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Genes Coding for Cytochrome *c* Oxidase in *Paracoccus denitrificans*

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Abstract

Several loci on the *Paracoccus denitrificans* chromosome are involved in the synthesis of cytochrome *c* oxidase. So far three genetic loci have been isolated. One of them contains the structural genes of subunits II and III, as well as two regulatory genes which probably code for oxidase-specific assembly factors. In addition, two distinct genes for subunit I have been cloned, one of which is located adjacent to the cytochrome c_{550} gene. An alignment of six promoter regions reveals only short common sequences.

Key Words: *P. denitrificans*, cytochrome *c* oxidase, mitochondria.

Introduction

Bioenergetics of *Paracoccus denitrificans* has been a popular object of study since the discovery that its aerobic electron transfer pathway very much resembles the mitochondrial respiratory chain (John and Whatley, 1975), but its respiratory system has typical bacterial characteristics as well. For instance, multiple alternative oxidases are expressed in response to environmental conditions (Ludwig, 1987; Bosma *et al.*, 1987a,b; Bosma, 1989). However, the caption that *P. denitrificans* is a "free-living mitochondrion" has gained new strength through detailed comparison of its respiratory enzymes to their mitochondrial counterparts. Here cytochrome *c* oxidase is a strikingly good example.

Over the past two decades an impressive amount of physiological, biochemical, and biophysical information has accumulated on the cytochrome

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c oxidase complex (cytochrome aa_3, EC 1.9.3.1; for recent reviews, see Chan and Li, 1990; Bisson, 1990; Saraste, 1990; and the related articles in this volume). Perhaps the most intricate current problem is its molecular catalytic mechanism. In mitochondria as well as in most bacterial cells (Ludwig, 1987), the reduction of dioxygen by cytochrome aa_3 is associated with vectorial translocation of protons across the membrane. An important aspect of the catalytic mechanism is the location of the enzyme's metal centres (see Azzi *et al.*, this volume).

Detailed knowledge on vectorial catalysis, including proton pumping, as well as the localization of the binding sites for four redox centers will require collaboration of many experimental disciplines. Application of molecular genetics (such as site-directed mutagenesis) and high-resolution structural analysis (electron microscopy, X-ray crystallography, and spectroscopic methods) will both be needed for proper mechanistic scenarios. In these efforts bacterial cells are much easier tools than mitochondria of even simple unicellular eukaryotes. Combination of mutant data, structural analysis, and spectroscopy has made bacteriorhodopsin the most advanced case in our understanding of vectorial catalysis in a molecular pump (Henderson *et al.*, 1990). We hope that cytochrome oxidase will soon become another enzyme that is understood in the same depth.

Another point of interest in the molecular biology of prokaryotic respiratory enzymes is the regulation of their biosynthesis. *Paracoccus denitrificans* is a facultative aerobic organism, able to grow under a variety of environmental conditions. Apparently, the induction of cytochrome *c* oxidase is regulated both by the nature of the carbon source and by the degree of aeration. Larger amounts of cytochrome *c* oxidase are synthesized during growth in poor media, and at elevated concentrations of oxygen. However, details of this regulation are not yet known.

The development of genetic work on the prokaryotic cytochrome oxidase started only recently but is progressing rapidly. This communication reviews briefly recent developments in the molecular biology of the *P. denitrificans* cytochrome *c* oxidase.

Purification of the Protein

A cytochrome aa_3-type oxidase from a bacterial source was first purified by Ludwig and Schatz (1980), who obtained a preparation containing two different subunits from *P. denitrificans*. The spectroscopic properties and molecular activity of the enzyme were found to be very similar to the mitochondrial oxidases (Ludwig, 1986; Steinrücke *et al.*, this volume). Moreover, immunological evidence (Ludwig, 1980) and direct protein

sequencing (Steffens *et al.*, 1983) showed that these two subunits correspond to subunit I (COI) and subunit II (COII) of the mitochondrial enzyme.

The second part of the *Paracoccus* oxidase story began when Berry and Trumpower (1985) tried to isolate the cytochrome *c* reductase complex from this bacterium. They solubilized the membranes with dodecyl maltoside, instead of the previously used Triton X-100 (Ludwig and Schatz, 1980), and found an ubiquinol oxidase which was a "supercomplex" of cytochrome *c* reductase, cytochrome c_{552}, and cytochrome *c* oxidase. The cytochrome $bc_1/c_{552}/aa_3$ complex could be further split into the individual enzymes with an additional gel-chromatographic step in the presence of dodecyl maltoside.

The most recent developments in the purification made use of the genetic information. DNA sequencing of the oxidase genes revealed that one of them codes for a homolog of the mitochondrial subunit III (COIII; Saraste *et al.*, 1986). This prompted an effort to isolate a three-subunit complex, which was achieved by Haltia *et al.* (1988). These authors modified and extended the procedure of Berry and Trumpower. The final separation by FPLC in the presence of a mixture of dodecyl maltoside and dodecyl dimethylaminoxide gave fractions containing either two or three subunits (Fig. 1). The identity of COIII was proven by specific labelling with dicyclohexyl carbodiimide (DCCD; Prochaska *et al.*, 1981) and by the N-terminal protein sequence (Haltia *et al.*, 1988).

There may be two explanations why COIII escaped detection for such a long time. First, Ludwig's rather long purification procedure uses extensively Triton X-100. This detergent may cause COIII to dissociate and separate

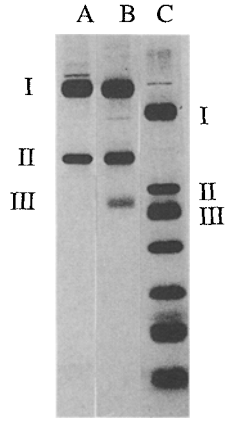

Fig. 1. Lanes A and B show SDS-PAGE of two preparations of the *Paracoccus* cytochrome oxidase. Lane C is the bovine mitochondrial enzyme. Roman numerals I–III refer to three major subunits. Note that ten smaller subunits of the eukaryotic enzyme are not well resolved in this gel. See Haltia *et al.* (1988) for details.

from the complex (Finel and Wikström, 1988). Second, the column steps in the Berry and Trumpower method do not resolve cytochrome c_{552} from the oxidase (Bolgiano *et al.*, 1988). This cytochrome migrates in the same position as COIII in SDS-PAGE, preventing its detection (Haltia *et al.*, 1988). The case of COIII in the *Paracoccus* oxidase demonstrates how genetic studies can produce good hypotheses for protein work.

Isolation of the Genes

The development of genetic manipulation on *P. denitrificans*—vectors, conjugation, and other cloning methods—is described by Harms elsewhere in this volume. Isolation of the oxidase genes from *P. denitrificans* was carried out using either synthetic oligonucleotides (Raitio *et al.*, 1987) or specific antibodies (Steinrücke *et al.*, 1987) as probes. The former were designed and targeted toward evolutionarily conserved regions in the COI and COII sequences taking into account the information on the sequenced authentic *Paracoccus* oxidase peptides (Steffens *et al.*, 1983). One of the mixed probes (probe C, Raitio *et al.*, 1987) has been subsequently used by us and others to isolate COI genes from other bacterial species.

Ludwig and his coworkers isolated the COII gene from an expression library constructed with a plasmid vector. They found that the COII precursor is expressed and correctly processed in *E. coli*. The processing has two aspects: the N-terminal signal sequence is removed and a 17-residue C-terminal peptide is trimmed off (Steinrücke *et al.*, 1987).

Three different loci, all of which contain structural genes for cytochrome oxidase, have so far been found. The first locus was cloned using a COII probe. It has the genes for COII and COIII, and three additional open reading frames (ORFs), two of which (ORF1 and ORF3) will be discussed below; ORF2 is a short frame, and it is not certain whether it is translated. The second locus was cloned when it became clear that the gene for COI is not adjacent to the COII and COIII genes (Raitio *et al.*, 1987). It contains the COIα gene. The existence of a third locus was deduced from the fact that a deletion of the entire COIα gene did not give rise to a phenotype that could be distinguished from the wild type. This locus was subsequently cloned and sequenced by Raitio *et al.* (1990) and shown to contain a gene for the second version of subunit I (COIβ) and another for cytochrome c_{550} (*cycA*). This region of the chromosome was independently isolated by Van Spanning *et al.* (1990), using oligonucleotide probes derived from the protein sequence of cytochrome c_{550}.

Figure 2 shows the organization of the genes in three *Paracoccus* oxidase loci. The assignment of the *ctaB-G* genes follows the nomenclature that has

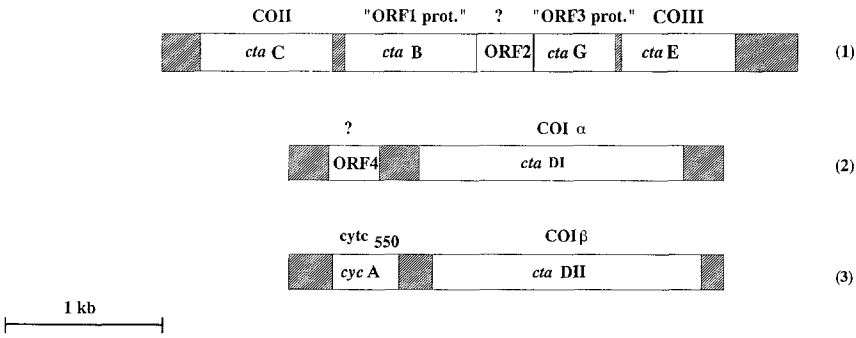

Fig. 2. Schematic illustration of the gene structure in three separate oxidase loci. *ctaC* and *ctaE* code for COII and COIII, respectively. Two iso-COIs are coded by *ctaDI* and *ctaDII*. *cycA* is the gene for cytochrome *c*550. *ctaB* and *ctaG* (ORF1 and ORF3) are probably genes for two assembly factors. See text for details and references.

been proposed for the *Bacillus subtilis* oxidase (Mueller and Taber, 1989; Saraste, 1990). *cta* comes from *cyt*ochrome *a*, and the genes are assigned according to their homologues in *B. subtilis*. *ctaA* is a regulatory gene (Mueller and Taber, 1989) that has not yet been identified in *P. denitrificans*. Apart from *ctaDI*, *ctaDII*, *ctaC*, and *ctaE* that code for the structural subunits (COIα, COIβ, COII, and COIII, respectively), and *ctaB* and *ctaG* that code for oxidase-specific assembly factors (see below), at least the genes responsible for heme A synthesis must be involved in the biosynthesis of cytochrome oxidase.

Assembly

The general picture of protein biosynthesis is currently becoming increasingly complicated. Even simple soluble proteins require cofactors and assistance to fold properly. Cytochrome oxidase, a membrane-bound complex of three hydrophobic, metal-, and heme-binding proteins (see Azzi *et al.* and Buse *et al.*, this volume), seems to need specific factors for assembly. Tzagoloff and his coworkers have recently demonstrated that two nuclear genes in yeast encode factors specifically involved in the formation of active cytochrome oxidase. After deletion of either of these genes, the synthesis of both the mitochondrial- and nuclear-encoded subunits continues but no correctly assembled oxidase is spectroscopically detectable. These yeast *cox10* and *cox11* genes code for proteins homologous to the *ctaB* and *ctaG* (ORF1 and ORF3) products (Nobrega *et al.*, 1990; Tzagoloff *et al.*, 1990).

The *ctaB* is also present in the *B. subtilis* oxidase operon (Saraste *et al.*, 1991), and a homologous gene (*cyoE*) is found in the *E. coli* cytochrome

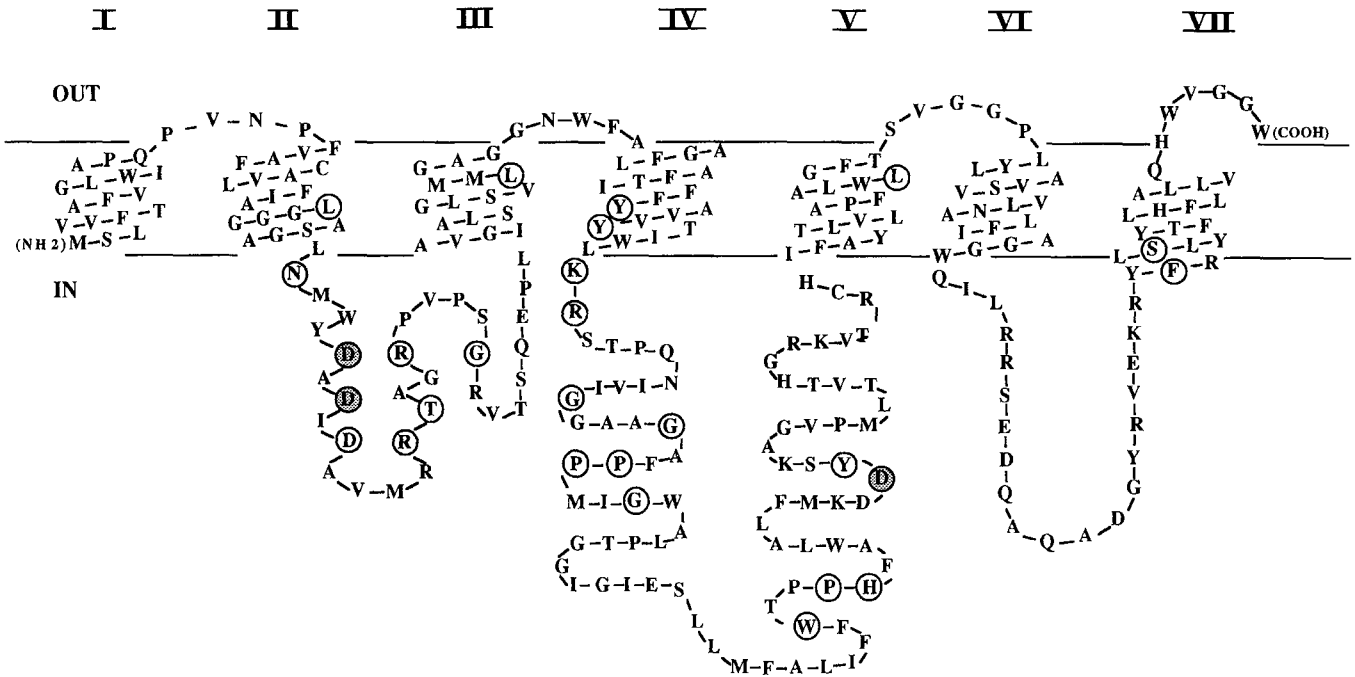

Fig. 3. A model of the *ctaB* product or ORF1 protein. Topological experiments of Chepuri and Gennis (1990) postulate seven transmembrane segments roughly in the locations shown. A large part of the sequence may not be embedded in the bilayer but is located on the cytoplasmic surface. Almost all conserved residues are in two predicted loops (circles; shaded circles are three asparates which are substituted with glutamates in the yeast protein). IN and OUT refer to the cytoplasmic and periplasmic space, respectively. See text for references.

bo operon (Saraste *et al.*, 1989; Chepuri *et al.*, 1990). Figure 3 shows a schematic model of its hydrophobic protein product. Chepuri and Gennis (1990) have proposed a topological model for the membrane-bound *cyoE* protein. They used random gene fusions to two marker enzymes, alkaline phosphatase and β-galactosidase, to map the external and internal loops, respectively, and concluded that the *cyoE* protein has seven transmembrane segments. The model of Fig. 3 has been adapted from their paper (Chepuri and Gennis, 1990). The circled amino acids are conserved in the *E. coli*, *B. subtilis*, *P. denitrificans*, and yeast sequences. Note that the conserved residues are predicted to reside on the cytoplasmic surface of the bacterial membrane and only short loops are predicted on the outer surface.

What is the function of the ORF1 protein? Does it assist COI to fold properly (Nobrega *et al.*, 1990) or does it catalyze the formation of the active site in the enzyme? Mutagenized, partially inactivated protein has to be produced and expressed *in vivo* to approach these questions. However, it is tempting to speculate that the ORF1 protein could also assist the binding of a metal center (Cu_B?) to the apoprotein. The predicted cytoplasmic domain has four conserved carboxylic acids and one histidine—these might form a metal-binding site. Three invariant tyrosines, two of which are in the transmembrane segment IV, might also have a part in the metal-center-assembling activity (see Fig. 3).

Deletion of the COIII gene leads also to defective assembly. COI and COII are not able to make a mature, enzymatically active complex in the absence of COIII. However, all metal centers appear to be present in the COI plus COII complexes which are formed in the mutant membranes (Haltia *et al.*, 1989; see also Nakamura *et al.*, 1990). Conversely, the assembly of the oxidase in yeast mitochondria does not proceed at all if, for example, the COIII gene in mtDNA is inactive or the genes coding for the minor subunits in nucleus have deletions (see Poyton *et al.*, 1989). COIII may have a scaffolding role in the bacterial oxidase complex, or the assembly defect may simply indicate that COI, COII, and COIII have evolved to assemble together.

Regulation

Paracoccus denitrificans can grow in the presence and absence of O_2. Moreover, it can use a broad spectrum of carbon compounds to drive catabolic and anabolic processes (e.g., mannitol, succinate, methanol, ethanol, acetate), and even autotrophic growth on CO_2 and H_2 has been demonstrated (see Harms, Stouthamer, this volume). Complex regulatory mechanisms are probably required to allow optimal metabolism in this bacterium.

The expression of cytochrome *c* oxidase must have two kinds of regulatory features in *Paracoccus*. The first can be seen as a general induction problem: how is the expression of the respiratory enzyme genes regulated? Transcription of the *E. coli cyo* promoter has been shown to be blocked by glucose (catabolite repression) as well as by low oxygen concentrations (Minagawa *et al.*, 1990). The second aspect deals more specifically with the synchronization of subunit synthesis from the different loci. Detailed experimental analysis of the promoter regions is needed for better understanding of these regulatory phenomena.

Cytochrome bc_1 and aa_3 complexes seem not to be expressed in a coordinated fashion, since the former enzyme appears to be synthesized constitutively (Trumpower *et al.*, this volume). The cytochrome *c* that mediates electron transfer between these complexes appears to be a 22-kDa membrane-associated protein (Bolgiano *et al.*, 1989). Its gene has not yet been analyzed. Under limiting oxygen concentrations a cytochrome *bo*-type enzyme seems to be the major oxidase, and the expression of both cytochrome aa_3 and its electron donor are repressed (Bosma *et al.*, 1987a).

Growth on methanol induces cytochrome aa_3 (Van Verseveld *et al.*, 1981), although this oxidase may not be absolutely necessary for this type of metabolism (see Harms *et al.*, 1987). However, the current consensus is that methanol oxidation requires methanol dehydrogenase, cytochrome c_{550}, and the cytochrome aa_3-type oxidase. Cytochrome c_{550} seems to be synthesized under all growth conditions, whereas the expression of methanol dehydrogenase and cytochrome oxidase may be commonly regulated under methylotrophic growth (see Harms *et al.*, 1987; Van Spanning *et al.*, 1990).

Figure 4 is an attempt to align the sequences of six promoter regions in *P. denitrificans*. They all control metabolically related enzyme activities. Three of these regions are upstream from the oxidase loci (*ctaC*, *ctaDI*, and *ctaDII*, Fig. 2). The *mdhA* sequence is in front of the methanol dehydrogenase gene (Harms *et al.*, 1987), *fbc* in front of the cytochrome *c* reductase operon (Kurowski and Ludwig, 1987), and *cycA* upstream from the cytochrome c_{550} gene (Raitio *et al.*, 1990; Van Spanning *et al.*, 1990). An arrowhead labels a G residue at the site where Kurowski and Ludwig (1987) have mapped the 5′-end of the *fbc* transcript. The consensus sequences are weak, and experiments are required before the promoter activities in these sequences can be pointed. Weak resemblances of the *Paracoccus* promoter sequences to the *Rhodobacter* promoters have been discussed by several authors (Kurowski and Ludwig, 1987; Harms *et al.*, 1987; Van Spanning *et al.*, 1990).

One interesting feature in Fig. 4 is that some promoter regions remind each other. We have tried to classify the sequences so that their place in the alignment reflects their pairwise similarities and have underlined related oligonucleotides in the neighboring sequences. *mdhA*, *ctaDI*, and *ctaC* or

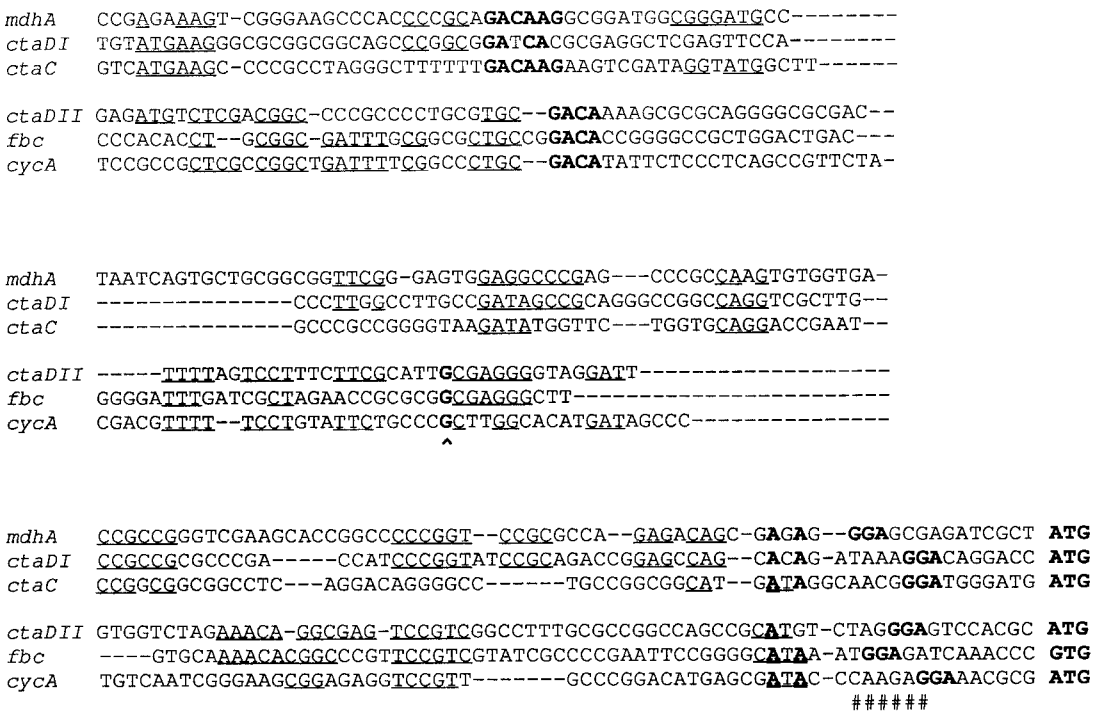

Fig. 4. An alignment of six *Paracoccus* promoter regions. Conserved characters found in all six are shown in bold. *ctaC*, *ctaDI*, and *ctaDII* refer to the upstream sequences in the front of three oxidase loci (Fig. 2). *cycA*, *mdhA*, and *fbc* refer to the promoter regions in the front of the cytochrome c_{550} and methanol dehydrogenase genes and the cytochrome *c* reductase operon. Underlining indicates identities in neighboring sequences. The initiation codons are shown in bold and the ribosomal binding site is underlined with #. An arrowhead shows a probable 5′-end of the *fbc* transcript. See text for references.

ctaDII, *cycA*, and *fbs* have mutual matches that are not found in the other pair. The alignment is not very convincing, but it may still indicate that, for instance, the *mdhA* and *ctaDI* promoters are under the same kind of control.

The presence of two copies of COI in *P. denitrificans* has not yet been explained in physiological terms, 89% of their amino acid sequences are identical, and all substitutions fall out of the general conservation pattern in COI (Raitio *et al.*, 1990). It is possible that their biosynthesis is differently regulated—at least the promoter regions show very little similarity (Fig. 4). COIα and COIβ might assemble with the standard copies of COII and COIII to make two isoenzymes. One of these might be involved in the oxidation of C1 compounds such as methanol and methylamine.

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