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MINI-REVIEW

The Cytochrome *c* Reductase/Oxidase Respiratory Pathway of *Paracoccus denitrificans*: Genetic and Functional Studies

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Abstract

Data are presented on three components of the quinol oxidation branch of the *Paracoccus* respiratory chain: cytochrome *c* reductase, cytochrome c_{552}, and the *a*-type terminal oxidase. Deletion mutants in the bc_1 and the aa_3 complex give insight into electron pathways, assembly processes, and stability of both redox complexes, and, moreover, are an important prerequisite for future site-directed mutagenesis experiments. In addition, evidence for a role of cytochrome c_{552} in electron transport between complex III and IV is presented.

Key Words: Cytochrome *c* oxidase, cytochrome aa_3, cytochrome bc_1 complex; site-specific recombination; deletion mutants.

Introduction

Over the past 20 years, a large body of data on *Paracoccus denitrificans* has been accumulated suggesting that this bacterium may be evolutionarily related to present-day mitochrondria. At the same time, it also turns out that it may serve as a simple prokaryotic model for electron transport and energy transduction mechanisms of mitochondria. Early indications even date back to 1957 (Vernon and White, 1957), when the oxidation of horse heart cytochrome *c* by membrane particles of *Paracoccus* (known as *Micrococcus* then) was described and its electron transport chain analyzed subsequently

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(Scholes and Smith, 1968). Its close homology to mitochondria, with special reference to its oxidative phosphorylation system, was reviewed in detail (John and Whatley, 1975, 1977), and confirmed later, at least basically, by phylogenetic considerations (Woese, 1987).

In the early eighties, data on the protein structure of individual respiratory complexes became available and agreed well with the above notion. Two main conclusions emerged from such studies: (1) Protein sequence similarities to the corresponding mitochondrial subunits suggested genuine structural homologies between the two systems. (2) As shown clearly for at least two respiratory complexes of *Paracoccus* (see below), their subunit structures are much simpler than those of their mitochondrial counterparts, without, however, an obvious loss of any major functional complexity.

With a simple prokaryotic genetic system and the genes for individual subunits at hand, the stage is set for application of site-directed mutagenesis experiments to gain an insight into molecular processes of electron transport and proton translocation. Assuming homologous function at least in basic terms for both the bacterial and the mitochondrial enzyme complexes, one would hope to transfer conclusions to the mitochondrial system as well.

As an important prerequisite for such an approach, techniques have to be developed to specifically delete subunit genes of interest in a *Paracoccus* strain to be used as a host for future mutagenesis experiments. Construction of such deleted host strains has been achieved recently and will be outlined below.

Cytochrome bc_1 Complex

Structural Data

Similar to other bacterial bc_1 preparations, a complex III has been isolated from *Paracoccus* membranes and shown to be active in electron transport and in generating a proton gradient upon reconstitution (Yang and Trumpower, 1986, 1988). It is composed of three different subunits carrying the well-known redox centers of all bc_1 complexes: a cytochrome c_1, of unusually high molecular weight in *Paracoccus* (Ludwig *et al.*, 1983), a cytochrome *b*, and an iron-sulfur center (FeS) associated subunit.

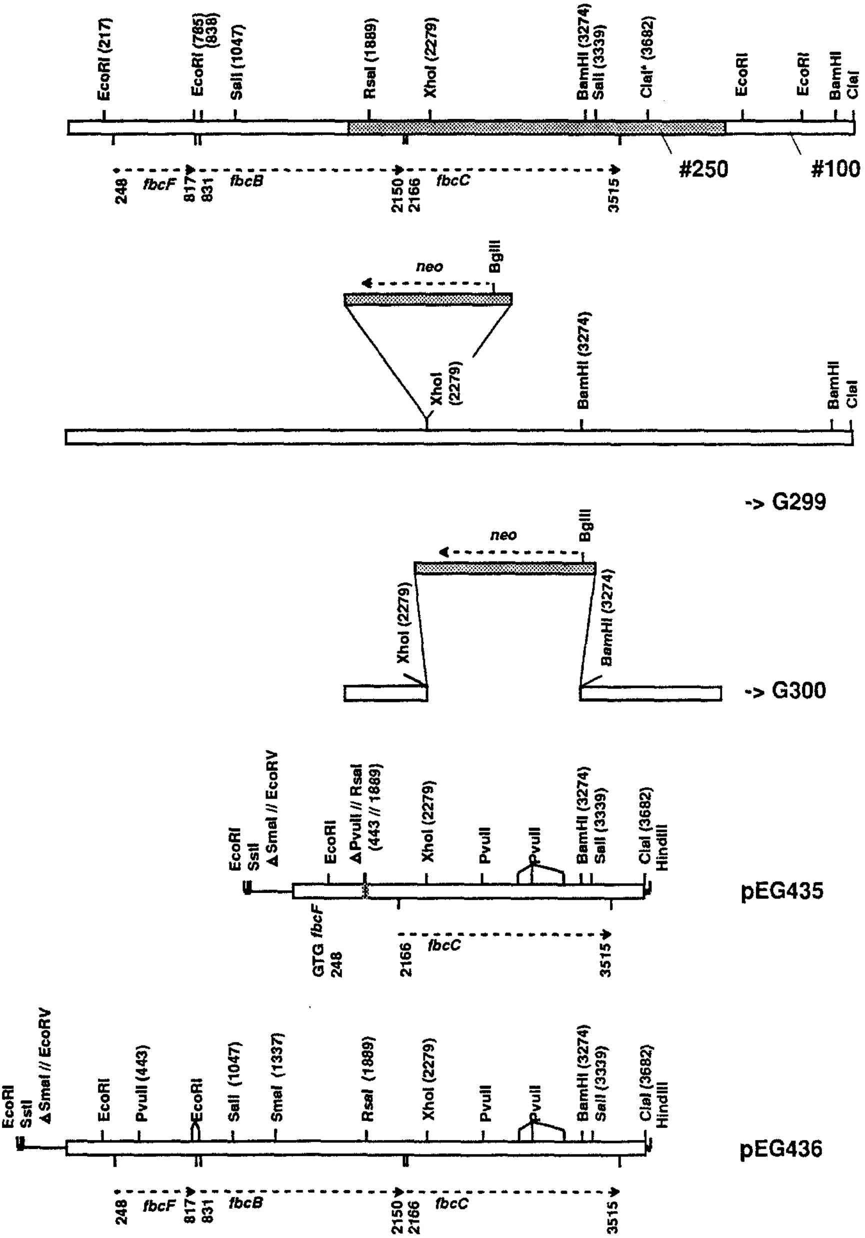
Using specific antibodies directed against the cytochrome c_1 polypeptide, a *Paracoccus* gene bank has been screened and the *fbcC* gene (coding for cytochrome c_1) located next to the genes for the other two subunits, in a typical operon structure for the coordinated expression of the three structural genes (Kurowski and Ludwig, 1987). A comparison of the DNA-deduced protein sequences with either bacterial or mitochondrial sequences for corresponding subunits revealed their close homology, with the exception of a long

domain of unassigned sequence of unusual amino acid composition at the N-terminal part of the cytochrome c_1 polypeptide.

Cytochrome c_1 Gene Deletion Experiments. As a first target, the gene for the cytochrome c_1, last in the *fbc* operon, was deleted in two different types of mutants by inserting a kanamycin resistance gene with or without previous removal of part of the coding region (see Fig. 1, upper half, and Gerhus *et al.*, 1990). The inactivated cytochrome c_1 gene(s) were introduced into a derivative of pSUP 202 (Simon *et al.*, 1983), a vector unable to replicate in *Paracoccus.* This "suicide plasmid" approach ensures that, after conjugational transfer into *Paracoccus*, any exconjugate resistant to kanamycin should carry the resistance gene in a chromosomal location as a result of homologous recombination taking place between the mutilated plasmid copy of the c_1 gene and the wild-type genomic copy. Whether or not this event is actually site-specific and a double crossover, is ascertained by probing for the loss of the (vector-coded) streptomycin resistance and by analyzing the immediate c_1 gene vicinity by Southern hybridization (results not shown). The two resulting types of mutants, represented by strains G299 and G300 (see Fig. 1), were compared to recipient strain membranes in their spectral, kinetic, and subunit pattern. In all mating experiments, the recipient strain was PD1222 (de Vries *et al.*, 1989), a wild-type derivative which lacks a major restriction system and exhibits enhanced conjugation frequencies.

Properties of Mutants. Spectral analysis of both mutants revealed a deficiency of the characteristic 553 nm absorption (red-ox) in the cytochrome *c* region (not shown). Likewise, compared to donor strain membranes, electron transport rates were severely reduced in all assays comprising the bc_1 complex, whereas complex IV activities were unaffected (see Gerhus *et al.*, 1990). When membranes were probed for the presence of bc_1 complex subunits by specific antibodies, the complete loss of the deleted gene product, cytochrome c_1, was evident (see Fig. 2). The concomitant absence of the two other gene products, cytochrome *b* and the FeS subunit, however, came as a surprise, since their genes are located upstream of the insertional mutation and therefore should not directly be affected in transcription of the operon (see also below).

Complementation of the Mutation. Using a broad host range plasmid as a propagatable vehicle, two different gene cassettes were introduced into the mutant strains *in trans*: the intact cytochrome c_1 gene, *fbc*C, under the regime of its own operon promoter, or the complete operon (see Fig. 1, lower section). Any one combination restored the wild-type spectral and enzymatic properties, and immuneblotting revealed the presence of all three gene products in all cases. We conclude from this that the expression of the *fbc*F and *fbc*B genes proceeds unhampered when they are followed by a disrupted *fbc*C gene copy in a chromosomal location, but both subunits (FeS and cytochrome *b*)


EcoRI (217)
EcoRI (785)
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RsaI (1889)
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ClaI* (3682)
EcoRI
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BamHI
ClaI
#250
#100
248
fbcF
817
831
fbcB
2150
2166
fbcC
3515
neo
BglII
XhoI
(2279)
BamHI (3274)
BamHI
ClaI
-> G299
neo
BglII
XhoI (2279)
BamHI (3274)
-> G300
EcoRI
SstI
ΔSmaI // EcoRV
EcoRI
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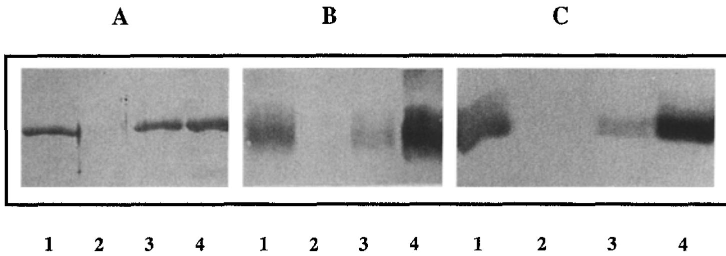

Fig. 2. Immunological detection of subunits of the *Paracoccus* cytochrome bc_1 complex in wild-type, mutant, and complemented mutant strain membranes. 50 μg each of membrane protein isolated from different strains were separated on 12% acrylamide SDS gels, blotted to PVDF membrane (Immunobilon-P), and antigens detected using specific antisera directed against the cytochrome c_1 (A), the cytochrome *b* (B), and the FeS subunit (C). Lane 1: wild type PD1222; lane 2: deletion mutant G300; lane 3: G300 with the *fbc*C gene *in trans* (c_1 complementation, see also Fig. 1); lane 4: G300 with the complete *fbc* operon *in trans*.

do not get inserted into or remain in a stable form in the membrane. [This assumption will have to be further substantiated by Northern blotting or by constructing gene fusions of the *fbc*F gene (coding for the FeS subunit) with the *lac*Z gene as marker gene.] Upon complementation, when only the c_1 gene is offered on a plasmid *in trans*, expression of c_1 resumes, and all three subunits form a stable and enzymatically active respiratory complex. This points to an important role of the c_1 in the assembly process of the complex, or to a shielding effect, protecting the two other subunits from proteolytic attack.

Cytochrome *c* Oxidase

Structural Data

Originally isolated as a two-subunit complex (Ludwig and Schatz, 1980), this oxidase has been characterized in great detail (see Ludwig, 1986, 1987). It has also been shown to pump protons in a reconstituted system, although only at a rate of $0.6\,H^+/e^-$ (Solioz *et al.*, 1982). Its subunits are

Fig. 1. Physical map of the *fbc* operon region of *Paracoccus*, coding for the three subunits of the cytochrome bc_1 complex, and constructs for deletion mutants and their complementation. Two clones, coding for part ("250") or the complete *fbc* operon ("100"), are shown with selected restriction sites and their coding region boundaries (as detailed in Kurowski and Ludwig, 1987). By inserting the *neo* gene cassette into the *Xho* I site within the *fbc*C gene (coding for cytochrome c_1) on clone "100" and conjugation of the construct on a noncompatible vector into the *Paracoccus* PD1222 host strain, mutant strain G299 with a disrupted c_1 gene is obtained (for further details, see text and Gerhus *et al.*, 1990). Mutant strain G300 is constructed by first deleting a portion of the c_1 gene in clone 250, followed by the insertion of the antibiotic gene. For complementation of mutant G300, two different cassettes on a broad host range plasmid were conjugated into this strain: pEG435 comprises the *fbc*G gene preceded by its operon promoter, whereas pEG436 contains the complete operon.

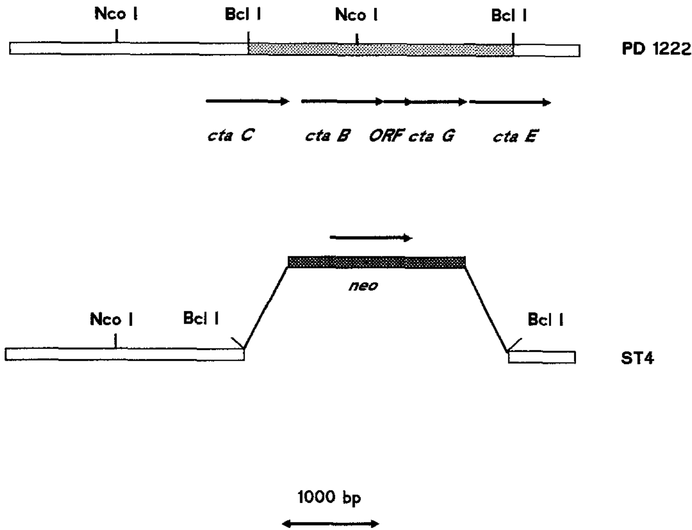

Fig. 3. Restriction map of the *Paracoccus* oxidase subunit II/III gene region (ctaC to ctaE) and insertional inactivation construct. PD1222: The *Bam* HI fragment representing the wild-type gene arrangement in the host strain (for details and nomenclature of genes, see Raitio *et al.*, 1987, and Saraste, 1990). Deletion strain ST4 was obtained by replacing the *Bcl* I fragment with the *neo* gene, and introducing it on a "suicide" plasmid into PD1222 (see text). For complementation experiments, either the *Nco* I fragment (leading to strain ST4N) or the complete *Bam* HI fragment as shown (leading to strain ST4B) were offered on a broad host range plasmid *in trans* (for details, see text).

immunologically cross-reactive with their mitochondrial counterparts. Cloning of the oxidase subunit genes (Steinrücke *et al.*, 1987, Raitio *et al.*, 1987) not only confirmed the assumed sequence homologies with the mitochondrial subunits, but also revealed a gene (*cta*E) corresponding to subunit III of the mitochondrial oxidase (Saraste *et al.*, 1986); it is separated from the *cta*C gene by a sequence of 1.8 kb containing 3 open reading frames (see Fig. 3) of as yet unknown function. Later attempts to isolate such a three-subunit oxidase complex were successful (Haltia *et al.*, 1988, Bolgiano *et al.*, 1988), but such a preparation did not yield any higher proton pump ratios than the two-subunit complex upon reconstitution (M. Wikström, personal communication). The gene for subunit I (*cta*D) did not map in the vicinity of this suspected operon region; it has been shown to be present in two different copies which differ slightly in sequence (*cta*DI and DII copies). Whether or not both copies contribute to the expression of subunit I is not known; deletions in the DI gene copy did not alter the oxidase$^+$ phenotype (Raitio *et al.*, 1990, and Steinrücke, unpublished).

Recent studies have shown that stripping the two-subunit complex of its subunit II by controlled protease digestion yielded a subunit I "complex"

with about 20% enzymatic activity when assayed with reduced mammalian cytochrome *c* (Müller *et al.*, 1988). This observation was taken as further evidence that both hemes and at least one of the three copper ions are associated with subunit I.

Deletion Mutant in the Subunit II/Subunit III Gene Region (ctaC to ctaE)

Construction of Mutant Strain ST4. A substantial deletion in the *cta*C to *cta*E gene region was obtained by removing a 2.7-kb *Bcl* I fragment and replacing it by the kanamycin resistance gene (*neo*). This deletion comprises a major part of the subunit II gene, all three open reading frames, and most of all the subunit III gene (see Fig. 3). This construct was ligated into a noncompatible ("suicide") vector and introduced into *Paracoccus* by conjugation; mutants created by site-specific recombination events were selected as detailed above (Fig. 3) and one such strain, ST4, analyzed further.

Characterization of Mutant and Complemented Mutants. The mutant strain ST4, deleted in the subunit II gene, was substantially decreased in its electron transport capacity from cytochrome *c* to oxygen: only 8.5% of the wild-type activity was reached. It was devoid of any spectrally detectable heme *a* (see Fig. 4) and, besides the expected loss of subunit II, also completely lacked subunit I, when membranes were probed with subunit-specific antibodies. Two explanations for this surprising result might be given: an assembly phenomenon, comparable to that seen in the bc_1 complex deletion mutants (see above), or a consequence of the complex genetic setup where the three open reading frames might contribute to the expression of an intact oxidase complex.

We therefore attempted to complement the mutation by offering *in trans* either an intact subunit II gene (preceded by its own presumed promoter region), or the complete subunit II to subunit III gene region (see Fig. 3). Interestingly, the full wild-type phenotype (in terms of electron transport capacity, spectrum, and presence of the two largest subunits) was only achieved when the complete gene locus was present on the complementing plasmid (strain ST4B; see also Fig. 4 and legend to Fig. 3). When the subunit II gene locus alone was offered on the plasmid *in trans*, only partial complementation was reached in strain ST4N. Under these circumstances, expression of subunit II was operative; the subunit was inserted into the membrane and visualized by Western blotting, even though with a tendency to form aggregates not easily cleaved upon SDS electrophoresis (not shown). However, subunit I was still not detectable to any extent, and, in accordance with present ideas of heme association, no *a*-type heme component was observed in the redox spectrum (see Fig. 4). As yet still unexplained, this

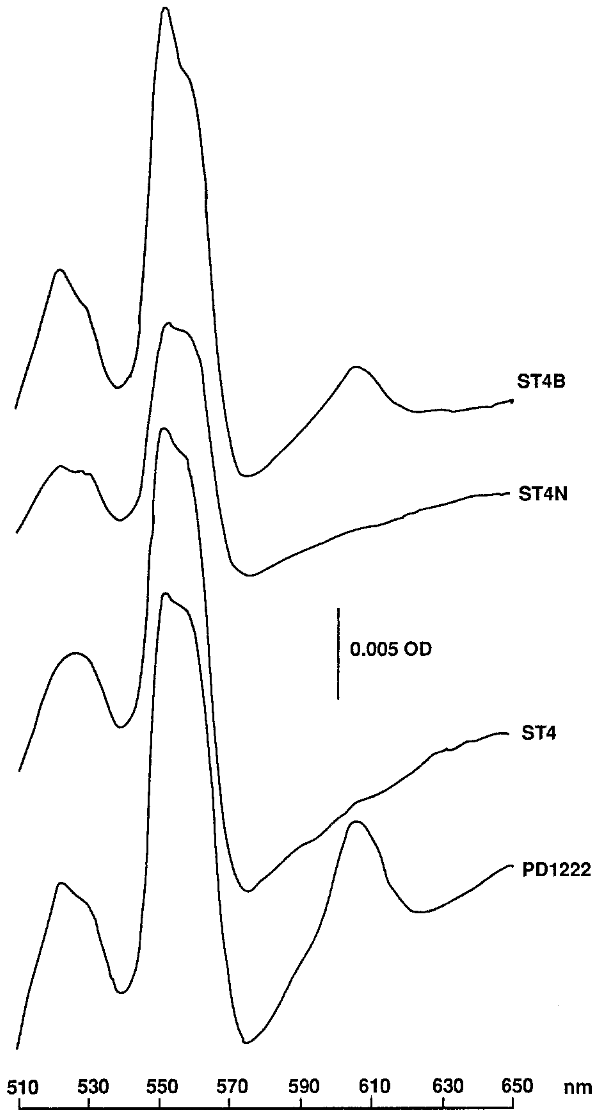

Fig. 4. Spectral properties of *Paracoccus* parent (PD1222), oxidase subunit II deletion mutant (ST4), and complemented mutant strains (ST4N and ST4B, see also Fig. 3). Membranes were isolated from succinate-grown cells of late log phase, solubilized with 0.5% Triton X-100, and redox spectra (dithionite *minus* air-oxidized) recorded in the α-region at room temperature at a protein concentration of 3.7 mg/ml.

complemented mutant ST4N exhibited 25% of the wild-type electron transport rate in membranes.

As has been documented also for the assembly of the yeast F_1F_0 complex recently (Ackerman and Tzagoloff, 1990), these findings suggest a participation of one of the open reading frame genes in the expression or assembly of *Paracoccus* oxidase: in the chromosomal background, transcription of genes downstream of the *cta*C gene will be arrested due to the marker gene insertion, if these genes are indeed arranged into an operon structure. Therefore, of the two alternatives tested here, only the complementation with the large fragment will lead to transcription and translation of the subunit II and the following open reading frame genes. Different deletion mutants and complementation approaches are currently being tested to gain further insight into the organization of genes and assembly processes for oxidase subunits (see also Haltia *et al.*, 1990).

Cytochrome c_{552}

Suggested Role of the c_{552} in the Redox Chain

Unlike the well-characterized, soluble cytochrome c_{550} of mitochondria which acts as a mediator between complex III and complex IV, a membrane-bound c-type cytochrome has been suggested to perform this function in *Paracoccus* (Berry and Trumpower, 1985). Evidence came from the isolation of an enzymatically active "supercomplex" consisting of the bc_1 and aa_3 complexes in tight association with a hydrophobic cytochrome c_{552} of M_r 22 kDa.

Characterization of Cytochrome c_{552} and Generation of Antibodies

The cytochrome was purified either via the ubiquinol oxidase supercomplex or by directly solubilizing membranes with Triton X-100, followed by a series of chromatographic steps (M. Jetzek, unpublished). In our hands, the purified cytochrome showed an absorption maximum at 551 nm in the α-region (reduced *minus* oxidized form, room temperature, using mammalian cytochrome c as an internal standard at 550 nm). On SDS gels, an apparent molecular weight of 20–22 kDa was determined, and the material occasionally migrated as two closely spaced bands. When the sample denaturation was performed under nonreducing conditions, two distinct bands of equal intensity in heme and protein staining at 22 kDa and approximately 16 kDa were observed. Antibodies raised against each individual band cross-reacted with both bands, suggesting different forms of the protein on denaturation. When peptide fragments were sequenced

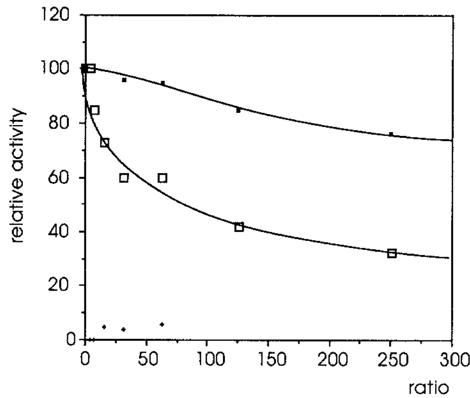

Fig. 5. Inhibition of electron transport in wild-type *Paracoccus* membranes by an anti-cytochrome c_{552} immuneglobulin fraction. Isolated membranes (5 nM aa_3) were assayed spectrally for NADH oxidase activity at 340 nm under aerobic conditions at 25°C in 20 mM KPi, pH 7.5 (NADH: 0.1 mM final concentration). For the immunological inhibition studies, membranes were preincubated for 30 min at 4°C with rising amounts (abscissa: weight ratio of immuneglobuline to membrane protein) of antibody specific for the membrane-bound *Paracoccus* cytochrome c_{552} (open squares) or, as a control, the soluble *Paracoccus* cytochrome c_{550} (filled squares). Activity is given in percent of the uninhibited rate. Small crosses indicate residual activity in the presence of 1 mM KCN.

(F. Buck and M. Jetzek, unpublished), typical *c*-type homology stretches were found.

Inhibition of Electron Transport Rates by Anti-c_{552} Antibodies

Effects on the electron transport rate of *Paracoccus* membranes exerted by various fractions of specific antibodies were determined. An immuneglobulin fraction (see Fig. 5) or a affinity purified fraction directed against the cytochrome c_{552} showed a significant decrease of the NADH oxidase activity of membranes derived from both succinate- or methanol-grown cells, whereas the same amount of an immuneglobulin fraction directed against the *Paracoccus* cytochrome c_{550} exerted only a minor inhibition. Further controls (not shown) using pre-immune sera, or the measurement of partial electron transport reactions (involving only complexes I *plus* III, and complex IV alone), showed no rate inhibitions and confirmed the specificity of the c_{552} antibody action. We conclude that the membrane-bound cytochrome c_{552} indeed plays a key role in transferring electrons between complexes III and IV, as suggested previously (Berry and Trumpower, 1985). Studies on cloning and deleting the gene for the c_{552} are in progress. In this context it should be noted that neither of the deletion mutants discussed above showed any change in the level of cytochrome c_{552}, which makes a coregulation unlikely.

Conclusions

The experiments outlined above represent an important prerequisite for future site-directed mutagenesis approaches toward structure and function of these respiratory complexes: using the *Paracoccus* system, they demonstrate that genes can be introduced into this bacterium and (1) either be maintained *in trans* and be expressed and their products incorporated into functional enzyme complexes, or (2) these genes, in an inactivated form, can exchange with their wild-type copies to yield site-specific mutants. Such deletion mutants may then be used to host and express genes altered by point mutations, thus preventing hybrid protein complexes from forming. For reasons of cofactor availability and differences in regulation of gene expression, such an approach would not be feasible in *E. coli*.

From a physiological point of view, these mutants allow a first glimpse into the complex assembly and stability phenomena governing the expression of such hydrophobic multi-subunit complexes. Some major questions, however, still remain unexplained, especially those on compensating routes of electron transport. With the bc_1 complex deletion mutants, no dramatic increase in the amount of cytochrome *o*, an alternative oxidase, is observed. This branch, shown to be competent in energy transduction also in *Paracoccus* (Puustinen *et al.*, 1989), could be expected to largely complement the mutation functionally, at least under aerobic conditions. Since no drastic increase in generation time is observed for this mutant strain under the growth conditions used here, the electron transport chain components may operate at rates much below maximum capacity and not be rate-limiting.

Another enigma is the ability of the aa_3 deletion mutants to grow on methylamine as the sole carbon source. Schemes of C_1 compound utilization (see, e.g., Stouthamer, 1980) rely exclusively on a functional *a*-type terminal oxidase to accept electrons, e.g., from methanol or methylamine dehydrogenase. One may hypothesize that electrons entering at the level of *c*-type cytochromes are transferred to oxygen via (1) another terminal oxidase that escaped detection so far (see also Henry and Vignais, 1979), (2) soluble enzymes able to react with oxygen in a side-reaction (like nitrite reductase), or (3) a shunt to the cytochrome *o* pathway, i.e., a *co* oxidase activity. Construction of double mutants (deleted in oxidase and reductase) will help elucidating this question.

The cytochrome c_{552} inhibition studies may be taken as further evidence for a contribution of this hydrophobic heme protein in the electron transport chain between complexes III and IV, a situation clearly different from that of the mitochondrial electron transport chain. The soluble c_{550} of *Paracoccus* (Scholes *et al.*, 1971), homologous to the mitochondrial cytochrome *c*, does

not seem to participate in this reaction (see also Kuo *et al.*, 1985), and may have other functions as electron mediator (van Spanning *et al.*, 1990). It will be interesting to obtain the full sequence for the *Paracoccus* c_{552} and further confirm its physiological role by gene deletion studies.

Note Added in Proof

Meanwhile we have been able to show that the genes *cta*B and *cta*G code for proteins obligatory for assembly of an intact cytochrome aa_3 (Steinrücke *et al.*, *J. Biol. Chem.*, submitted 1990).

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