A gene in *Paracoccus* for subunit III of cytochrome oxidase

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Received 7 July 1986

The region of *Paracoccus denitrificans* chromosome where the genes coding for cytochrome oxidase (cytochrome aa_3) subunits are located has been cloned. DNA sequencing revealed an open reading frame that codes for a protein homologous to the subunit III of the eukaryotic, mitochondrial enzyme. This subunit is absent from the isolated *Paracoccus* oxidase. It now seems that it is part of the native enzyme in the bacterial cytoplasmic membrane. This may explain the observed discrepancies in the function of the isolated enzyme.

Cytochrome oxidase *Subunit III* (Paracoccus) *DNA sequencing*

1. INTRODUCTION

Aerobic bacteria are able to use many different terminal oxidases in cellular respiration. One of these, cytochrome aa_3, is structurally and functionally similar to the mitochondrial enzyme [1,2]. This enzyme catalyses electron transfer from cytochrome *c* to dioxygen and coupled to this transfers two protons/electron across the membrane, one of which is used in formation of water [3]. The bacterial oxidase has been isolated from a number of species and shown to contain 2 or 3 different subunits. These correspond to the functionally central subunits I, II and III in the mitochondrial enzyme [4] that are coded for by three genes in the mitochondrial DNA itself; the subunits coded for by nuclear genes appear to be absent in the bacterial enzymes [1–3].

The first known bacterial cytochrome aa_3 was purified by Ludwig and Schatz [5] from *Paracoccus denitrificans*. The isolated enzyme contains two different subunits which are known to be homologous to the subunits I and II of the mitochondrial enzyme [2,6]. It lacks, however, the third subunit. In mitochondria and some other bacteria [1,3] this subunit III is retained in the isolated oxidase. It seems to be important in proton translocation, directly or indirectly. Dicyclohexylcarbodiimide (DCCD) modifies a glutamic acid residue [7] residing in one of its membrane-spanning segments [4]. This leads to inhibition of net proton translocation while electron transfer is not greatly affected [8].

It has been difficult to demonstrate effective proton translocation with the purified *Paracoccus* cytochrome oxidase reconstituted to liposome membranes [9]. The enzyme in situ clearly has this activity [10]. We have been cloning the genes of the *Paracoccus* cytochrome oxidase. Here, we have now found a gene in the genomic DNA that codes for the subunit III. This subunit must be lost during the traditional isolation procedure [5], and its loss renders the isolated enzyme a poor proton translocator as seems to be the case for the mitochondrial oxidase in the equivalent situation [11,12].

2. MATERIALS AND METHODS

P. denitrificans S1657 was a gift from Dr Henk van Verseveld. A DNA library was constructed by cloning partially *Sau*3A-digested DNA fragments with the λ EMBL-3 vector in *E. coli* NM539. The library was screened with two synthetic oligonucleotide probes (see below). DNA hybridization, isolation of λ DNA and restriction mapping is described in [13]. *Sal*I restriction fragments hybridizing to the probes were purified

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

by gel electrophoresis and subcloned into M13mp8 and M13mp19. For sequencing DNA was randomly fragmented by sonication, followed by shotgun cloning into *Sma*I-cut M13mp8 [14] or by *Exo*III deletions [15]. DNA sequences were determined by the dideoxynucleotide method as modified in [16].

3. RESULTS AND DISCUSSION

Two synthetic oligonucleotides were designed as mixed probes on the basis of the evolutionary conservation of the protein sequence [4] and the known peptide sequences of the *Paracoccus* proteins [6]. They were both directed to the gene of subunit II. Hybridization was carried out with two probes in parallel, and λ clones positive with both were selected for further analysis by restriction mapping and DNA sequencing. The gene for the subunit II was first found by sequencing the ends of two *Sal*I fragments shown in fig.1. The figure gives a rough map of the *Paracoccus* chromosome in the region of the subunit II and III genes (COII and COIII). The latter was found by analysis of the 3′-end of the insert in a particular clone (Paλ5). The DNA sequence approaching this *Sau*3A site is shown in fig.1.

The COIII gene probably begins at nucleotide 36 in the shown sequence. The N-terminal of the predicted protein matches with the mitochondrial subunit III (fig.2). The first ATG codon is also preceded by a sequence similar to the ribosome binding site (Shine-Dalgarno box [17]) in *E. coli* genes; it is underlined in fig.1. The N-terminal half of subunit III is aligned in fig.2 with two mitochondrial proteins. The oxidase subunits III from yeast, beef and *Paracoccus* have 34 identical residues among the 151 aligned. Most of the C-terminal half of the subunit III is known from other λ clones. It shows also clear homology to the mitochondrial proteins, and the entire protein is very similar to them in molecular size as well (not shown).

The DCCD-binding site in subunit III has been identified by Prochaska et al. [7]. It is a glutamic

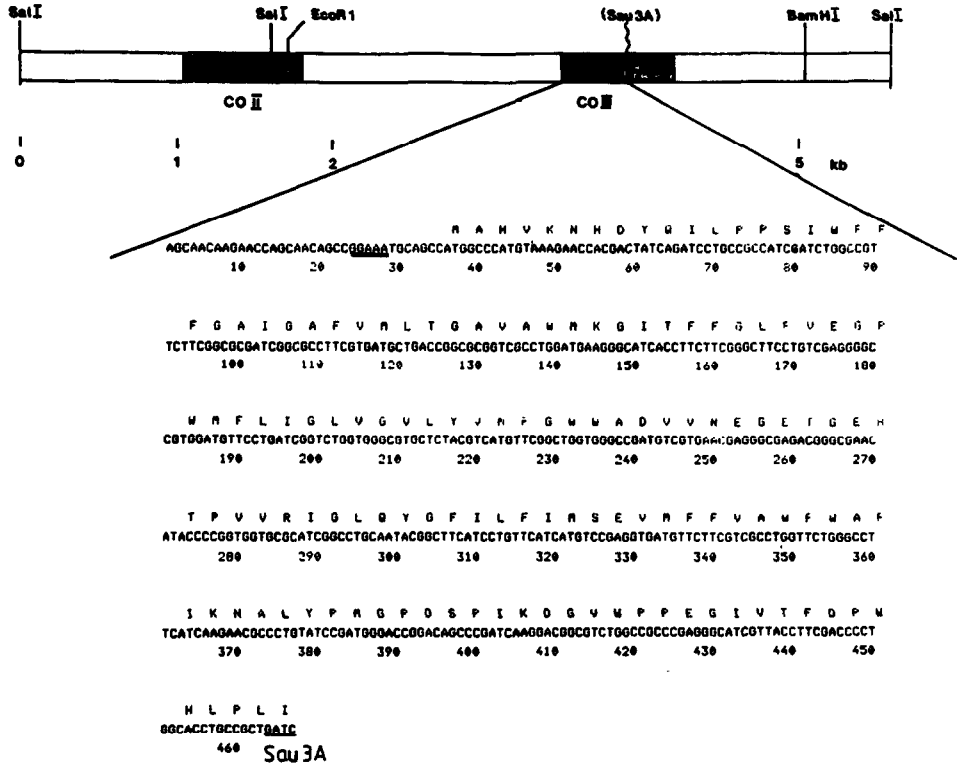

Fig.1. A map of the *Paracoccus* chromosome in the region of COII and COIII. Restriction sites for *Sal*I, *Eco*RI and *Bam*HI are shown. The 3′-end of the insert in the λ clone Paλ5 is an *Sau*3A site in the middle of COIII. 467 bp of DNA sequence approaching this site is shown below. A possible ribosome-binding site in the front of the gene and the *Sau*3A site at the end are underlined.

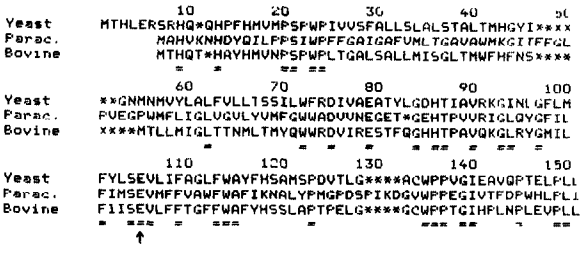

Fig.2. Alignment of N-terminal amino acid sequences of bovine [20], *Saccharomyces* [21] and *Paracoccus* subunit III. Identical residues in all three are underlined. The arrow points to the DCCD-binding glutamic acid.

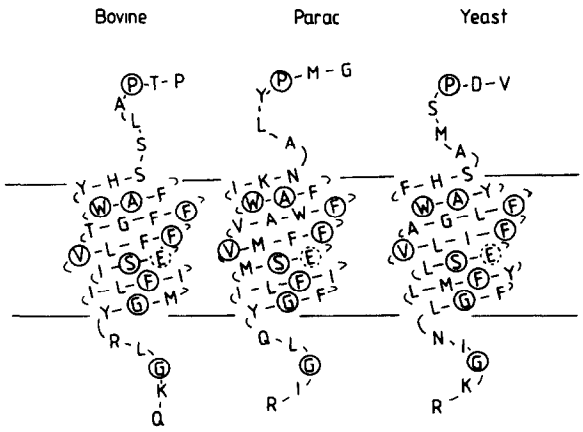

Fig.3. Transmembrane segment containing the DCCD-binding site in yeast, bovine and *Paracoccus* subunit III. Identical residues are circled, and the DCCD-binding site is indicated with a dashed circle.

acid residue inserted into a long hydrophobic segment, one of the proposed transmembrane helices in this protein [4]. This glutamic acid is present in the *Paracoccus* protein as well (dashed circle in fig.3). Conservation of the amino acid sequence shows helical geometry in this segment: one side of the predicted helix has an array of invariant amino acids, notably aromatic residues (circled in fig.3).

We conclude that subunit III is probably present in the *Paracoccus* cytochrome oxidase as it is found in some other bacterial cytochromes aa_3 [1,18]. Alternative purification procedures [19] may yield preparations that retain this subunit. Its absence from the conventional preparation is likely to be the reason why the reconstituted enzyme shows a low proton/electron transport stoicheiometry [9,12].

ACKNOWLEDGEMENTS

We are grateful to Katri Sundell for excellent technical assistance and to Drs K. Krab and M. Wikström for useful comments. This work has been supported by a research contract from the Academy of Finland (Science Research Council).

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