Isolation, Analysis, and Deletion of the Gene Coding for Subunit IV of Cytochrome c Oxidase in Paracoccus denitrificans

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The gene coding for subunit IV of the cytochrome c oxidase in Paracoccus denitrificans has been cloned and sequenced. The derived amino acid sequence shows no significant homology to any known protein. Gene deletion has no consequences for the integrity of the complex and its spectral and enzymatic properties. Complementation of the deletion mutant in trans results in expression of subunit IV; sequence analysis of the 5' non-coding region leads to the identification of a putative promoter sequence.

Cytochrome c oxidase (cytochrome aa$_3$; E.C.1.9.3.1) is one of the terminal oxidases in Paracoccus denitrificans (for reviews, see Refs. 1-3). Subunit I contains three redox active centers, heme a and the binuclear site (heme a$_3$Cu$_b$). Cu$_a$, the entry point for electrons from cytochrome c, is located in subunit II which represents the predominant binding site for this electron donor. The electrons are transferred from Cu$_a$ to heme a and then to the binuclear site where the oxygen reduction takes place. The function of subunit III is still obscure. All three subunits show strong homologies to the mitochondrialy coded subunits of the eukaryotic enzyme.

Several years ago, Haltia discovered a small polypeptide copurifying with the oxidase, which was partially sequenced (4, 5). A fourth component was found in the crystalline oxidase as well (6), and this small subunit could be characterized from the x-ray structure (7) as a polypeptide consisting of a single transmembrane helix and an N terminus protruding into the cytoplasmic space.

Here we present the isolation and analysis of the gene coding for this fourth subunit. In addition, the purified 3-subunit enzyme complex resulting from a deletion of the gene is characterized. Furthermore, evidence for a putative promoter sequence is shown.

MATERIALS AND METHODS

Synthesis of Oligonucleotide Probes—The N-terminal sequence of the putative subunit IV (4) was taken to synthesize two degenerate oligonucleotides: primer A4U (5'-GCRAGCCGGTCYTGGT-3') and primer A4D (5'-CAYCAAGAWATCAGSGA-3'), the latter one as the reverse complement.

DNA Manipulations—General cloning techniques and DNA manipulations were performed essentially as described in Ref. 8. Genomic DNA was obtained from P. denitrificans 1222 as described earlier (9). Polymerase chain reaction was used to amplify P. denitrificans genomic DNA with the primers A4D and A4U with an annealing temperature of 55°C. The sequence of the 65-bp fragment obtained by polymerase chain reaction was used to synthesize a 30-mer oligonucleotide which was labeled with a dCTP/ligG(11)-dUTP-tail for Southern and colony hybridization, which were performed as described by the manufacturer (Boehringer Mannheim) with a hybridization temperature of 42°C. A 1.9-kbp EcoRI/SphI and later a 3.5-kbp SstII/SalI fragment were isolated from a partial gene library derived from genomic digests with the respective enzymes. The second fragment was then cloned into pUC18 as a HindIII/SalI fragment (pHW5; see Fig. 2). Subcloning of various fragments, exon/mung digestion, and synthesis of specific primers were applied for sequence determination, using double-stranded DNA and T7 polymerase with 7-deaza-dGTP as described by the manufacturer (Pharmacia), according to the dideoxynucleotide chain termination method of Sanger (10).

Construction of Deletion Mutants and Complementation—The 815-bp EcoRI/Ascl fragment from pHW5 (see Fig. 2) was replaced by the 1.1-kb kanamycin resistance gene from pHP4511 (11) resulting in pHW14. The HindIII/SalI fragment of pHW15 was then ligated blunt-end into the Smal site of the suicide vector pHVS1 (12) leading to pHW15 which was mated into PD1222. Homologous recombinants were selected for kanamycin resistance and loss of β-galactosidase activity. Two representative strains HW11/24 and 55 (see Fig. 3A) were characterized; for all subsequent steps, HW11/24 was used and referred to as HW21.

Complementation Constructs—The promoterless broad host range vector pBR326 was used to complement HW21 with different fragments for promoter probe studies. The 683-bp PoulI fragment and the 1.7-kbp EcoRI/SalI fragment of pHW5 were ligated into pRG, resulting in pHW21 and pHW22, respectively (see Fig. 2). These were used to complement HW21 leading to HWK21 and HWK22.

Strains, Growth Conditions, and Enzyme Preparation—P. denitrificans strain PD1222 (13) and the deletion strain HW21 (see above) were grown on TY medium for DNA isolation or on succinate medium (14) for enzyme isolation, including kanamycin (50 µg/ml), where appropriate. Complemented strains were grown in the presence of streptomycin sulfate (25 µg/ml). Membrane isolation, enzyme purification, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and cytochrome difference spectra were performed as described in Ref. 15. Western blots were performed with monoclonal antibodies directed against subunit IV (6).

Ligand Binding Spectra with Cyanide and Carbon Monoxide—All spectra were recorded on an Uvikon 941 (Kontron Instruments) between 380 and 650 nm. Difference spectra of cytochrome a and cytochrome a$_3$ were performed as follows: 10 µl of a 10 mM potassium ferricyanide solution were added to 700 µl of a 3 mM oxidase solution. The sample was divided between two cuvettes. The sample cuvette was incubated with 20 µl of 350 mM KCN for 2 h. All volume changes were balanced with buffer in the reference cuvette, and absolute spectra were recorded. In the next step, N,N,N',N'-tetramethyl-1,4-phenylenediamine and ascorbate were added to sample cuvette to a final concentration of 60 µM and 2 mM, respectively. The reference was reduced with dithionite. Again absolute spectra were measured, and difference spectra calculated according to Ref. 16. Carbon monoxide difference spectra were obtained by subtracting the dithionite-reduced from the (reduced + CO) sample spectrum.

Catalytic Activity—The spectrophotometric assay was performed at 25°C with reduced horse heart cytochrome c at 20 µM as described before (15).

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1 The abbreviations used are: bp, base pair(s); kb, kilobase pair(s).

2 O. Richter, unpublished data.

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RESULTS

Sequence Analysis—Polymerase chain reaction with two degenerate primers derived from the N terminus of the putative fourth subunit lead to the isolation of a 65-bp fragment yielding an amino acid sequence identical to the published N terminus (5). This sequence was taken to synthesize a labeled 30-mer oligonucleotide which hybridized to a 1.9-kb EcoRI/SphI and a 3.8-kb SacII/SalI fragment from two partial gene libraries. Sequence analysis of both strands (Figs. 1 and 2) lead to the identification of the gene encoding subunit IV, the ctaH locus.

The open reading frame codes for a polypeptide of 50 amino acids (Fig. 1) while the mature protein lacks the N-terminal methionine (4, 5). A molecular mass of 5364 daltons is calculated for the mature form, which is in good agreement with its migration behavior on SDS gels (see Fig. 3A).

Construction of the Mutant Deleted in Subunit IV and Characterization of the Mutant Oxidase Complex—In the cloned 3.8-kb HindIII/SalI fragment, the ctaH gene plus 170 bp of the upstream and 380 bp of the downstream sequence were replaced by the kanamycin resistance gene, offering flanking regions of 2.1 and 0.95 kb for homologous recombination. After conjugation into PD1222, strains were selected for resistance to kanamycin and for loss of β-galactosidase activity. Membranes of two putative deletion strains were tested for the loss subunit IV using monoclonal antibodies (Fig. 3A). Subunit IV is not expressed in either of the deletion mutants HWΔ1/24 or 55.

Deletion of the fourth subunit does not affect the assembly of the remaining 3 larger subunits. An intact 3-subunit enzyme complex can be isolated from HWΔ1. On SDS gels all 3 subunits are visualized with their normal migration behavior (not shown). The enzyme was then characterized further to check whether the loss of subunit IV has any consequences on the enzymatic properties of the oxidase. Measuring the catalytic activity revealed no differences for the turnover numbers for the 3- and 4-subunit (wild type) enzymes. The turnover numbers at 20 μM cytochrome c are 418 s⁻¹ for wild type and 404 s⁻¹ for HWΔ1. Any direct influence of subunit IV on proton pumping can be excluded as well since it was shown that the wild type and a 2-subunit complex (lacking subunits III and IV) exhibit similar energy-transducing capabilities (17). Spectral analysis of ligand-bound oxidase, using cyanide or carbon monoxide, was carried out to look for changes in the heme surroundings. Fig. 4 presents the results of the cyanide spectra. No differences are observed for the 4-subunit and 3-subunit

![DNA and derived amino acid sequence](image1)

![Restriction map of the cloned HindIII/SalI fragment comprising the ctaH gene](image2)

**Fig. 1.** DNA and derived amino acid sequence of the ctaH locus coding for subunit IV of cytochrome c oxidase of P. denitrificans. The mature protein lacks the first methionine. The putative -35 and -10 promoter sequences are underlined, and the putative ribosome-binding site is boxed.

**Fig. 2.** Restriction map of the cloned HindIII/SalI fragment comprising the ctaH gene. A, wild type gene locus as cloned insert (pHW5). B, gene deletion construct obtained by replacing the EcoRI/AciI fragment with the kanamycin resistance gene, indicated by dotted lines. C, PvuII and EcoRI/SalI fragments used for complementation, yielding strains HWK21 and HWK22 (see Fig. 3B and "Materials and Methods"). Arrow, direction of transcription; MCS, multiple cloning site.
Paracoccus Subunit IV

The gene *ctaH* encoding subunit IV of the *P. denitrificans* cytochrome *c* oxidase was isolated and analyzed. Fifty amino acids were derived from the DNA sequence but the N-terminal methionine does not appear in the mature protein. According to the crystal structure, residues 16–47 form a transmembrane helix (7). Surprisingly, a lysine residue is found within this helical region, with its side chain protruding into the hydrophobic membrane phase. In a sequence comparison, no similarity with other known subunits of bacterial quinol and cytochrome *c* oxidases was found, nor any significant homology to any other known protein.

Deletion of the fourth subunit leads to the isolation of a 3-subunit enzyme complex which is obviously not affected by the loss in its functional properties. The catalytic activity is the same for wild type and the 3-subunit enzyme, indicating that subunit IV has no influence on the electron transfer reactions. In addition, both heme centers are left intact as judged from cyanide and carbon monoxide spectra which reveal no difference to wild type. The isolation of a functional 2-subunit complex from wild type membranes, leaving the two smallest subunits behind (17, 18), is additional evidence that the C-terminal two-thirds of this subunit are required for the functional expression of the quinol oxidase. Recently, a projection map obtained from two-dimensional crystalline arrays of cytochrome *bo* 3 was fitted to the *Paracoccus* structure, showing that subunit IV of cytochrome *bo* 3 basically maintains the same position as subunit IV of cytochrome *aa* 3.

In contrast to previous assumptions (7), subunit IV is slightly shorter (49 instead of 56 amino acids). The N-terminal half of this subunit makes extensive contacts with residues in subunits I and III, while no protein contacts are seen for the upper half of the transmembrane helix, pointing to the periplasm; in this region a tightly bound phospholipid molecule is in close vicinity to all four subunits and establishes a number of contacts with subunits I, III, and IV. This nicely explains the fact that, upon genetic deletion or *in vitro* removal of subunit III, subunit IV is lost along with subunit III. An interesting

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**FIG. 3.** Western blot analysis of membranes (40 µg) of different *P. denitrificans* strains, using monoclonal antibodies directed against subunit IV; for details see “Materials and Methods.” A: lane 1, wild type strain PD1222; lanes 2 and 3, deletion strains HWΔ1/24 and 55. Arrows, *M* of marker proteins. B, two complemented mutants, HWK21 and HWK22, lanes 1 and 2, respectively.

**FIG. 4.** Spectral separation of the heme a and a₃ contribution from cyanide difference spectra (16) for the isolated 4-subunit wild type (—) and 3-subunit mutant complex (----); enzyme concentration 3 µM. A, heme a spectra. B, heme a₃ spectra.

\[ \text{M. Saraste, personal communication.} \]
question is whether subunit IV is under the control of its own promoter, or part of a larger transcriptional unit. To test this, promoter probe studies were carried out. Cloning two different fragments into the promoterless vector pRG lead to expression of subunit IV. As both contain at least the first 170 bp upstream of the start codon, this is clear evidence that a promoter must be located within this region.

Fig. 5 suggests a promoter sequence about 90 bp upstream of the translational start (see also Fig. 1). This –10 to –35 region is compared to 5 promoter regions mapped in P. denitrificans, and is in agreement with corresponding putative sequences of 14 other Paracoccus genes as well as with an earlier suggestion (22).

In summary, we have shown that subunit IV in the cytochrome aa₃ from P. denitrificans can be excluded to (i) act as an assembly factor, (ii) be required for cofactor insertion, or (iii), being in contact with subunits I and III, should provide some general stabilization of the complex (7). While the considerably larger subunit IV of quinol oxidases obviously is strictly required during the assembly process, the single transmembrane helix of this subunit in the P. denitrificans cytochrome c oxidase may be speculated to be an evolutionary remnant.

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FIG. 5. The putative catH promoter sequence fits a consensus sequence derived from 5 mapped promoter regions of P. denitrificans. AN gives the number of base pairs gapping the –35 and the –10 region. References are given as accession numbers of the EMBL nucleotide sequence data base: catC (coding for subunit II of the cytochrome c oxidase (X05828)); fbc (coding for the ubiquinol-cytochrome c oxidoreductase (X05799)); cycA (cytochrome c₅₅₀ (Y07533)); tpp (putative sugar transport protein; Footnote 2); sdhC (succinate dehydrogenase cytochrome b subunit (U31902)).

H. Witt, unpublished data.