

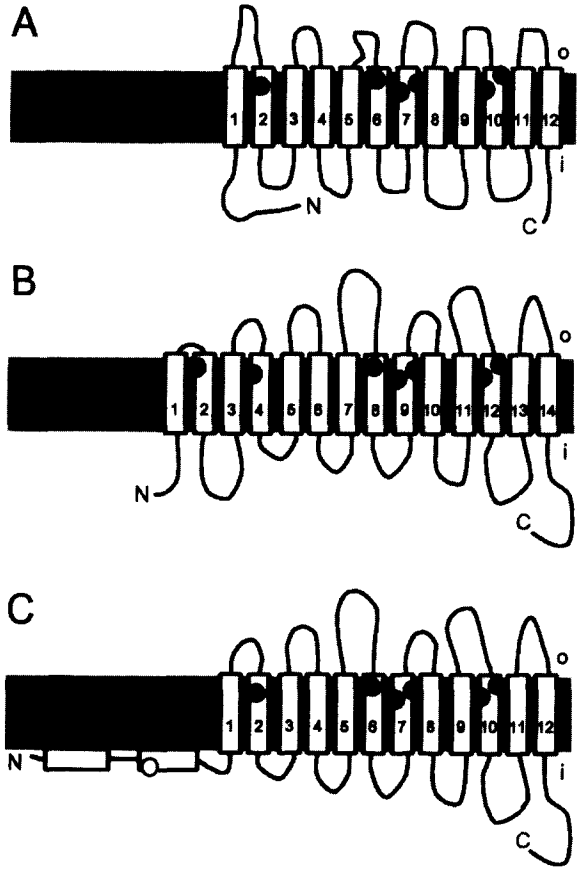

Fig. 1. Topological models of subunit I of heme-copper oxidases. A: Subunit I of the conventional heme-copper oxidases. B: Previous model of subunit I of the cbb_3-type oxidase based on domains predicted from the primary amino acid sequence. C: New topological model of subunit I of the cbb_3-type oxidase (see text). Numbered white rectangles designate the transmembrane helices. Black circles represent conserved histidines; gray circles in B designate two candidate low-spin heme B ligands and the white circle in C represents the histidine which was found not to be a heme B ligand. i, inside; o, outside.

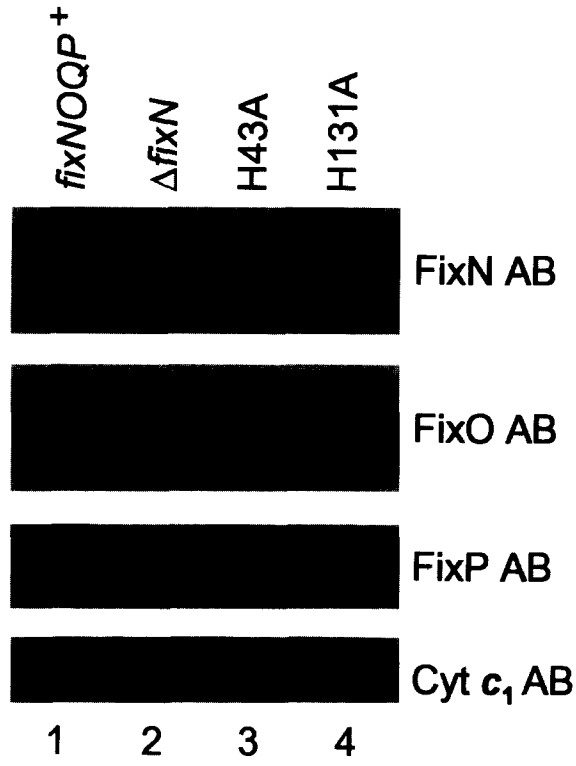

Fig. 2. Western blot analysis of membrane proteins. Four similar blots were developed using antibodies (AB) against FixN, FixO, FixP and cytochrome c_1, as indicated on the right. Membranes were prepared from anaerobically grown cells. Approximately 40 μg membrane protein was loaded in each lane. The following strains were tested: Bj4504 (*fixNOQP*$^+$ by complementation, lane 1), Bj4526 (Δ*fixN*, lane 2), Bj4593 (H43A, lane 3) and Bj4568 (H131A, lane 4).

3. Results

3.1. Influence of the H43A and H131A mutations on the presence of the subunits

In order to assess the possible role of the H43 or H131 residues in FixN as low-spin heme B ligands, these histidines were changed by site-directed mutagenesis (see Section 2) to the small, apolar amino acid alanine. The phenotype of the corresponding mutants with respect to the presence of the cbb_3 oxidase subunits was checked by Western blot analysis with antibodies specific for FixN, FixO and FixP. The presence of membrane-bound cytochrome c_1 was used as an internal control using similar amounts of proteins loaded in each lane (Fig. 2). As expected, the FixN, FixO and FixP subunits were present in the positive control stain Bj4504 (*fixNOQP*$^+$, lane 1), whereas they were absent in the Bj4526 mutant used as a negative control (Δ*fixN*, lane 2) [5]. In mutant Bj4593 (amino acid exchange H43A, lane 3) the three subunits were found in similar amounts as in the *fixNOQP*$^+$ strain. By contrast, FixN protein was absent in the H131A mutant (Bj4568, lane 4), and FixO and FixP were only weakly detectable, suggesting that the His-to-Ala replacement at this position led to a defect in assembly or stability of the oxidase subunits. Heme staining of the membrane-bound *c*-type cytochromes indicated that FixO and FixP were synthesized as holoproteins (data not shown).

Table 1
Phenotypes of *B. japonicum* wild-type and mutant strains

Strain	Mutation	TMPD oxidase activity[a]	Cytochrome *c* oxidase activity[b]	Symbiotic N_2 fixation[c]
Bj4504	*fixNOQP*$^+$	100 ± 8	100 ± 5	100 ± 8
Bj4526	Δ*fixN*	38 ± 8	8 ± 3	6 ± 1
Bj4593	H43A	116 ± 7	105 ± 1	142 ± 13
Bj4568	H131A	43 ± 10	19 ± 9	16 ± 2

[a]At least three measurements were made with cultures grown anaerobically to an A_{550} of 0.7.
[b]At least two measurements were made with membrane fractions of two independent anaerobic cultures.
[c]Acetylene reduction in soybean roots collected from at least five plants per strain.
All values are given in percent and represent the means ± standard deviation.

3.2. *Consequences of the H43A and H131A replacements for anaerobic respiration and symbiotic nitrogen fixation*

The consequences of the His-to-Ala exchanges for respiration were tested using TMPD as an artificial electron donor to measure oxygen consumption as a function of cytochrome *c*-dependent oxidase activity from anaerobically grown cells (Table 1). Strain Bj4593 (H43A) had a similar TMPD oxidase activity as the complemented strain Bj4504 (*fixNOQP*$^+$). The H131A and Δ*fixN* mutants showed approximately 40% residual oxidase activity, indicating that the cbb_3-type oxidase is responsible for more than half of the total TMPD oxidase activity present in wild-type cells grown under anaerobic conditions. This relatively high residual activity may be caused by an alternative cytochrome *c* oxidase [25] or by enzymes of the denitrification pathway [26,27]. In addition, we measured cytochrome *c* oxidase activity in vitro with reduced horse heart cytochrome *c* as electron donor and obtained similar results (Table 1); however, the H131A and the Δ*fixN* mutants displayed a more severe reduction of cytochrome *c* oxidase activity as compared with the TMPD oxidase activity.

As shown previously [1], the cbb_3-type oxidase is essential for symbiotic nitrogen fixation because it supports energy conservation under the extremely microaerobic conditions that prevail in soybean root nodules. Therefore, we analyzed the ability of the mutants to fix nitrogen in symbiosis (Fix phenotype; Table 1). While the *fixNOQP*$^+$ strain and the H43A mutant were Fix$^+$, the Δ*fixN* and H131A mutants showed only 6–16% residual Fix activity. Nodules of plants infected with the two latter mutant strains were greenish inside, indicating the absence of functional leghemoglobin. The results suggest that the histidine at position 131 of FixN is essential for the cbb_3-type oxidase to energetically support nitrogen fixation in root nodules, whereas the histidine at position 43 is not essential.

3.3. *Membrane topology of the N-terminal segment of FixN*

Since the non-essential H43 is apparently not involved in liganding the low-spin heme B, it is no longer compelling that this residue is located on the periplasmic side of the cytoplasmic membrane. Considering that 14 transmembrane α-helices can be predicted for FixN [2], whereas subunit I of most bacterial heme-copper oxidases contain only 12 α-helices [3], we attempted to characterize the topology of the N-terminal domains. Translational β-galactosidase and alkaline phosphatase fusions were created at the 35th amino acid of *fixN*. This amino acid was initially assumed to be located in the first periplasmic loop between the predicted helices 1 and 2 (Fig. 1B). The β-galactosidase and alkaline phosphatase activities were measured from microaerobically grown cells, as the *fixNOQP* operon is maximally expressed under this condition [5]. As shown in the Table 2, only the β-galactosidase gene fusion was strongly expressed. This does not support the existence of a 'periplasmic loop' between the hydrophobic domains 1 and 2 but rather suggests a cytoplasmic location for this segment. Furthermore, the two N-terminal hydrophobic stretches are probably not transmembranous domains (Fig. 1C).

4. Discussion

The FixN-type subunit I of cbb_3-type heme-copper oxidases differs from the classical subunit I of other heme-copper oxidases by having two additional hydrophobic domains at the N-terminus, resulting in a total of 14 rather than 12 putative transmembrane helices. At each of the previously presumed periplasmic ends of helices 2 and 4, a histidine is present that might serve as low-spin heme B ligand. However, the first of these histidines is not conserved in the CcoN proteins of *Rhodobacter capsulatus* and *P. denitrificans* which are homologs of FixN [28,29]. In the present work we addressed two questions: (i) Which of the two histidines might serve as the heme B ligand? (ii) Are the first two hydrophobic segments of FixN indeed transmembrane helices?

To identify the hitherto questionable N-terminal heme B ligand, both candidate histidines (H43 and H131) were changed to alanine by site-directed mutagenesis. The H43A mutation led to a wild-type phenotype with regard to assembly and function of the cbb_3-type oxidase. Thus, H43 cannot be the low-spin heme B ligand, which is corroborated by the fact that it is not strictly conserved in all FixN-like proteins known

Table 2
Membrane topology analysis of the *B. japonicum* FixN protein

Strain	Relevant genotype	β-Galactosidase activity	Alkaline phosphatase activity
Bj4504	*fixNOQP*$^+$	61 ± 14	60 ± 4
Bj4598	*fixN'-'lacZ*	218 ± 19	nd
Bj4604	*fixN'-'phoA*	nd	71 ± 20

The *B. japonicum* mutant strains were grown microaerobically. Values (Miller units) are the means ± standard deviation of at least three measurements from three independent cultures grown to an $A_{550} = \sim 0.3$; nd, not determined.

[28,29]. By contrast, the H131A mutation led to a functionally inactive enzyme, proving the importance of this residue. Furthermore, the H131A mutant had a defect in assembly or stability of the cbb_3 oxidase complex, as shown by Western blot analyses. Unfortunately, we are currently unable to distinguish between oxidase complex assembly and subunit stability. Our result can therefore be interpreted in two ways: either the incorporation of the low-spin heme is a prerequisite for the formation of a stable FixN protein, which in turn is known to be essential for complex formation [5], or the His-to-Ala mutation at position 131 causes an alteration in the tertiary structure of the protein that subsequently leads to an unstable polypeptide. In contrast to the situation in the *ΔfixN* mutant in which all three oxidase subunits were completely missing, the H131A mutant contains small, but clearly detectable amounts of FixO and FixP proteins in membrane preparations (Fig. 2). However, the fully assembled cbb_3-type oxidase is not present in sufficient amounts in this mutant to allow its spectroscopic characterization.

As H43 turned out to be a non-essential residue of FixN, there was no need to postulate its location on the periplasmic side of the lipid bilayer. In fact, with the help of β-galactosidase and alkaline phosphatase fused in close proximity to H43, we could show that the intervening loop between hydrophobic segments 1 and 2 resides in the cytoplasm. This led to a new model for the topology of the N-terminus of the FixN subunit (Fig. 1C), which is in perfect agreement with our results from the histidine mutants. The functionally important residue H131 is now located at the end of a newly defined helix 2 (formerly helix 4) resembling the situation in classical subunits I of heme-copper oxidases (Fig. 1A). Moreover, the crystal structure of the *P. denitrificans* aa_3-type oxidase has revealed a threefold symmetry based on 12 transmembrane domains [8], which now can also be postulated for the FixN-type subunit of cbb_3 oxidases.

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