The EMBO Journal vol.8 no.12 pp.3571 - 3579, 1989

Deletion of the gene for subunit III leads to defective assembly of bacterial cytochrome oxidase

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Communicated by M.Wikström

COIII is one of the major subunits in the mitochondrial and a bacterial cytochrome *c* oxidase, cytochrome aa_3. It does not contain any of the enzyme's redox-active metal centres and can be removed from the enzyme without major changes in its established functions. We have deleted the COIII gene from *Paracoccus denitrificans*. The mutant still expresses spectroscopically detectable enzyme almost as the wild-type, but its cytochrome *c* oxidase activity is much lower. From 50 to 80% of cytochrome *a* is reduced and its absorption maximum is 2–3 nm blue-shifted. The EPR signal of ferric cytochrome *a* is heterogeneous indicating the presence of multiple cytochrome *a* species. Proteolysis of the membrane-bound oxidase shows new cleavage sites both in COI and COII. DEAE-chromatography of solubilized enzyme yields fractions that contain a COI + COII complex and in addition haem-binding, free COI as well as free COII. The mutant phenotype can be complemented by introducing the COIII gene back to cells in a plasmid vector. We conclude that cytochrome oxidase assembles inefficiently in the absence of COIII and that this subunit may facilitate a late step in the assembly. The different oxidase species in the mutant represent either accumulating intermediates of the assembly pathway or dissociation products of a labile COI + COII complex and its conformational variants.

Key words: cytochrome aa_3/gene deletion/membrane protein assembly/*Paracoccus denitrificans*/subunit III

Introduction

Assembly of membrane protein complexes that are composed of several different subunits, sometimes in odd stoichiometries, probably requires specific nucleation and a unique pathway. In the bacterial photosynthetic reaction centre, subunit H, an amphipathic protein which is one of its four subunits, may act as such a nucleating centre (Chory *et al.*, 1984), and an organizing role has been postulated (Friedl *et al.*, 1981; Walker *et al.*, 1982) to a similarly amphipathic subunit b during biosynthesis of the *Escherichia coli* F_1F_0 ATP-synthase.

Cytochrome *c* oxidase (EC 1.9.3.1) is a multisubunit membrane protein essential for eukaryotic aerobic life. In mitochondria it has three major subunits (COI, COII and COIII), the genes for which are invariably found in mtDNA. Several other subunits (up to 10) are encoded by nuclear genes (Kadenbach *et al.*, 1987). The bacterial enzyme appears to be composed of subunits homologous to COI, COII and COIII. There may be, however, additional proteins that have a function in its structure or biosynthesis (Raitio *et al.*, 1987; Saraste *et al.*, 1989). *Paracoccus denitrificans*, a 'free-living mitochondrion', has an aerobic respiratory chain that is related to the mitochondrial one, and its cytochrome *c* oxidase closely resembles the mitochondrial enzyme (Ludwig, 1987).

Cytochrome *c* oxidase catalyses reduction of molecular oxygen to water. Four electrons needed for this reaction are donated by cytochrome *c* on the outer surface of the membrane. The electrons are transferred via two metal centres, Cu_A and cytochrome *a*, to the Fe–Cu bimetallic centre formed by cytochrome a_3 and Cu_B. This active site may reside approximately in the middle of the membrane dielectric (Holm *et al.*, 1987; Wikström, 1988). Four protons consumed in the reduction of oxygen to water are taken from the inside and, coupled to this reaction, four additional protons are 'pumped' across the membrane from the inside to its outside (Wikström, 1988,1989). Both processes contribute to the generation of proton electrochemical gradient across the membrane.

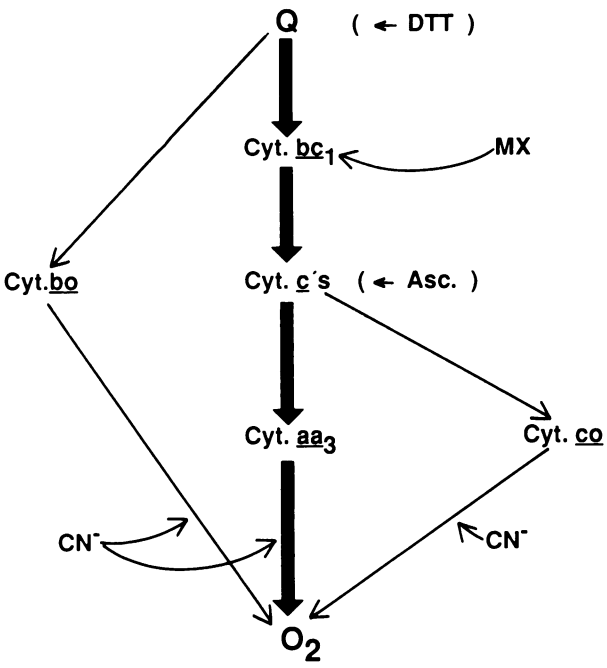

Fig. 1. Branched electron transfer chain of *Paracoccus denitrificans*. Diagram shows the terminal part of electron transfer chain in oxygen-respiring paracocci. Cytochromes *bo* and *co* are two alternative oxidases. The reaction sites of the inhibitors myxothiazol (MX) and cyanide as well as of the electron donors ubiquinol (Q) plus dithiothreitol (DTT) and ascorbate (Asc) plus cytochrome *c* are shown (see the section 'Cytochrome *c* oxidase activity' in Results).

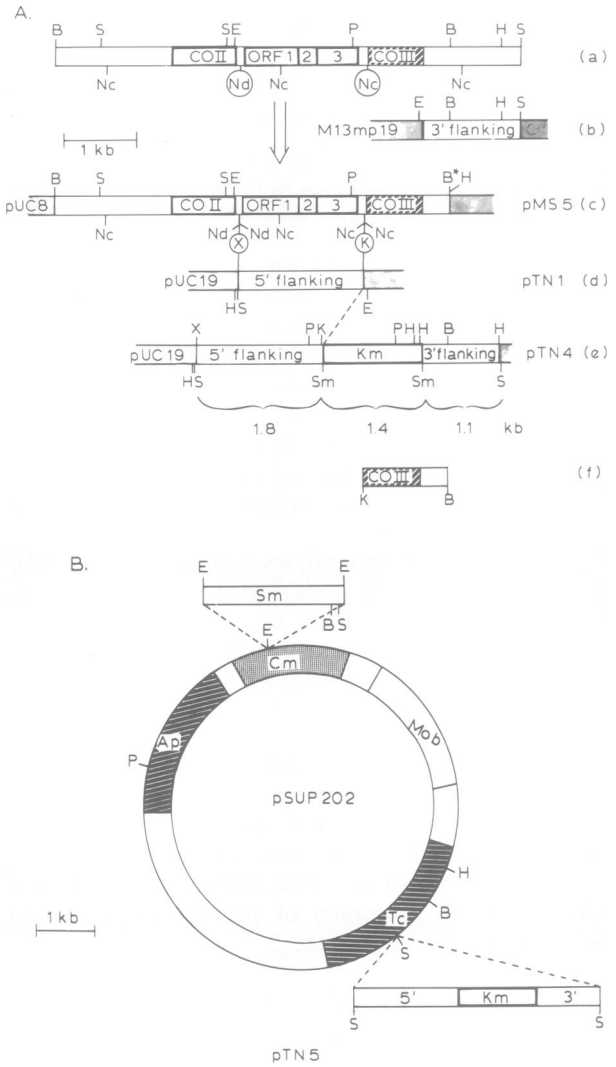

Fig. 2. Construction of the plasmid for gene replacement. (**A**) The map of the *cox1* locus is shown in (**a**); the COIII gene is dark-shaded. Two new restriction sites, *Xba*I and *Kpn*I, were constructed upstream of the ORF1 and COIII gene, respectively, inserting linkers into *Nde*I (Nd) and *Nco*I (Nc) sites. The 5′ flanking region in gene replacement was this *Xba*I–*Kpn*I fragment (**d**). The 3′ flanking region was obtained from an *Exo*III deletion clone earlier used for sequencing [(**b**); Raitio *et al.*, 1987]. The Km^R-marker was isolated as a *Sma*I fragment from pUC4-KIXX. Constructions were carried out in *E.coli* JM101 using pUC vectors. Restriction sites; B, *Bam*HI [B* is an inactivated *Bam*HI site in pMS5; (**c**)]; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; Nd, *Nde*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I. (**f**) shows the *Kpn*I–*Bam*HI fragment that was used in the complementation of TN-57. (**B**) Plasmid pTN5 used in homologous recombination. The insert of pTN4 [(**e**) in **A**] was inserted into the *Sal*I site and a Sm-resistance marker (see Materials and methods) into the *Eco*RI site of pSUP202 (Simon *et al.*, 1983).

It is currently thought that COI carries both haem A groups and Cu_B (Holm *et al.*, 1987; Müller *et al.*, 1988). It contains the bimetallic active site for oxygen reduction. The fourth metal, Cu_A, may be located in COII, which is also involved in cytochrome *c* binding. COIII can be removed from the purified enzyme without major alterations in the spectroscopic or catalytic properties (Penttilä, 1983; Püttner *et al.*, 1985; Finel and Wikström, 1986). Thus it certainly does not contain any of the redox centres. In fact, the bacterial cytochrome oxidase was originally purified without COIII (Ludwig and Schatz, 1980), and this subunit was found only after its gene had first been discovered (Raitio *et al.*, 1987; Haltia *et al.*, 1988).

The role of COIII in the oxidase has been widely discussed (Brunori *et al.*, 1987; Prochaska and Fink, 1987; Finel, 1989). It has been implied to participate in proton translocation. Dicyclohexylcarbodiimide (DCCD) modifies a membrane-buried, conserved glutamic acid residue in COIII causing a specific loss in proton translocation activity (Casey *et al.*, 1980), and removal of COIII has been reported to abolish this activity as well (Penttilä, 1983). However, H^+-translocation was subsequently demonstrated in liposomes reconstituted with the mitochondrial or bacterial oxidases lacking COIII (Solioz *et al.*, 1982; Puttner *et al.*, 1985; Finel and Wikström, 1986). A subunit homologous to COIII is also a component of the *E.coli* cytochrome *o* (an alternative terminal oxidase in bacteria; see Anraku and Gennis, 1987; Saraste *et al.*, 1989).

Although the aerobic respiratory chain of *P.denitrificans* closely resembles the mitochondrial one, this organism also displays clear bacterial characteristics (Figure 1). Up to five different terminal oxidase have been reported (Ludwig, 1987; Bosma, 1989). Oxygen tension and carbon source are thought to regulate their amounts in the cytoplasmic membrane (Poole, 1988). In the context of this paper, the possibility of having alternative oxidases has two corollaries. On one hand it allows a radical manipulation of one of these, because the organism survives by employing another. On the other, the presence of several oxidases with partially overlapping spectral and enzymological properties complicates the analysis of mutants.

We decided to investigate the function of COIII in cytochrome aa_3 by deleting its gene from the chromosome *P.denitrificans*. As we shall show, the absence of COIII leads to interesting defects in the assembly of cytochrome oxidase. The mutant can be complemented in *trans* when the COIII gene is introduced back into it in a plasmid vector.

Results

Construction of mutants lacking the COIII gene

In *Paracoccus*, the structural genes for cytochrome aa_3 are found in three separate loci (Raitio *et al.*, 1987,1989). Two of these contain variants of the COI gene; the third, *cox1*, encodes COII, three ORFs and COIII. We used homologous recombination to replace the COIII gene in *cox1* with a kanamycin resistance marker. The strategy of the gene replacement is shown in Figure 2. A Km^R marker was inserted between the flanking regions of COIII gene and a narrow host-range replicon pSUP202 (Simon *et al.*, 1983) was employed to harbour this construct, pTN5. A Sm^R marker was needed for discrimination of those *Paracoccus* clones that had acquired Km^R phenotype via a single crossing-over.

A suitable *E.coli* strain (S17-1, Table II) was first transformed and the plasmid further transferred via conjugation to the *P.denitrificans* 1222-strain, which has an enhanced conjugation frequency (Harms, 1988; de Vries *et al.*, 1989). In this host pTN5 is not able to replicate but, as it contains sequences homologous to the host cytochrome, it may integrate into the genome. Mutants created by double crossing-over should have a Km^R, Sm^S phenotype. Final screening was performed by Southern blotting. Ten double crossing-overs were identified among 120 trans-conjugants

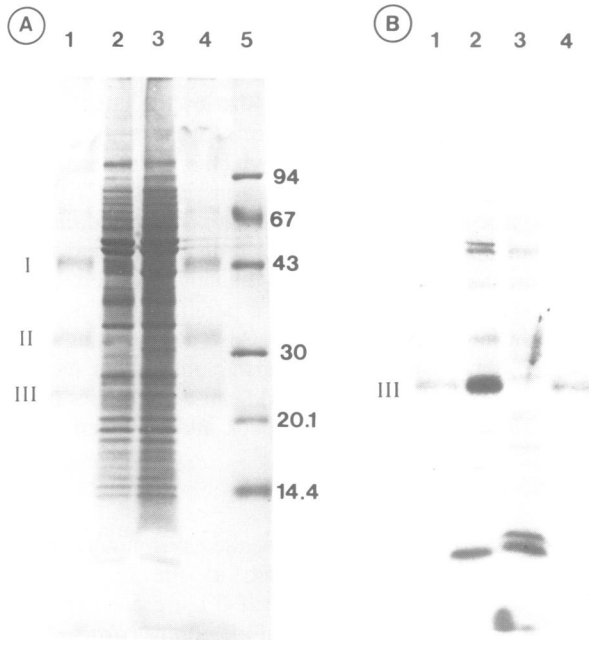

Fig. 3. DCCD-labelling of COIII. Membranes or cytochrome oxidase reconstituted into liposomes were labelled with radioactive DCCD and proteins analysed by SDS−PAGE (see Materials and methods). **A** is a Coomassie blue-stained gel, **B** an autoradiogram. **Lanes 1** and **4** are the isolated *Paracoccus* cytochrome oxidase, **lanes 2** and **3** membranes from the parent strain PD 1222 and COIII-minus mutant TN-57. The positions of subunits I, II and III are marked. **Lane 5**: mol. wt markers.

screened. One of them, TN-57, was selected for further analysis.

Antibodies raised against the purified three-subunit *Paracoccus* cytochrome oxidase (Haltia *et al.*, 1988) do not recognize COIII. Instead, we have used DCCD-labelling to detect this protein in membranes (Prochaska *et al.*, 1981; Haltia *et al.*, 1988). Membranes isolated from TN-57 and the parent strain (both of which contain spectroscopically detectable oxidase, see Figure 4) were labelled with [^{14}C]DCCD, solubilized with dodecyl maltoside and analysed by SDS−PAGE and autoradiography (Figure 3). There is no labelled protein migrating as the apparent 23 kd band (III) in the mutant membranes (lane 3), while this band is clear in the PD 1222 membranes (lane 2) or in the isolated enzyme (lanes 1 and 4). This verifies the COIII-minus phenotype of the mutant.

Optical spectra

Figure 4 shows reduced minus oxidized spectra of the membranes isolated from the mutant and parent strains. In A the spectra are recorded so that the total amount of cytochromes is detected: oxidation with ferricyanide (FIC) and reduction with dithionite. The characteristic α-band of cytochrome aa_3 at ~605 nm shows that the mutant still expresses cytochrome oxidase in amounts comparable to the parent strain (Figure 4A). The mutant has significantly increased absorption at 550−560 nm, which can be in part attributed to enhanced expression of cytochrome *o* (the *b*-type haems of this enzyme absorb around 560 nm; see Poole, 1988).

The redox centres in cytochrome oxidase get spontaneously oxidized when air is present. This is the case for the wild-type enzyme (upper trace in Figure 4B). It contains

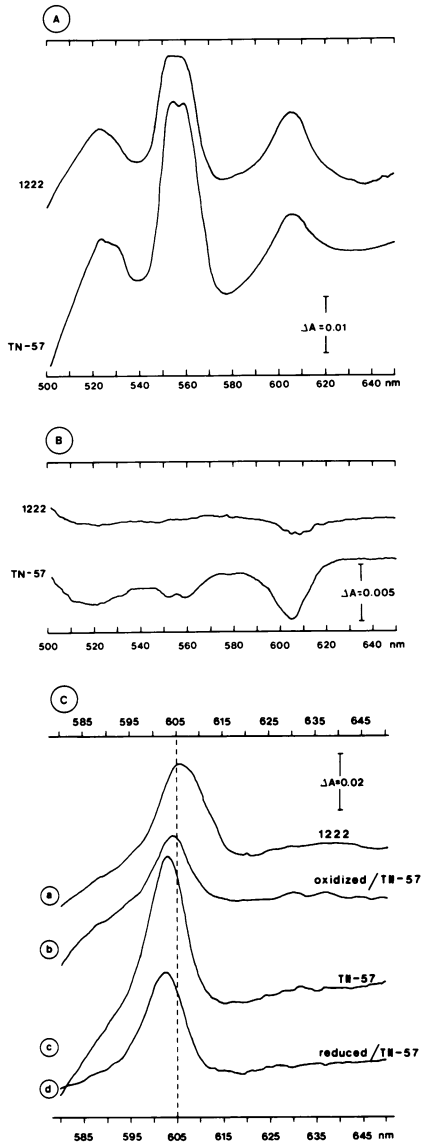

Fig. 4. Optical spectra. In **A** and **B** membrane samples were solubilized with dodecyl maltoside as described in Materials and methods. 1222 and TN-57 refer to the wild-type and mutant samples. (**A**) Reduced minus oxidized difference spectra at 500−650 nm. Samples were first oxidized by addition of solid FIC for recording of the baseline, then reduced with dithionite. Protein concentrations were 1.2 and 2.2 mg/ml in PD 1222 and TN-57. (**B**) FIC-oxidized minus auto-oxidized spectra. Baselines were recorded using auto-oxidized samples which were then further oxidized with solid FIC. Samples are the same as in **A**. (**C**) Difference spectra at liquid nitrogen temperature. (**a**) is the reduced (dithionite) minus oxidized (FIC) spectrum of the parent strain membranes; (**b**), (**c**) and (**d**) are spectra of the mutant cytochrome oxidase. (**b**) Dithionite-reduced minus autoxidized. (**c**) Dithionite-reduced minus FIC-oxidized. (**d**) Auto-oxidized minus FIC-oxidized. Protein concentrations of PD 1222 and TN-57 membranes were 12.4 and 21.8 mg/ml, respectively.

only a small amount of reduced cytochrome aa_3, revealed by a trough at 605 nm in the FIC-oxidized versus auto-oxidized spectra. In contrast, a major part of the mutant enzyme is reduced in the isolated membranes (lower trace).

Two facts suggest that it is cytochrome *a* and not cytochrome a_3 which stays reduced in the membranes: (i) cytochrome *a* accounts for 80% of the absorption at 605 nm; and (ii) carbon monoxide does not bind to the autoreduced mutant enzyme (data not shown); it is known

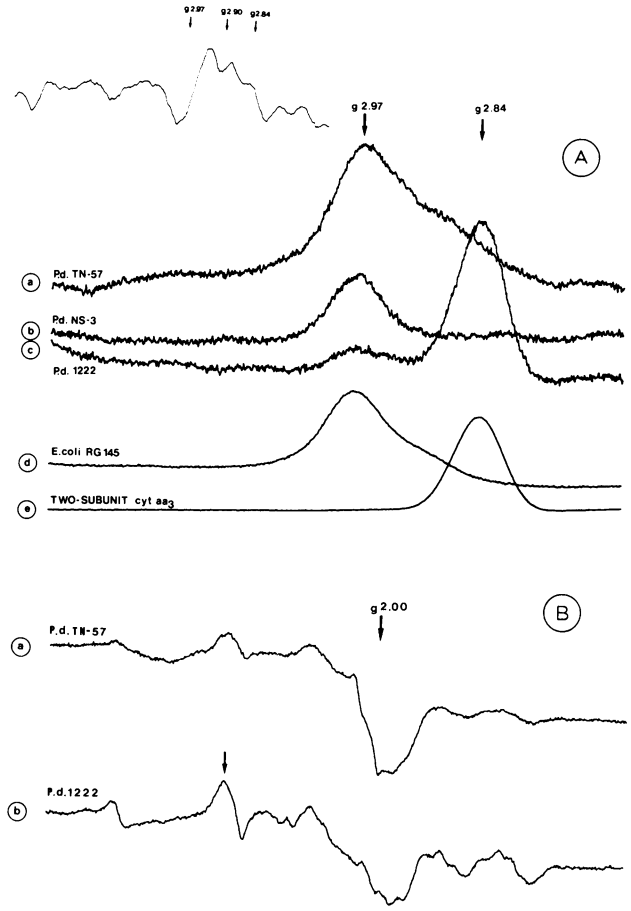

Fig. 5. EPR spectra. (**A**) g = 3 region in the EPR spectra of membrane vesicles from four bacterial stains and of the purified two-subunit *Paracoccus* cytochrome oxidase. g2.97 and g2.84 are two reference points discussed in the text. *Paracoccus* mutants TN-57 [(**a**), COIII-minus] and NS-3 [(**b**), cytochrome aa_3-minus] should be compared to the parent strain (**c**) and to the purified enzyme (**e**). Membranes from *E.coli* RG145 (**d**) are included for assignment of the cytochrome *o*-derived signal at g2.97. Instrumental conditions: modulation amplitude 3.1 mT, microwave power 0.5 mW, microwave frequency 9.44 GHz, time constant 0.164 s, temperature 6°K [except for (**d**) 8°K]. (**a**)–(**d**) are sums of four scans; a single scan from 0.2 to 0.25 T took 336 s. (**e**) is a sum of two scans. (**d**) was plotted on an eight times and (**e**) sixteen times less sensitive absorption derivative scale than (**a**)–(**c**). Cytochrome aa_3 (μM) and protein (mg/ml) concentrations were 10 μM in (**a**), 56 mg/ml in (**b**), 12 μM in (**c**), 48 mg/ml in (**d**) and 130 μM in (**e**). The insert (upper left corner) shows spectrum (**a**) after the contribution of cytochrome *o* at g2.97 has been subtracted. The difference spectrum was smoothed using a filtering method described in Biermann and Ziegler (1986); filter width was 4.8 mT and the resolution enhancement parameter α 6.0. (**B**) g = 2 region of the EPR spectra of the mutant (**a**) and parent strain (**b**) membranes. Instrumental conditions: modulation ampitude 1.0 mT, microwave power 10.0 mW, time constant 0.0410 s, temperature 31 K. Spectra are sums of four scans from 0.28 to 0.38 T; each scan took 168 s. The arrow at (**b**) points to the most prominent manganese line; g2.00 is marked for reference. Cytochrome oxidase concentrations were 11 μM (**a**) and 8.4 μM (**b**).

to bind only to the ferrous cytochrome a_3 (see Wikström *et al.*, 1981). CO-difference spectra measured after complete reduction suggests that the cytochrome a_3 site has been formed both in the autoreduced and auto-oxidized mutant enzyme. Thus the presence of reduced cytochrome *a* may indicate that the internal electron transfer is blocked or defective in the mutant.

Low-temperature spectroscopy of the reduced α-band in the mutant membranes is summarized in Figure 4C. Both the autoreduced and auto-oxidized enzyme species have slightly shifted peak positions. The α-maximum of the former is more blue-shifted than that of the latter.

The α-band arises from an electronic transition of the porphyrin macrocycle inner ring which is influenced by the molecular orbitals of the central iron (Brill, 1977; Adar, 1978). Cytochrome *a* is bis-imidazole coordinated, and the molecular orbitals around the iron reflect both the distance and orientation of the histidine ligands (Peisach, 1978; Palmer, 1985). A change in these parameters is probably the reason for the observed shift. Also a weakening of the hydrogen bond that the formyl group substituent of haem A makes with the apoprotein could cause the higher transition energy of the α-band (Babcock and Callahan, 1983; Babcock, 1988).

EPR spectra

EPR is a sensitive method to probe the environment around a paramagnetic haem iron such as the bis-imidazole coordinated Fe^{3+} of cytochrome *a*. Figure 5A shows the g = 3 region in the EPR spectra of membrane particles from various strains of bacteria. The low spin signal at g2.84 is the g_z of cytochrome *a* (Erecinska *et al.*, 1979; Albracht *et al.*, 1980), whereas the g2.97 signal is likely to belong to the hexacoordinated haem in cytochrome *o*. The latter assignment is based on the spectrum (b) of a cytochrome aa_3-deficient *Paracoccus* mutant NS-3 (Willison *et al.*, 1981). It has been grown aerobically and contains only *b*-type cytochromes and must use terminal oxidases other than cytochrome aa_3. The assignment is confirmed by spectrum (d) of the membranes from *E.coli* RG145 that (over)expresses cytochrome *o* (Au and Gennis, 1987). The EPR parameters of this enzyme have been determined by Hata *et al.* (1985) using a purified preparation.

Comparison of the cytochrome *a* spectrum of TN-57 (a) to the parent strain (c) reveals a clear difference. The dominant feature in the former is an asymmetric signal that begins before g2.97 and ranges beyond g2.84. The parent strain membranes give a minor signal at g2.97 and a large, symmetric one at g2.84. The former resonance, due to the cytochrome *o*, has increased in the mutant, but in addition new signals have appeared between this and g2.84 (i.e. the normal cytochrome *a* signal). The intensity at g2.84 in the TN-57 membranes is much lower than expected on the basis of their oxidase concentration. The mutant membranes contain autoreduced enzyme (Figure 4B), and the signal around g2.90 increases slightly after oxidation with FIC; a complete oxidation cannot be achieved since FIC has not access to the inside of sealed vesicles (oxidation was maximally ~70%). This suggests, however, that the autoreduced fraction might give rise to the absorption between cytochrome *o* and normal cytochrome *a* signals.

The spectrum in the g2.90 region is better resolved after the contribution of cytochrome *o* to (a) was eliminated (the insert in the upper left corner of Figure 5A). This was accomplished by subtracting an appropriately scaled cytochrome *o* spectrum (b). One of the three resolved maxima (g2.84) can be attributed to the normal cytochrome *a*.

The EPR spectrum at $g = 3$ of the purified wild-type *Paracoccus* cytochrome oxidase which contains only two subunits, COI and COII, is shown in (e). It has a symmetric

Table I. Cytochrome oxidase activity with different electron donating systems

	Activity inhibitable with antibodies (e^-/sec)		Activity sensitive to myxothiazol (%)
	Asc + TMPD	Asc + cytochrome *c*	DTT + Q_1
PD1222	290	213	64
TN-57	8	37	0
TN-57/pMS13	366	260	70

The measurements with membrane vesicles were performed as described in Materials and methods. Molecular activities (e^-/s) are mean values of three to six experiments. PD1222 is the wild-type, TN-57 the COIII-lacking mutant, and TN-57/pMS13 is the complemented mutant.

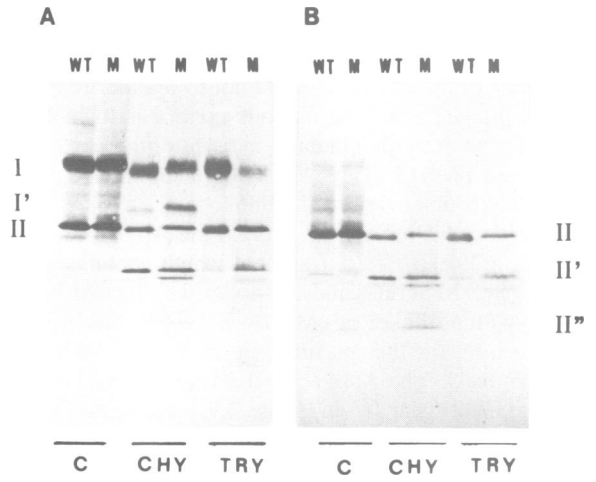

Fig. 6. Proteolysis of membranes. Membranes isolated from the wild-type (WT) and mutant (M) bacteria were digested with chymotrypsin (CHY) or trypsin (TRY). Undigested controls (C) are in **lanes 1** (PD 1222) and **2** (TN-57). After SDS–PAGE, immunoblots were developed either with antibodies against the COI + COII complex (**A**) or COII (**B**). I', II' and II" refer to proteolytic fragments produced from COI and COII.

g2.84 signal indicating that the ferric cytochrome *a* is completely normal [compare (a) and (e)]. This reflects the apparent paradox of our experiment: COIII can be removed from the properly assembled oxidase without any change in the environment of cytochrome *a*.

EPR spectra of the g = 2 region (Figure 5B) show that the mutant and parent strain enzymes bind Cu_A in comparable amounts. This metal gives rise to a characteristic trough in EPR spectrum around g2.00. In this region copper and manganese signals overlap. Tightly bound manganese has been shown to be located either in COI or COII; it could be removed only after partial denaturation of the oxidase (Seelig *et al.*, 1981). The general shape of the mutant spectrum has changed because of the weaker manganese signals [the arrow in (b) shows the position of the major Mn line]. Thus manganese may be involved in formation of the properly assembled subunit complex.

Cytochrome c oxidase activity

In order to investigate the terminal segment of the respiratory chain, oxidase activity was measured polarographically using three different electron donating systems: (i) ascorbate plus tetramethyl-*p*-phenylenediamine (TMPD, an electron mediator), (ii) ascorbate plus cytochrome *c* and (iii) dithiothreitol (DTT) plus ubiquinone-1 (Q_1) (see Figure 1). There is evidence for another cytochrome *c* oxidizing enzyme, cytochrome *co*, in *Paracoccus* (Bosma, 1989). The activity due to cytochrome aa_3 was therefore defined as that inhibited with a specific antiserum (Table I). These antibodies inhibited practically all cyanide-sensitive cytochrome *c* oxidation in both the parent strain and mutant membranes. Myxothiazol, a specific inhibitor of the cytochrome *c* reductase (cytochrome bc_1; Figure 1), was found to inhibit the oxidation of Q_1 in the wild-type but not in the mutant membranes (Table I).

Three points emerged. First, the mutant has a dramatically lowered oxidase activity especially with ascorbate plus TMPD. Secondly, cytochrome *c* slightly enhanced the activity of the mutant enzyme. Thirdly, there is no myxothiazol-sensitive respiration in the mutant.

In *Paracoccus*, the physiological electron donor to cytochrome aa_3 is thought to be a membrane-bound cytochrome c_{552} (Bolgiano *et al.*, 1989). The low ascorbate-TMPD-oxidase activity which can be enhanced by addition of external cytochrome *c*, may indicate that the absence of COIII perturbs the interaction between cytochrome c_{552} and the oxidase. Also the insensitivity of the respiration to myxothiazol in the mutant shows that electrons cannot enter the oxidase physiologically, that is via cytochromes bc_1 and c_{552}. In addition, it suggests that another oxidase takes part in the oxygen consumption of TN-57 membranes. This is most likely the ubiquinol-oxidizing cytochrome *bo* (Parsonage *et al.*, 1986; see Figures 1 and 5A and Table I).

Proteolysis in situ

In the isolated or membrane-bound wild-type enzyme the extreme N-terminus of COI and the loop that connects two membrane-spanning segments of COII are accessible to chymotrypsin and trypsin (Finel, 1988). Proteolytic cleavage of the COII loop produces a characteristic fragment labelled II' in Figure 6.

New fragments were generated from COI and COII, when the mutant membranes were subjected to a similar proteolysis. Figure 6 shows immunoblots of membrane proteins after cleavage with chymotrypsin and trypsin *in situ*. The blottings were carried out using either an antiserum against the purified two-subunit enzyme (A) or against the purified COII (B). Undigested controls (C) of PD 1222 and TN-57 membranes (labelled WT and M) are shown for comparison. A shorter version of II' and a new proteolytic fragment II" are produced with chymotrypsin (CHY) from COII in the mutant membranes (middle lanes). Trypsin (TRY) appears to digest the mutant COI more effectively (lanes on the right in Figure 6A): although mobility of the COI band does not clearly increase, weak antibody-binding of the digested mutant protein may reflect a diminished immunological reactivity which is apparently caused by the N-terminal cleavage (M.Finel, unpublished results). Chymotrypsin produces a truncated COI, band I' (Figure 6A), in the mutant sample. (Note that all proteolytic digestions are partial.)

The exposure of new cleavage sites in COI and COII of the TN-57 membranes indicates that at least a part of these proteins either does not assemble into the normal quaternary structure or, in their complexes, has a conformation different from the mature one.

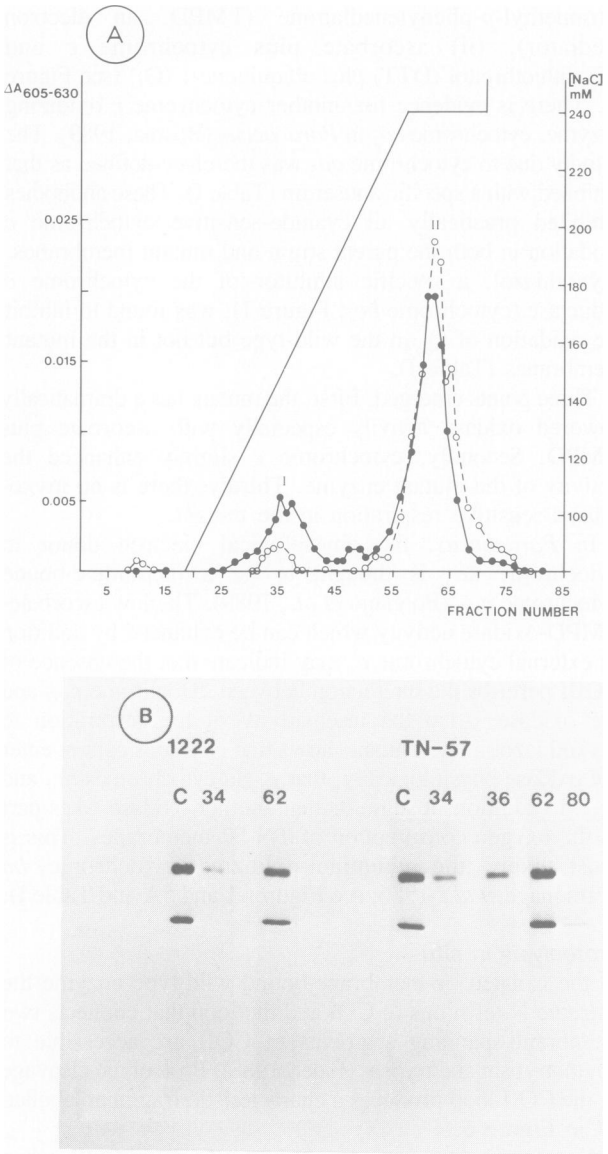

Fig. 7. Fractionation of solubilized cytochrome oxidase on a DEAE–Sepharose column. (**A**) Column conditions are described in Materials and methods; NaCl gradient is shown on the left. The wild-type (dashed line) and mutant (solid line) enzymes were followed by difference spectra at 605–630 nm (samples were first oxidized with FIC for base-line measurement, then reduced with dithionite for difference spectra). (**B**) Immunoblots of column fractions from (**A**) were developed with the anti-holoenzyme serum. C indicates control purified wild type oxidase samples. Numbers refer to the fractions in (**A**). Both subunits I (**top**) and II (**bottom**) bind the antibodies; COIII in the peak II of PD1222 sample is not detected. Note that on the right, both fractions 34 and 36 belong to peak I of the mutant sample.

Anion-exchange chromatography of solubilized oxidase

Up to this point we have studied the membrane-bound enzyme. The heterogenous optical and EPR signals as well as new sites accessible to proteolysis suggest, *ad hoc*, that multiple species of the mutant oxidase exist in the membranes. These might be isolated after solubilization. Figure 7A shows elution profiles of solubilized proteins from the parent strain and mutant membranes on a DEAE–Sepharose column. Cytochrome oxidase (monitored at 605 nm) elutes at two different positions. The elution profiles of the wild-type and TN-57 oxidases are qualitatively similar. However, the peak eluting at low ionic strength is larger in the mutant and corresponds to 22% of the total oxidase (4% in the control).

Immunoblots of the column fractions (Figure 7B) reveal that peak I contains only COI (fractions 34 and 36), whereas the major peak II contains both COI and COII (fractions 62). After the latter, fractions containing only COII are present in the mutant but not in the parent strain (fraction 80). (We mark that the antiserum does not detect COIII.)

In peak I, cytochrome *a* is 80–90% reduced and has a blue-shifted α-maximum regardless of the source of the membranes. In contrast, while the mutant oxidase in the major peak II is >50% reduced, the parent strain enzyme is >90% oxidized. The auto-oxidized component of the mutant peak II can be purified by further chromatographic steps (T.Haltia, unpublished results).

Complementation of the mutation

It is important to confirm that the mutant phenotype is caused by deletion of the COIII gene and not by another rearrangement in the genome. The obvious control is to return the deleted gene into the mutant in a plasmid vector. We constructed pMS13 (Table II) using a multi-host plasmid pMMB67 (Fürste *et al.*, 1986) as a vector. Since *P.denitrificans* is not able to express the Ap marker of this plasmid, a 2.4 kb *Eco*RI-fragment which contains a Sm^R-marker (See Materials and methods) was ligated into the mp18-type multilinker of pMMB67. This marker was used for selection of the plasmid in *E.coli* as well as in *P.denitrificans*. The *Kpn*I–*Bam*HI fragment that contains the entire COIII gene [(f) in Figure 2A] was inserted adjacent to the marker, *E.coli* strain SM10 (Table II) transformed and the plasmid further transferred to TN-57 via conjugation.

The mutant phenotype of TN-57 became fully complemented. Its oxidase activity, optical spectrum and EPR signals at g = 3 and g = 2 changed back to the wild-type. As it is shown in Table I, all three measured activities became similar to those of the wild type membranes.

Discussion

Our hope was to elucidate the role of COIII in cytochrome oxidase. Deletion of the COIII gene did not, however, yield a mutagenized, homogeneous enzyme with altered properties. Instead it lead to a population of defectively assembled cytochrome oxidases.

An important result of this study is that all metal centres of cytochrome aa_3 can be formed in the absence of COIII. However, most of the enzyme lacks activity, has an abnormal cytochrome *a* and a defect in the internal electron transfer. *Paracoccus* seems to feel this defect and compensates the physiological inactivity of cytochrome aa_3 by making more of another terminal oxidase, cytochrome *o*, which has a related function (Puustinen *et al.*, 1989).

Free COI that has probably bound two haems (and perhaps also Cu_B) appears to be one species of the mutant enzyme (D in Figure 8). Its cytochrome *a* is most probably reduced, has a blue-shifted α-peak and may give rise to one of the EPR-signals around g2.90 (the insert in Figure 5). The second population may be a COI + COII complex which still has cytochrome *a* in an unusual conformation (resulting in the second additional EPR signal) and a defect in the internal electron transfer to the binuclear centre. The third

Table II. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E.coli		
SM10	Km^R, *thi, thr, leu, lacY, tonA, phx, supE,* RP4-2 integrated (Tc :: Mu)	Simon *et al.*, 1983
S17-1	Sm^R, *pro*, res^-, mod^+, RP4-2 integrated (Tc :: Mu) (Km :: Tn7)	Simon *et al.*, 1983
RG145	Ap^R, cyt d^- contains pRG101 for overproduction of cytochrome *o*	Au and Gennis, 1987
P.denitrificans		
PD 1222	Rif^R, Spc^R, mod^+, enhanced conjugation frequency	De Vries *et al.*, 1989
TN 57	PD 1222, Km^R, $COIII^-$	This study
NS-3	cyt. aa_3^-	Willison *et al.*, 1981
Plasmids		
pSUP202	Ap^R, Cm^R, Tc^R, mob^+	Simon *et al.*, 1983
pTN5	pSUP202, Ap^R, Sm^R, Km^R-marker embedded with the flanking regions of COIII gene	This study
pMMB67EH	Ap^R, $lacI^Q$, *tacP, rrnB, bla,* multilinker mp18	Furste *et al.*, 1986
pMS13	pMMB67EH, Ap^R, Sm^R, COIII inserted as *KpnI*–*Bam*HI fragment	This study

[a]Rif, rifampicin; Spc, spectinomycin; Sm, streptomycin; Km, kanamycin; Ap, ampicillin; Tc, tetracyclin; Cm, chloramphenicol; res, host-specific restriction, the indices a and b refer to two different systems present (Harms, 1988); mod, host-specific modification; mob, conjugative transfer functions (Simon *et al.*, 1983).

oxidase species may resemble the active two-subunit enzyme that can be prepared from or is spontaneously created during isolation of the *Paracoccus* cytochrome oxidase (Ludwig and Schatz, 1980; Haltia *et al.*, 1988).

An alternative explanation of our data is that a two-subunit oxidase is primarily formed in the absence of COIII. This complex may not acquire a proper conformation, has a low electron transfer activity, a tendency to be autoreduced and to dissociate into COI and COII. Although this alternative cannot be ruled out, the facts that the g = 3 EPR region appears to contain two maxima in addition to the normal cytochrome *a* signal and that at least two species of mutant enzyme can be chromatographically isolated makes us favour the first scenario.

In any case COIII can be seen as a 'catalyst' of the assembly. This property of COIII is reminiscent of 'molecular chaperons' (Ellis, 1987; Hemmingsen *et al.*, 1988), which are crucial in the assembly of some oligomeric proteins. The scheme in Figure 8 illustrates this. It shows multiple variants of the (mutant) oxidase, some of which may also be true intermediates of the assembly pathway. These are a COI + COII complex which has not reached the conformation of the mature oxidase core (A), the mature three-subunit enzyme (B) and a two-subunit enzyme that already has found such a stable conformation (C). COIII is needed to enhance the transition from A to B. From the latter COIII can dissociate without conformational changes in the residual COI + COII complex (C). The A to C transition may also take place in the absence of COIII, although with lower probability (dashed line in Figure 8).

COIII enhances the assembly, but its role in cytochrome oxidase may not be restricted to this. Despite the fact that COIII can be easily lost during purification, it is most probably a stoichiometric component of the oxidase complex *in situ* (Haltia *et al.*, 1988; Bolgiano *et al.*, 1988). Cytochrome *c* binds to a cleft that is located between COII and COIII of different monomers in the dimeric bovine enzyme (Fuller *et al.*, 1981; Hall *et al.*, 1988). Studies on the oligomeric state of the enzyme have suggested that COIII resides in the interphase between the monomers (Finel and Wikström, 1988). The COIII-depleted isolated oxidase tends

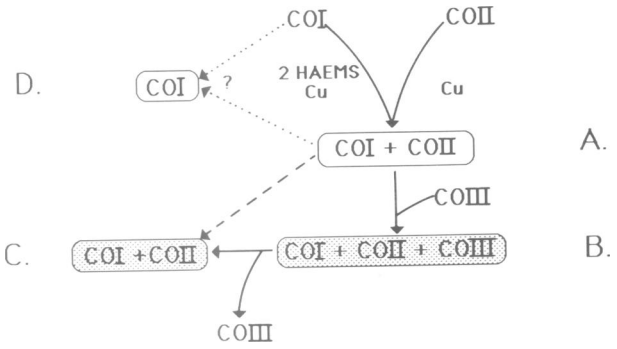

Fig. 8. Different species of the (mutant) cytochrome oxidase. Shaded complexes are active. See text for further explanation.

to be monomeric (Ludwig *et al.*, 1982; Finel, 1989). It is therefore possible that the absence of COIII influences the capability of the enzyme to dimerize and/or interact correctly with cytochrome *c*. The proton pump in cytochrome oxidase may need a dimeric structure to function (Finel, 1989). If this is true, COIII might have a very central controlling function both in the assembly and enzymic catalysis of cytochrome oxidase.

In yeast mitochondria the assembly of cytochrome oxidase is not at all nucleated in the mutants that lack nuclear-coded subunits (Poyton *et al.*, 1989). COI, COII and COIII are synthesized but are not able to form a haem-binding complex. Since the *Paracoccus* COI, COII and COIII are very similar to those in mitochondria, it seems that additional factors affect their assembly in eukaryotes.

Materials and methods

Materials

Bacterial strains and plasmids obtained elsewhere or constructed in this study are listed in Table II. Kanamycin- and streptomycin-resistance markers were taken from pUC4-KIXX (Pharmacia) and transposon Tn*1831* (see Harms, 1988), respectively. Restriction endonucleases were purchased form Promega. T4-DNA ligase was purified according to Panet *et al.* (1973). Preparation of competent *E.coli* and their transformation is described by Hanahan (1983).

Dodecyl maltoside was purchased from Boehringer, [^{14}C]DCCD from Amersham and ubiquionone from Hoffman-La-Roche. Antisera against the purified two-subunit *Paracoccus* cytochrome oxidase and COII were gifts from Professor B.Ludwig (Lübeck).

Deletion mutagenesis

General recombinant DNA methods are described by Maniatis *et al.* (1982). *E.coli* S17-1 transformed with pTN5 (Figure 1B) and the recipient *Paracoccus* PD1222 were grown overnight in selective media. Cells were harvested, washed thoroughly with medium without antibiotics and concentrated 5-fold. Donor (100 μl) and recipient (200 μl) cells were spread on thick LB plates, and conjugation was allowed to proceed for 48 h at 35°C (see Harms, 1988; de Vries *et al.*, 1989). Conjugation frequency was 8×10^{-7}. Clones (120) were grown two times overnight without selection on the plasmid markers. After this they were screened on replica plates containing Km + Rif or Sm + Rif. Ten mutants resistant to Km but sensitive to Sm were found. These were finally tested by Southern blotting using the Km-marker, COIII gene and the 5′ flanking *Xba*I-*Kpn*I fragment [(d) in Figure 1A] as probes.

Membrane preparations

All strains of *Paracoccus denitrificans* were grown with strong aeration in a 9 l or 400 l fermentor at 30°C using succinate as carbon source in the minimal medium of Ludwig (1986) modified so that pH was 7.1-7.4 at the time of inoculation and biotin was substituted for 0.1 g/l yeast extract. Cells were harvested after 7-10 h below Klett number 200 at 690 nm. No antibiotics were used in the fermentor cultures of TN-57. *E.coli* RG145 was grown to early log phase in the presence of ampicillin (50 μg/ml). Cells were frozen with dry ice and stored at −20°C.

Cells converted to spheroplasts were lysed with osmotic shock according to Berry and Trumpower (1985). Phenylmethylsulfonylfluoride (PMSF; 1 mM) and 0.5 mM benzamidine were used to inhibit proteolysis. After 15 min of lysis, 10 mM $MgCl_2$ and 0.2 mg pancreatic DNase I (Sigma) per g wet cells were added. Formation of spheroplasts from *P.denitrificans* NS-3 required overnight incubation with lysozyme.

Activity measurement

Oxygen consumption was measured polarographically using a Clark-type oxygen electrode at 25°C. The buffer (50 mM K-Hepes, 50 mM KCl, 1 mM EDTA, pH 7.2) was supplemented with 11 mM ascorbate, 24 μM horse heart cytochrome *c* (Sigma, type VI), 1.1 mM TMPD, 3.1 mM DTT and 63 μM Q_1 in the combinations indicated in Table I. Myzothiazol (300 nM) or antibodies (25 μg/ml) were used as inhibitors and finally KCN (56 μM) was added to determine baseline drift.

Spectrophotometry

Membranes (5-10 mg protein/ml) were solubilized in 2% dodecyl maltoside (DM). After 5 min at 4°C samples were centrifuged in Eppendorf tubes and white pellets discarded. The supernatants were diluted 1:4 in 200 mM Tris-HCl, 0.05% DM, pH 7.4, and spectra recorded using a Shmimadzu UV-3000 spectrophotometer. Membranes were diluted 1:1 with 200 mM K-HEPES, 2 mM EDTA, pH 7.4 for spectrophotometry at liquid nitrogen temperature. Cytochrome aa_3 concentrations were calculated with a millimolar extinction coefficient 23.4 cm^{-1} (Ludwig and Schatz, 1980).

EPR spectroscopy

Membranes for the EPR spectroscopy were suspended into 50 mM K-HEPES, 2 mM EDTA, pH 7.4, oxidized with FIC (PD 1222 and TN-57 membranes) and centrifuged for 20 min at 45 000 r.p.m. in a Beckman Ti60 rotor at 4°C. The membranes were washed until the supernatant was colorless. A Bruker ESP 300 X-band spectrometer equipped with an Oxford Instruments ESR 900 cryostat was used. Modulation frequency was 100 kHz. The exact instrumental conditions are found in the legend to Figure 5. Bruker ESP 1600 software version 2.0 was used in data processing.

Anion-exchange chromatography

Membranes (14 nmol of cytochrome aa_3, 10 mg protein/ml) were solubilized in 20 mM Tris-HCl, 1 mM PMSF, pH 8.0, 2% DM. After incubation on ice for 15 min, the samples were centrifuged in Eppendorf tubes for 10 min. The supernatants were applied to a DEAE-Sepharose CL-6B column (volume 6 ml) equilibrated with 20 mM Tris-HCl, 80 mM NaCl, 0.5 mM PMSF, 0.1% DM, pH 8.0 at 4°C. The elution was performed as shown in Figure 6A; ~1 ml fractions were collected and analysed by recording difference spectra analogous to those in Figure 3A.

Miscellaneous

Proteolysis experiments were performed as in Finel (1988) and DCCD-labelling as in (Haltia *et al.*, 1988). Protein concentrations were measured according to Markwell *et al.* (1978). SDS-PAGE, carried out using buffer system of Laemmli (1970) in 10-20% gradient acrylamide gels containing 5 M urea, and immunoblotting have been described by Finel and Wikström (1988). *Paracoccus* cytochrome aa_3 was purified according to Bolgiano *et al.* (1988) and Haltia *et al.* (1988).

Acknowledgements

This work has been supported by research contracts from Academy of Finland and the Sigrid Juselius Foundation. T.H. has received further support from the Emil Aaltonen Foundation and the Magnus Ehrnrooth Foundation. We are grateful to Mirja Leppäkoski and Hilkka Vuorenmaa for skilful technical assistance and to Dr Joel Morgan for discussions.

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Received on August 7, 1989