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Terminal oxidases in *Paracoccus denitrificans*

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Introduction

Unlike the highly specialized organization of the electron transport pathway in the mitochondrion, bacterial respiratory chains in general are far more complex (for recent reviews, see Refs. 1–4), and their organization reflects the requirement of microorganisms for adaptation to changing environmental conditions. Therefore, branching points and alternative routes in redox pathways are often found as characteristic features, providing metabolic flexibility to the prokaryotic cell. In particular, this is true for terminal oxidases: in many cases, two (or more) different types of oxygen-reducing enzymes have been described for different growth conditions in the same organism, sometimes even coexisting to various levels of expression. As a classical example, *E. coli* synthesizes two quinol oxidases, cytochrome *o* (*bo*) and cytochrome *d*, depending largely on oxygen availability [1,4,5,6].

In past years, many reports documented that, in comparing mitochondrial redox enzymes with their bacterial counterparts, the latter components showed a much lower complexity in terms of number of subunits. This made such bacterial systems excellent models to study structural and functional aspects of electron transport and energy transduction. An major additional advantage is the fact that only a single genetic system is required for expression, and it is usually readily accessible to mutagenesis manipulations.

Quite recently, sequence comparisons revealed that the former strict separation between the aa_3-type ('mitochondrial') cytochrome *c* oxidases and the cytochrome *o*-type ('bacterial') quinol oxidases, carrying heme *o* [7], was no longer justified. The notion emerged that both types of oxidases are structurally related, belonging to a larger protein family [5,8]. Their members can be furnished with different heme moieties, and are able to work with either quinol or cytochrome *c* as electron donor, while their oxygen reducing sites and, apparently, their proton-pumping properties as well [9], may remain unchanged.

Paracoccus denitrificans

Nominated early on as a potential candidate in support of the endosymbiotic theory, this Gram-negative soil bacterium has been a rewarding object for studying electron transport complexes. Several of its respiratory chain complexes have indeed been shown to be related in various ways to those of mitochondria,

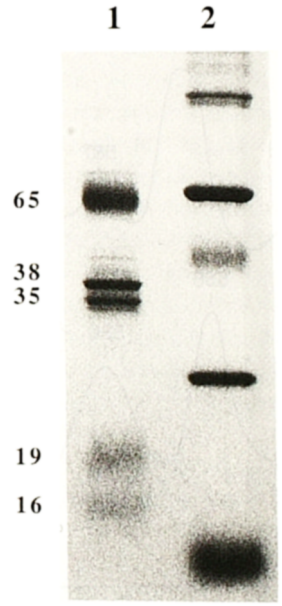

Fig. 1. Subunit pattern of the purified *Paracoccus* cytochrome ba_3 (quinol oxidase). 15 μg of purified enzyme (lane 1) were electrophoresed on a 12% polyacrylamide gel in the presence of SDS, and stained with Coomassie blue. Numbers indicate estimated M_r $\times 10^3$. Lane 2: molecular-weight marker proteins: phosphorylase *b* (98 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa), cytochrome *c* (12.4 kDa).

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but, even more importantly, they exhibit a much simpler subunit pattern (e.g., see Refs. 10–12).

Cytochrome *c* oxidase (aa_3)

This oxidase was originally isolated in a fully functional form as a two-subunit enzyme [10], but was later shown to contain a third subunit [13]. While two structural genes of largely identical nucleotide sequence seem to code for the largest subunit of the complex, the subunit II and subunit III genes are organized in an operon structure (*cta*) and are intervened by three open reading frames [14]. By deleting the subunit II gene (*cta*C) and/or inserting the kanamycin marker gene, we have shown that an aa_3-*minus* phenotype can be generated in *Paracoccus*, i.e., besides the expected loss of subunit II, no *a*-type heme nor the subunit I polypeptide can be found in the cytoplasmic membrane [15]. Interestingly, this phenotype cannot be complemented by the intact *cta*C gene alone, but only by offering the complete *cta* operon *in trans*. If the subunit II gene plus fragments of different length, extending into the open reading frame region, are used for complementation, expression and assembly proceeds to various levels. We therefore concluded that the first open reading frame gene, *cta*B, has a chaperon function in assisting the membrane insertion of subunit I, whereas the third open reading frame (*cta*G) gene product must be responsible for heme (or copper) insertion during assembly of the complex. Homologous genes have been found in other bacterial systems and in yeast as well (e.g., see Ref. 16).

Alternative oxidase

Apart from the aa_3 cytochrome oxidase, *Paracoccus* has been shown to contain *b*-type terminal oxidases which were interpreted as cytochrome *o* (e.g., see Ref.

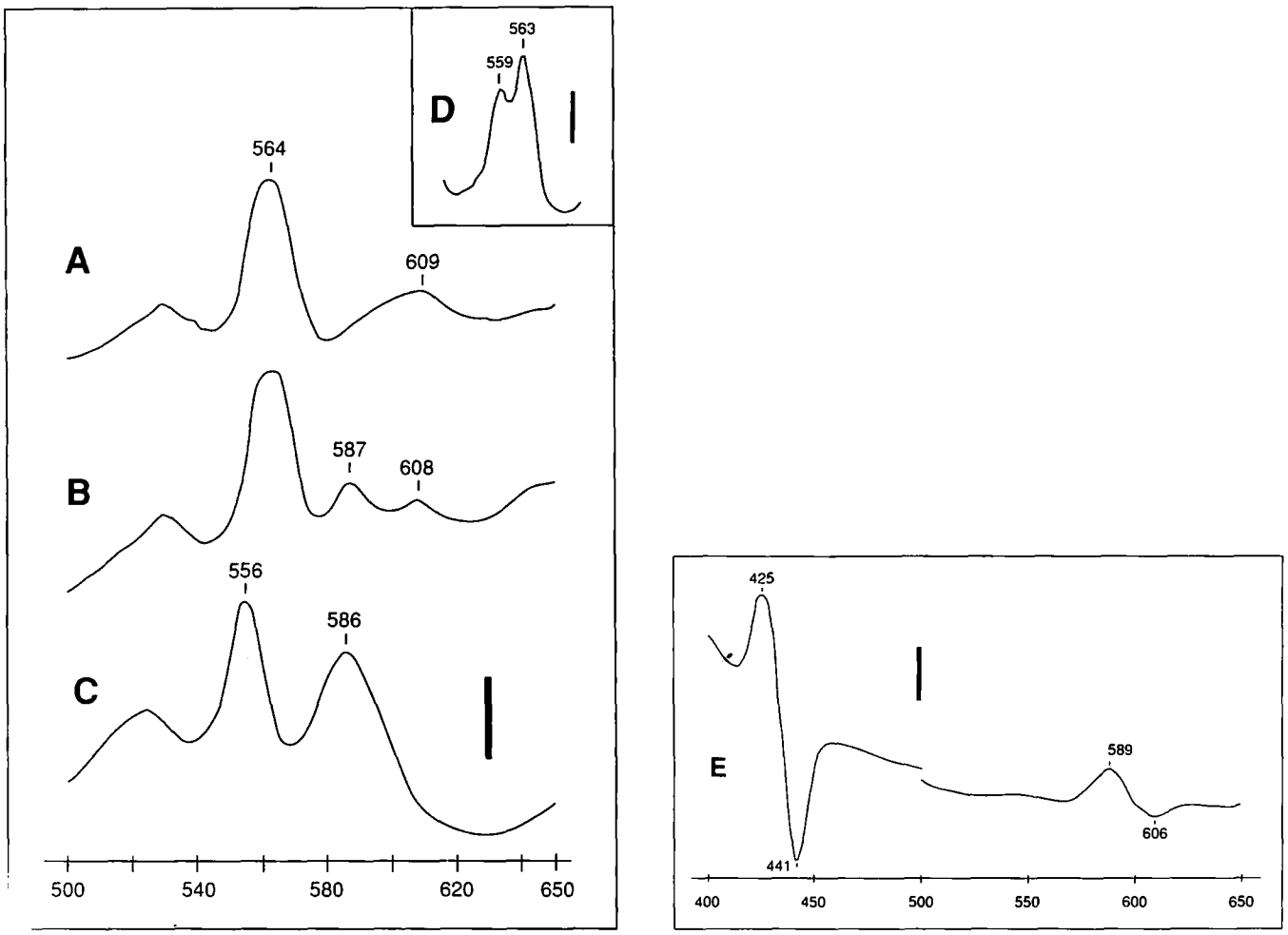

Fig. 2. Spectral identification of the ba_3 oxidase. Traces A to D: difference spectra (dithionite-reduced *minus* ferricyanide-oxidized) of the purified enzyme; bar represents 0.005 ΔA. (A = arbitrary absorbance unit.) (A) Room temperature spectrum, protein concentration 0.094 mg/ml; (B) as A, but in the presence of 1 mM KCN; (C) pyridine hemochrome difference spectrum, protein concentration 0.075 mg/ml; (D) as A, but recorded at −196°C at 0.1 mg/ml; (E) CO-reduced *minus* reduced difference spectrum; protein concentration 0.033 mg/ml; bar represents 0.005 ΔA for the Soret and 0.002 ΔA for the α region.

17). Reports of an a_1-type cytochrome [18] and induction of a *d*-type terminal oxidase under specialized growth conditions [19] have appeared as well. In most cases, however, these cytochromes were characterized only by spectroscopic means in intact cells or membranes. We have used a mutant strain of *Paracoccus* lacking the cytochrome bc_1 complex (Gerhus, E. and Ludwig, B., manuscript in preparation) to purify a mixed heme oxidase [20]. This alternative oxidase is an integral membrane protein consisting of 5 different subunits, ranging in molecular weight between 65 and 16 kDa (Fig. 1); it acts solely as a ubiquinol oxidase, and is spectrally characterized as a copper-containing ba_3 hemoprotein (with a 1:1 ratio of both hemes, see Fig. 2). Its specific heme content suggests a theoretical M_r of approx. 185000, which is in good agreement with the sum of M_r of its five subunits. Apart from its functional identity, it seems to be structurally related to the oxidase family of genes as judged from preliminary sequencing data and its immunological properties (crossreacting with an anti-*E.coli* subunit I antiserum). Its relation to other *b/a*-type bacterial oxidases (e.g., see Refs. 21,22) appears likely, but requires further characterization.

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