Heme C incorporation into the *c*-type cytochromes FixO and FixP is essential for assembly of the *Bradyrhizobium japonicum* cbb_3-type oxidase

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Abstract The monoheme and diheme *c*-type cytochromes FixO and FixP are two of the subunits of the respiratory cbb_3-type oxidase of *Bradyrhizobium japonicum*. The cysteines of the respective heme C binding motifs CXXCH were changed to serines by site-directed mutagenesis, which led to inactive oxidases in all mutants. Western blot analyses showed that an intact heme binding site in the FixO polypeptide is a prerequisite not only for the synthesis of holo-FixO protein but also for the formation of the entire cbb_3-type oxidase complex. Both heme binding sites of FixP were essential for maturation and assembly of this subunit. It was not possible to create stable FixP variants that contained only one heme C.

Key words: Assembly of membrane proteins; Cytochrome oxidase; Heme C binding site; Monoheme cytochrome *c*; Diheme cytochrome *c*

1. Introduction

The *Bradyrhizobium japonicum* cbb_3-type oxidase is a high-affinity cytochrome *c* oxidase that terminates the microaerobic respiratory chain in this organism [1–3]. It belongs to the superfamily of heme-copper oxidases and is encoded by the *fixNOQP* operon [1,3,4]. The cbb_3-type oxidase differs from the common heme-copper oxidases by the composition of its subunits and cofactors. While it possesses a classical subunit I, the FixN protein, with a Cu_B cofactor and a low- and a high-spin heme, here of the B-type, it lacks a Cu_A-containing subunit II [5,6]. Instead, two *c*-type cytochromes are present as membrane-anchored, periplasmically oriented subunits II (FixO) and III (FixP). FixO is a 28-kDa protein with one, and FixP a 32-kDa protein with two heme-binding-site motifs (CXXCH), suggesting that FixO is a monoheme and FixP a diheme cytochrome *c* [1]. The *B. japonicum* cbb_3-type oxidase appears to contain a fourth, small subunit (FixQ) which, however, is not essential (Fig. 1; [1,7]).

The posttranslational maturation pathway which cells use to convert apocytochrome to holocytochrome *c* includes a heme ligation step [8]. A cytochrome *c* heme lyase is thought to catalyze the formation of two thioether bonds between the vinyl side chains of heme and the cysteine thiols of the heme binding site in the apoprotein. *B. japonicum* mutants affected in genes for cytochrome *c* biogenesis lack all of the cellular *c*-type cytochromes, including FixO and FixP [9,10]. Thus, heme C binding appears to be an essential step also in the maturation of the cbb_3-type oxidase complex. In a previous report we have shown the role of the individual subunits of the cbb_3-type oxidase in assembly and function of the enzyme [7]. A model was proposed in which FixN and FixO form a core complex which allows the subsequent association with FixP to form an active enzyme. Here, we address the question of whether or not heme binding is a prerequisite for assembly and stability of FixO and FixP polypeptides in the membrane. In particular, we were interested to know whether a FixP subunit lacking just one of the two heme C binding sites might be detectable in the membrane complex as a monoheme cytochrome *c*.

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Abbreviations: PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Recombinant DNA work and construction of mutants

Standard procedures were used for cloning, Southern blotting and hybridization [11]. Chromosomal DNA of *B. japonicum* was isolated as described previously [12]. DNA hybridization probes were radioactively labeled using the nick-translation technique [11]. DNA sequence analyses were performed using the chain-termination method [13] and the equipment for automated DNA sequencing (Sequencer model 370A and fluorescent dye terminators from Applied Biosystems, Foster City, CA).

The mutations in the *fixO* gene (C68S/C71S; strain Bj4594) and in the *fixP* gene (C122S/C125S and C219S/C222S; strains Bj4583 and Bj4582, respectively) were introduced by polymerase chain reaction (PCR)-based mutagenesis as described previously [7] using the following mutagenic primers: primer S4 for mutations C68S/C71S (′5-CGTCCGCGAAGGCAGCTATCTCAGC-3′), primer S5 for mutations C122S/C125S (′5-GAATTCCGCGCCGTCCCACGG-3′) and primer S2 for mutations C219C/C222S (′5-TCCGTCGCCTCCCACGGCGATGGC-3′). To create a mutant FixP protein in which both heme binding sites were altered (strain Bj4584), the 5′- and 3′-halves of the corresponding mutant *fixP* genes were fused by cloning. Fig. 1 depicts the positions and mutational alterations of the heme C binding sites. All mutant constructs were sequenced to confirm the mutations and then subcloned into pSUP202 pol6K [7]. Details of the cloning strategies can be obtained from the authors upon request. Like the wild-type construct, pRJ4504 [7], the plasmids pRJ4582, pRJ4583, pRJ4584 and pRJ4594 carry the 4.7-kb *Kpn*I-*Xba*I fragment (*orf277*, *orf141*, *fixNOQP*) in pSUP202pol6K, but contain the C219S/C222S, the C122S/C125S and the C219S/C222S plus C122S/C125S mutations in FixP, or the mutations C68S/C71S in FixO, respectively. They were cointegrated into the chromosome of *B. japonicum* Bj4503 (Δ*fixNOQP*) as described previously [7]. The cointegrates were verified by Southern blot hybridization.

2.2. Enzymatic assays

Cytochrome *c* oxidase activity with reduced horse heart cytochrome *c* as the electron donor was performed as described [7].

2.3. Membrane preparation and Western blotting

B. japonicum cells were grown anaerobically in YEM medium supplemented with 10 mM KNO_3 [14] and harvested in the late exponential growth phase. Membrane fractions were isolated as described elsewhere [7]. Protein concentration, SDS-PAGE according to Laemmli [15], heme stains and Western blot analyses with antibodies

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against FixN, FixO, FixP and cytochrome c_1, were performed as described previously [7].

2.4. Purification and mass spectrometry of FixP

Six histidines were fused to the C terminus of FixN by genetic means. The *fixN*$_{His6}$*OQP* operon containing the additional histidine codons was integrated into the chromosome of *B. japonicum* Bj4503 (Δ*fixNOQP*) as described above. The construction of this insertion mutant and the purification of the engineered oxidase will be described elsewhere (Zufferey, R., Arslan, E., Thöny-Meyer, L. and Hennecke, H., unpublished results). The purified, His-tagged oxidase was precipitated with 10% trichloroacetic acid and the pellet was resuspended in 70% formic acid. The molecular mass of FixP was determined by electrospray mass spectrometry.

3. Results

3.1. An intact heme binding site in FixO is required for assembly or stability of the cbb$_3$-type oxidase

The presence of the FixO protein had previously been shown to be essential for assembly of the *cbb*$_3$-type oxidase [7]. We now tested whether the formation of the enzyme complex required an intact heme binding site in FixO. Both cysteines of the heme C binding motif CYLCH in FixO were changed to serines by site-directed mutagenesis (Fig. 1). The effect of the C68S/C71S exchanges on the enzymatic function was studied using the cytochrome *c* oxidase assay with membranes from anaerobically grown *B. japonicum* cells. Like the Δ*fixO* in-frame deletion mutant (Bj4518), the C68S/C71S mutant caused a more than 90% decrease of cytochrome *c* oxidase activity as compared with the wild-type control, strain Bj4504 (Table 1).

Western blots of membrane proteins isolated from anaerobically grown cells were then probed with antibodies specific to FixN, FixO, FixP and cytochrome c_1 (Fig. 2A). Comparable levels of membrane-bound cytochrome c_1 (control) were found in membranes of all strains tested, thus demonstrating that similar amounts of protein were loaded in each lane (Fig. 2A, bottom panel). The FixN, FixO and FixP subunits were present in the *fixNOQP*$^+$ strain (Bj4504, lane 2), whereas all three of them were absent not only in the Δ*fixO* in-frame deletion mutant (Bj4518, lane 1) [7], but also in the C68S/C71S mutant (Bj4594, lane 3). This result explains the loss of enzymatic activity observed in the mutant strain (Table 1).

3.2. FixP is a diheme cytochrome c

FixP has been predicted to be a diheme cytochrome for two reasons: (i) there are two heme C consensus binding sites CXXCH in its amino acid sequence ([1]; see also Fig. 1), and (ii) heme staining of the FixP protein in a denaturing polyacrylamide gel consistently gave a stronger signal than with the monoheme cytochrome FixO [2]. To determine the number of covalently bound heme cofactors, we analyzed the FixP holoprotein by mass spectrometry. The theoretical molecular mass of the FixP apoprotein is 31 023 Da, those of a monoheme and diheme FixP would be 31 642 and 32 261 Da, respectively [1]. The latter two values must be corrected by deduction of 131 Da, because the N-terminal methionine is missing in the mature FixP protein [2], resulting in 31 511 and 32 130 Da as the predicted molecular masses for mono- and diheme FixP. The *cbb*$_3$-type oxidase was purified as an enzyme complex with a His$_6$-tagged FixN subunit. The molecular mass of the FixP subunit was determined in two independent experiments and was found to be 32 218 ± 4.6 Da in the first and 32 225 ± 102 Da in the second experiment. The data are in good agreement with the assumption that FixP is a diheme cytochrome *c*.

3.3. Both heme binding sites of FixP are essential for oxidase assembly in the membrane

Next, we asked whether attachment of the two heme cofactors to FixP apoprotein follows a sequential pathway. If such a process occurs, one might expect to find a monoheme intermediate in at least one of the single-heme-binding-site mutants, provided that such mutant variants are stable enough to be detectable in membranes of steady state cells. The pairs of cysteines in each of the two CXXCH consensus motifs of FixP were changed to serines by site-directed mutagenesis (strains Bj4582 and Bj4583; Fig. 1). In addition, a mutant in which all four cysteines of both heme binding sites were replaced by serines (strain Bj4584; Fig. 1) was constructed.

The functional consequence of these alterations was tested with the cytochrome *c* oxidase assay of isolated membranes from anaerobically grown cells (Table 1). Strain Bj3618 (*fixP*::*aphII*; negative control) showed a 85% decreased cytochrome *c* oxidase activity as compared with the wild-type control, strain Bj4504. Like strain Bj3618, the mutants Bj4582, Bj4583 and Bj4584 caused an approximately 85% decrease of cytochrome *c* oxidase activity. Hence, both heme binding sites are required for the activity of the *cbb*$_3$-type oxidase.

Western blot analyses with membrane proteins of anaerobically grown cells was performed to check if the mutated FixP proteins can be assembled in the membrane. Fig. 2B shows the results obtained in immunoblots probed with FixN-, FixO- and FixP-specific antibodies. One blot was incubated with anti-cytochrome c_1 serum to control the similar amounts of protein loaded for each strain (bottom panel). As expected, strain Bj4504 (*fixNOQP*$^+$, lane 2) possesses the FixN, FixO and FixP proteins in its membrane. Bj3618 (*fixP*::*aphII*, lane 1) lacks only FixP, whereas FixN and FixO are present [1,7]. Likewise, FixN and FixO were also detected in each of the

Table 1
Cytochrome *c* oxidase activity of *B. japonicum* mutants strains

B. japonicum strain	Relevant genotype	Cytochrome *c* oxidase activity[a]
Bj4504	*fixNOQP*$^+$	100 ± 2.5
Bj4518	Δ*fixO*	9.9 ± 0.4
Bj4594	C68S/C71S in *fixO*	5.0 ± 0.7
Bj3618	*fixP*::*aphII*	15.4 ± 4.3
Bj4582	C219S/C222S in *fixP*	14.8 ± 1.6
Bj4583	C122S/C125S in *fixP*	14.5 ± 3.1
Bj4584	C122S/C125 plus C219S/C222S in *fixP*	16.4 ± 1.7

[a]At least two measurements were made with membrane fractions of two independent, anaerobically grown cultures. All values are given in percent and represent the means ± standard deviation. 100% cytochrome *c* oxidase activity equals 1.1 μmol cytochrome *c* min^{-1} per mg protein.

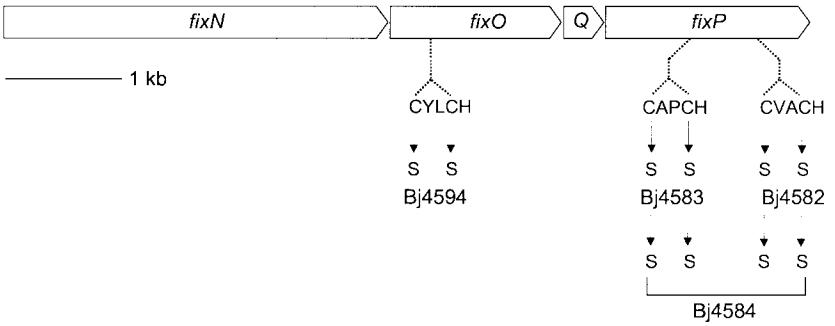

Fig. 1. Heme binding site mutants of FixO and FixP. The *fixNOQP* operon is depicted on top. Below the gene map, heme C binding motifs in the derived polypeptides and the Cys-to-Ser amino acid exchanges in the mutant proteins are shown together with the designations of *B. japonicum* strains.

new mutant strains, Bj4582 (C219S/C222S, lane 3), Bj4583 (C122S/C125S, lane 4) and Bj4584 (C122S/C125S plus C219S/C222S, lane 5), although in slightly decreased amounts as compared with Bj4504. The FixP protein, however, was absent not only in Bj3618 [1,7] but also in mutants Bj4582, Bj4583 and Bj4584, suggesting that both heme binding sites are required for assembly or stability of FixP. Heme stains of the membrane fractions showed that FixO was present as a holoprotein in all *fixP* mutant strains (data not shown).

4. Discussion

Cofactor incorporation is an important step in the biogenesis of holoproteins and may occur in a defined pathway, as it has been proposed for maturation of *c*-type cytochromes [8]. Mutation of the critical amino acids in heme C binding sites can block cofactor linkage and leads to unstable apoproteins which are degraded rapidly [8,16]. In the present work, we show that heme insertion into both the FixO and FixP proteins is necessary for formation of the cbb_3-oxidase complex.

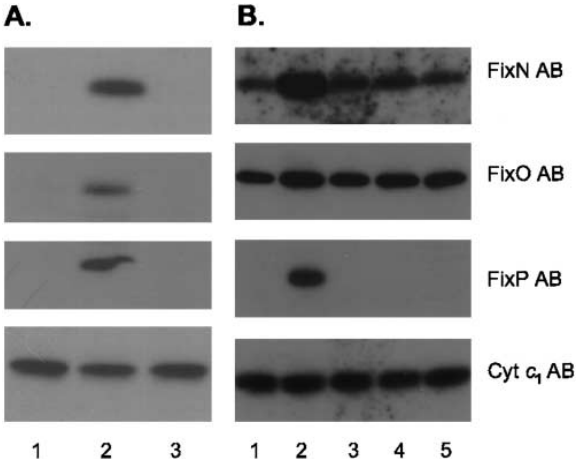

Fig. 2. Western blot analysis of membrane proteins. Four similar blots were probed with antibodies specific to FixN, FixO, FixP and cytochrome c_1, as indicated on the right. Membranes were prepared from anaerobically grown cells. Approximately 20 μg membrane protein was loaded in each lane. (A) Analysis of the heme C binding site mutant of FixO. The following strains were tested: Bj4518 (Δ*fixO*, lane 1), Bj4504 (*fixNOQP*$^+$ by complementation, lane 2) and Bj4594 (C68S/C71S in *fixO*, lane 3). (B) Analysis of the heme C binding site mutants of FixP. The following strains were tested: Bj3618 (*fixP*::*aphII*, lane 1), Bj4504 (*fixNOQP*$^+$, lane 2), Bj4582 (C219S/C222S, lane 3), Bj4583 (C122S/C125S, lane 4) and Bj4584 (C122S/C125S plus C219S/C222S, lane 5).

The C68S/C71S mutant in FixO displayed a strongly reduced cytochrome *c* oxidase activity, an effect that could be explained by the lack of all cbb_3-type oxidase subunits. We conclude that attachment of heme C to the FixO polypeptide is an early step in the biogenesis of the cbb_3-type oxidase and occurs before, or concomitantly with, the formation of the FixNO subcomplex because the latter contains FixO as a holoprotein [7]. The requirement of covalent heme binding for cytochrome assembly has also been observed in other respiratory membrane protein complexes, such as the bc_1 complex of *B. japonicum* and *Rhodobacter sphaeroides*, and the b_6f-complex of *Chlamydomonas reinhardtii* [16–18].

From its amino acid sequence, FixP has been predicted to be a diheme cytochrome *c*. To test this assertion, we performed mass spectrometry with the holoprotein. In two independent experiments, molecular masses of 32218 and 32225 Da were obtained which is consistent with FixP being a diheme cytochrome *c*. These values deviate by +88 and +95 Da, respectively, from the theoretical value of 32130 Da. However, such deviations are not uncommon with membrane proteins, and in the case of FixP they may have arisen due to the hydrophobicity of the N-terminal membrane anchor of FixP (P. James, personal communication).

The fact that FixP was now unambiguously shown to be a diheme cytochrome *c* corroborates previous results of biochemical analyses done with purified cbb_3-type oxidases: a heme B to heme C ratio of 2 to 3 was calculated for the *R. sphaeroides* cbb_3-type oxidase, and a similar value was obtained for the *B. japonicum* enzyme [2,5]. Considering that two hemes B are present in the catalytic subunit I, and that two subunits of the cbb_3-type oxidase are cytochromes *c* [5,6], one of them being a diheme cytochrome *c*, supports the idea that the two different cytochromes *c* are present in a 1:1 stoichiometry.

It has been postulated that the two heme C domains of FixP have evolved by tandem duplication of a gene for a low-molecular-weight monoheme cytochrome *c* [1]. Therefore, we were interested to see whether monoheme variants of the diheme cytochrome *c* (FixP) might be isolated from mutants in which only a single heme binding site was altered. The present work shows that blocking the covalent binding of heme to one or the other cytochrome *c* domain prevents the formation of a stable FixP protein. It is therefore possible that each of the two bound heme groups functions as a nucleation

site for folding of a stable, three-dimensional structure. It seems as if the heme C-containing domains cannot stabilize the protein independently of each other. Since we failed to detect variants of FixP containing only one heme cofactor, a potential order of the two heme attachment events could not be uncovered.

It is important to note that the lack of apoprotein stability in the FixO and FixP heme binding site mutants cannot be explained simply by the Cys-to-Ser exchanges as the sole cause for destabilization of the polypeptide. Other types of mutations known to block heme C attachment to cytochrome polypeptides by affecting enzymes of the cytochrome *c* maturation machinery have also led to unstable FixO and FixP proteins [8,9]. In those cases the FixO and FixP polypeptides did not carry amino acid substitutions. We conclude that covalent heme binding per se stabilizes the *c*-type cytochrome, and in the cases of FixO and FixP, this is a prerequisite for assembly of the cbb_3-type oxidase complex.

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