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Subunit III of cytochrome *c* oxidase is not involved in proton translocation: a site-directed mutagenesis study

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Subunit III (COIII) is one of the three core subunits of the aa_3-type cytochrome *c* oxidase. COIII does not contain any of the redox centres and can be removed from the purified enzyme but has a function during biosynthesis of the enzyme. Dicyclohexyl carbodiimide (DCCD) modifies a conserved glutamic acid residue in COIII and abolishes the proton translocation activity of the enzyme. In this study, the invariant carboxylic acids E98 (the DCCD-binding glutamic acid) and D259 of COIII were changed by site-directed mutagenesis to study their role in proton pumping. Spectroscopy and activity measurements show that a structurally normal enzyme, which is active in electron transfer, is formed in the presence of the mutagenized COIII. Experiments with bacterial spheroplasts indicate that the mutant oxidases are fully competent in proton translocation. In the absence of the COIII gene, only a fraction of the oxidase is assembled into an enzyme with low but significant activity. This residual activity is also coupled to proton translocation. We conclude that, in contrast to numerous earlier suggestions, COIII is not an essential element of the proton pump.

Key words: cytochrome aa_3/DCCD-binding glutamic acid/*Paracoccus denitrificans*/proton pump/site-directed mutagenesis/subunit III

Introduction

Subunit III (COIII) is a product of a mitochondrial gene and is one of the invariant protein constituents of the eukaryotic, proton-translocating cytochrome oxidase (cytochrome aa_3, EC 1.9.3.1). The homologous bacterial oxidases were originally thought to contain only two subunits (COI and COII), but COIII or its gene has been recently found in a number of species (for a review, see Saraste, 1990). COIII is thus likely to be a universal part of all aa_3-type oxidases (Haltia *et al.*, 1988; Buse *et al.*, 1989; Ishizuka *et al.* 1990; Saraste *et al.*, 1991). Moreover, a homologous protein is present in the cytochrome *o* terminal oxidase complex of *Escherichia coli* (Chepuri *et al.*, 1990). We have shown that COIII is necessary for the biosynthesis of functional cytochrome oxidase by deleting its gene from *Paracoccus denitrificans* (Haltia *et al.*, 1989).

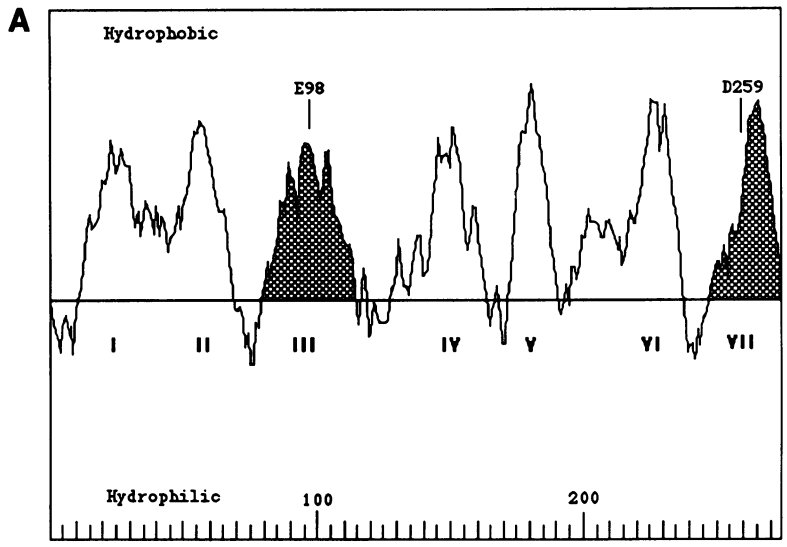
COIII is easily lost during isolation of the oxidase, yielding preparations composed of COI and COII only. However, careful purification using a mild non-ionic detergent such as dodecyl maltoside maintains COIII in the oxidase complex (Bolgiano *et al.*, 1988; Haltia *et al.*, 1988). The functional role of COIII has remained enigmatic: its removal from the purified enzyme does not have a large effect on the electron transfer or on the structure of the redox centres located in COI and COII. However, and most importantly, the removal of COIII invariably brings about a significant reduction in the proton translocation efficiency of the bovine enzyme (for reviews, see Prochaska and Fink, 1987; Brunori *et al.*, 1987). COIII contains a conserved glutamic acid residue (E98 in *P.denitrificans*) in the middle of a hydrophobic sequence that is likely to form a membrane-spanning helix (Raitio *et al.*, 1987; see Figure 1). A special property of this glutamic residue is that dicyclohexyl carbodiimide (DCCD) modifies it to a bulky dicyclohexyl-*N*-glutamyl urea derivative (Casey *et al.*, 1980; Prochaska *et al.*, 1981; Haltia *et al.*, 1988). The modified enzyme was reported to have lost its proton translocation activity. Consequently, E98 has been assigned an important role as a possible H^+ transfer element in proton pumping.

The hydropathy profile of COIII (Figure 1A) displays seven putative membrane-spanning segments. Most of the invariant residues reside in segment III (e.g. E98) and in the two C-terminal segments VI and VII (Figure 1B; Saraste, 1990). An invariant aspartate (D259) is located in the putative helix VII. Since ionizable residues in an otherwise hydrophobic environment are expected to have important structural and/or functional roles (Khorana, 1988; Gennis, 1989; Henderson *et al.*, 1990), both E98 and D259 were chosen as targets for site-directed mutagenesis. Our results show that the mutagenized oxidases are active in electron transfer and fully competent in proton translocation. In contrast to the site-specific mutants, the $COIII^-$ strain has only low oxidase activity, but even this is shown to be normally coupled to proton translocation. We conclude that

Table I. Electron transfer activities and proton translocation stoicheiometries

Strain	Activity (e^-/s)	H^+/e^-
pMS13(wt)	246	2.9
TN-57	8*	2.9
pTH2B(Glu98Gln)	272	2.9
pTH5B(Glu98Ala)	249	2.8
pTH7(Glu98Asp)	249	2.7
pTH12(Glu98Lys)	315	3.0
pTH11(Asp259Ala)	332	2.8

Electron transfer activities were determined using ascorbate plus TMPD as electron donors; the activity was defined as that sensitive to a cytochrome aa_3 antiserum (10 μl of antiserum was used in each assay). Note that in the proton translocation experiments succinate was used as the substrate.
*Higher activity (40–60 e^-/s, ~15% of control) was observed when cytochrome *c* was included in the assay; this activity was also confirmed with a purified preparation.



B

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                    III                                   VII
IN >>>>>>>>>>>>>>>>>>>> OUT          IN >>>>>>>>>>>>>>>>>>>> OUT
                  E98                                   D259
                   *                                     *
89   GFILFIMSEVMFFVAWFWAFIKNALYP   247   GFEAAAWYWHFVDVVWLFLFVVIYIWG   COX3$PARDE
89   GVGLFIISEVFFFLAIFWAFFHSAISP   242   GYESGILYWHFVDVVWLFLYISVYYWG   COX3$ASPNI
81   GMILFIISEVLFFTGFFWAFYHSSLAP   234   GFEAGAWYWHFVDVVWLFLYVSIYWWG   COX3$BOVIN
81   GMILFITSEVFFFLGFFWAFYHSSLAP   234   GFEAAAWYWHFVDVVWLFLYVSIYWWG   COX3$CYPCA
82   GMILFILSEVLFFVSFFWAFFHSSLSP   235   GFEAAAWYWHFVDVVWLFLYITIYWWG   COX3$DROME
82   GMILFILSEVLFFVSFFWAFFHSSLSP   235   GFEAAAWYWHFVDVVWLFLYITIYWWG   COX3$DROYA
81   GMILFITSEVFFFAGFFWAFYHSSLAP   234   GFEAAAWYWHFVDVVWLFLYVSIYWWG   COX3$HUMAN
83   GMILFIASEVLFFASFFWAFFNSSLAP   236   GFEAAAWYWHFVDVVWLFLYLSIYWWG   COX3$LOCMI
84   GsILFIVSEVMSFFLFFWAssHSSLAP   237   GFEAAAWYWHFVDVVWLFpFVSIYWWG   COX3$MAIZE
81   GMILFIVSEVFFFAGFFWAFYHSSLVP   234   GFEAAAWYWHFVDVIWLFLYVSIYWWG   COX3$MOUSE
89   GIILFIVSEALFFLAIFWAFFHSALTP   242   GFEGGILYWHFVDVVWLFLYISVYYWG   COX3$NEUCR
84   GsILFIVSEVMFLFAFFWAssHSSLAP   237   GFEAAAWYWHFVDVVWLFLFVSIYWWG   COX3$OENBE
84   GsILFIVSEVMFLFAFFWAssHSSLAP   237   GFEAAAWYWHFVDVVWLFpFVSIYWWG   COX3$ORYSA
81   PMILFITSEVCFFFAFFWAFFHSSLAP   234   GFEAAAWYWHFVDVVWLFLYVCIYWWG   COX3$PARLI
81   GMILFIVSEVFFFAGFFWAFYHSSLVP   234   GFEAAAWYWHFVDVVWLFLYVSIYWWG   COX3$RAT
87   GFILFIMSEVFAFISVFWAYFHSALSP   240   GTETSITYWHFVDTVWLFLFTVVYFWG   COX3$SCHCO
87   GFMLFTISETFLFASIFWAFFHSSLSP   242   GFECGIYYWHFCDVVWLFTYLTIYIWG   COX3$SCHPO
84   GsIpFIVSEVMFLFAFFWAssHSSLAP   237   GFEAAAWYWHFVDVVWLFLFVSIYWWG   COX3$SOYBN
80   GMILFITSEVCFFFAFFWAFFHSSLAP   233   GFEAAAWYWHFVDVVWFVLYWLIYWWG   COX3$STRPU
84   GsILFIVSEVMFLFAFFWAssHSSLAP   237   GFEAAAWYWHFVDVVWLFpFVSIYWWG   COX3$WHEAT
81   GMILFITSEVFFFIGFFWAFYNSSLAP   234   GFEAA-WYWHFVDVVWLFLYVSIYWWG   COX3$XENLA
89   GFLMFVLSEVLIFAGLFWAYFHSAMSP   242   GYETTIIYTHVLDVIWLFLYVTFYWWG   COX3$YEAST
28   GFWLFLGGETVLFASLFATFLAASNSN   179   KFYVASLYWHFIDVVWVFIFTVVYLMG   Cox3/B.sub
29   GFWLFLGGETVLFASLFATYLALKDKT   179   KFYVASLYWHFIDVVWVFIFTVVYLMG   Cox3/PS3
27   GFWIYLMSDCILFSILFATYAVLVNGT   178   RIMCLSLFWHFLDVVMICVFTVVYLMG   CyoC/E.coli
     ## ### ##   #   #  #                #     ############  ##  #
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Fig. 1. Target residues for mutagenesis. **A**: The hydropathy profile of *P.denitrificans* COIII, calculated using the Kyte and Doolittle (1982) index with a window length of 11. Seven predicted transmembrane segments are labelled I–VII, and the locations of E98 and D259 are marked. **B**: Sequence alignment demonstrating the invariant features in segments III and VII. The sequences were taken from the SwissProt database (release no. 15.0, the sequence identifiers are on the right) except for those of *Bacillus subtilis* (B.sub), the thermophilic bacillus PS3 (PS3) and *E.coli* (E.coli) which are from Saraste *et al.* (1991), Ishizuka *et al.*(1990) and Chepuri *et al.* (1990), respectively. The *P.denitrificans* COIII sequence is in the top line. The amoeban sequences predicted from the kinetoplast genes were omitted due to the extensive mRNA editing in this organelle (see Feagin *et al.*, 1988). Similar editing takes place in plant mitochondria. It is likely that the apparent prolines (lower case) at positions 93 (soy bean) and 265 (maize, rice and wheat) are changed to leucines, and the apparent serines at positions 91, 109 and 110 (maize, primrose, rice, soy bean and wheat) are actually phenylalanines or leucines due to C → U conversions in the middle position of triplets (see Gualberto *et al.*, 1989; Covello and Gray, 1989). The plant sequence identifiers are underlined. In the *E.coli* CyoC protein, E98 is substituted with an aspartate. The two invariant carboxylic acids are marked with an *, and arrow-heads on the top indicate the orientation of the segments in the membrane (IN refers to the cytoplasmic and OUT to the periplasmic side; this topology is taken from Chepuri and Gennis (1990). The sequence positions well conserved both in the mitochondrial and bacterial proteins are underlined with #.

COIII is not essential for the proton pump. This result should disprove the >10 year-old hypothesis about the involvement of COIII in the cytochrome oxidase proton pump (Casey *et al.*, 1980; Solioz, 1984; Brunori *et al.*, 1987; Prochaska and Fink, 1987; Chan and Li, 1990).

Results and discussion

Deletion of the COIII gene from *P.denitrificans* results in poorly assembled, heterogeneous cytochrome oxidase complexes. The activity of the enzyme in the COIII$^-$ strain is low but detectable (Table I; Haltia *et al.*, 1989). As we shall show below, even this residual activity—which may be due to a small amount of properly formed COI–COII complex—is coupled to proton translocation. However, to ascertain this result, we have modified two residues, the DCCD-binding E98 and another invariant carboxylic acid, D259 (Figure 1), and introduced COIII genes bearing these mutations back to the COIII$^-$ strain. The mutagenized subunits complement the deletion phenotype and produce enzyme with electron transfer activities comparable with that of the wild type (Table I). The mutants are therefore more suitable than the deletion strain for proton translocation experiments.

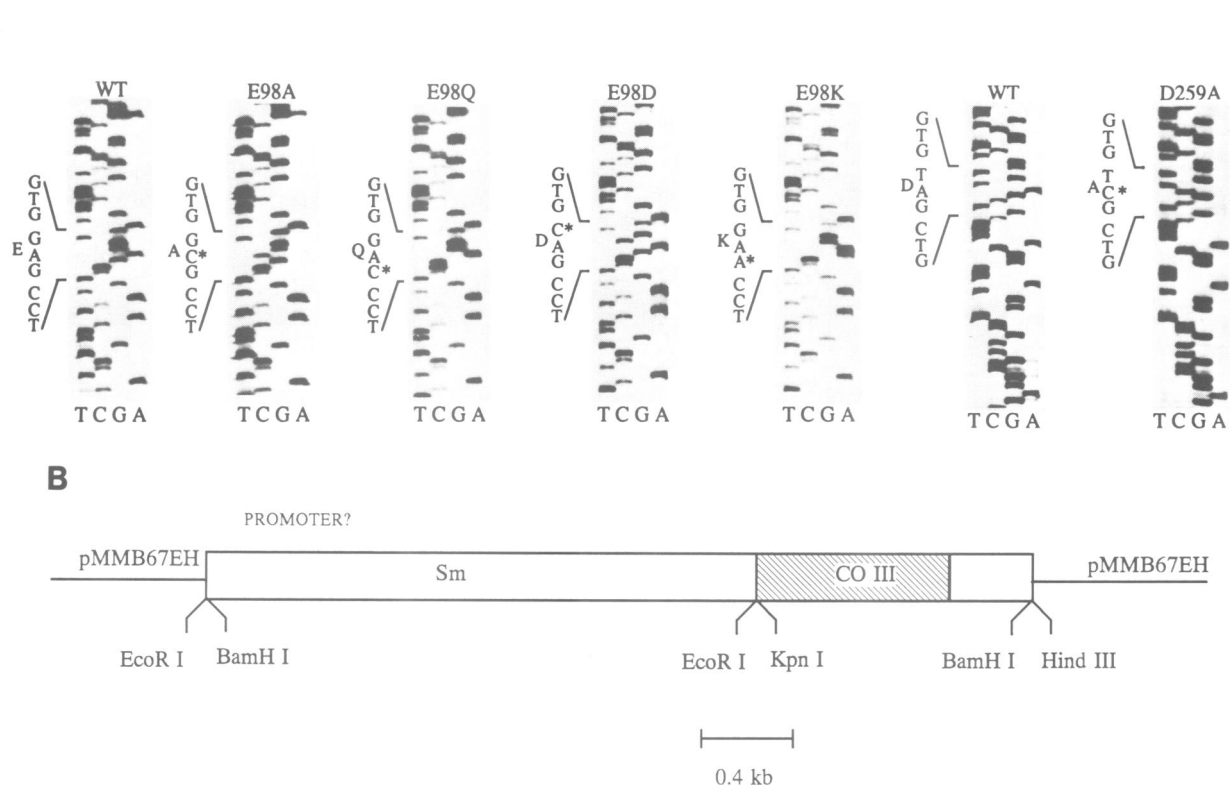

Fig. 2. (**A**). An autoradiogram of a DNA sequencing gel verifying each mutation. The relevant part of the COIII sequence is shown on the left of each sequence ladder. The desired mutation and the resulting amino acid change are indicated. WT = wild type. (**B**). The construct used to express the mutagenized COIII gene in *P.denitrificans* TN-57. Sm denotes a streptomycin marker derived from Tn1831 (Harms, 1988). The expression of COIII was observed to be dependent on the orientation of the Sm marker.

Four E98 mutants with different types of side chains were constructed (Figure 2A). The glutamate was replaced with alanine, glutamine, aspartate or lysine. D259 was substituted with alanine. The expression plasmid construct is shown in Figure 2B. The orientation of the streptomycin resistance marker (see Materials and methods) appears to be important for the expression of the COIII gene. The marker probably contains a promoter that, in the correct orientation, allows the transcription of a downstream gene.

Mutagenesis of E98 and D259 does not affect H^+ translocation

The turnover numbers in Table I show that all mutants exhibit activities comparable with that of the wild type strain, when ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) were used as electron donor. An advantage of this substrate system is that it yields very low activity with the $COIII^-$ strain, while high activities are observed with the wild type enzyme. This assay can thus be used to characterize the mutant phenotypes. However, a population of cytochrome aa_3 in the $COIII^-$ strain has a turnover number as high as 60 e^-/s (about 15% of control). This activity, which is observed in the presence of added cytochrome *c*, was also verified by purifying the auto-oxidizable cytochrome aa_3 fraction from the deletion mutant (see Haltia *et al.*, 1989).

The low spin haem of cytochrome oxidase has an EPR signal at $g = 2.84$ (Albracht *et al.*, 1980). In the $COIII^-$ mutant TN-57, this signal is changed and appears to be heterogeneous, reflecting improper assembly of cytochrome aa_3 in the absence of COIII. Moreover, most cytochrome aa_3 is not functional in the deletion mutant, which therefore over-expresses an alternative terminal oxidase, cytochrome *o*. The low spin haem in this enzyme gives rise to an EPR signal at $g = 2.98$ (see Haltia *et al.*, 1989). The $g = 3$ EPR spectra from the membranes of the site-directed mutants (Figure 3) show, first, that after the introduction of the mutagenized COIII genes back to the deletion strain, cytochrome aa_3 again becomes the dominant terminal oxidase. Secondly, there are only slightly elevated levels of free COI in the mutants (see Haltia *et al.*, 1989), as inferred from the low amplitude of the resonance at $g = 2.94$. This implies that there is no longer a major assembly defect during the biosynthesis of cytochrome oxidase. The deletion mutant appears to have a significant amount of uncomplexed COI, producing the $g = 2.94$ shoulder upfield from the cytochrome *o* resonance at $g = 2.98$. This assignment is based on the spectrum of isolated COI (Figure 3, spectrum h).

Figure 4A schematically illustrates proton translocation linked to electron transfer from the quinol pool to oxygen via two co-existing terminal oxidases. Our assay for proton translocation of cytochrome aa_3 assumes that there are no other proton pumps coupled to cytochrome *c* oxidation in the cells. To control this, we have used anti-cytochrome aa_3 serum. This inhibits 90–100% of cyanide-sensitive respiration, suggesting that alternative cytochrome *c* oxidases are not present.

Figure 4B shows typical proton translocation data with the *P.denitrificans* spheroplasts using different electron donors and acceptors. The results shown were the same, within experimental error, in wild type, mutated and $COIII^-$ strains of *Paracoccus*. Trace A demonstrates that with succinate as the substrate, the pulse of O_2 yields an H^+/e^- ratio near 3, which is consistent with earlier findings

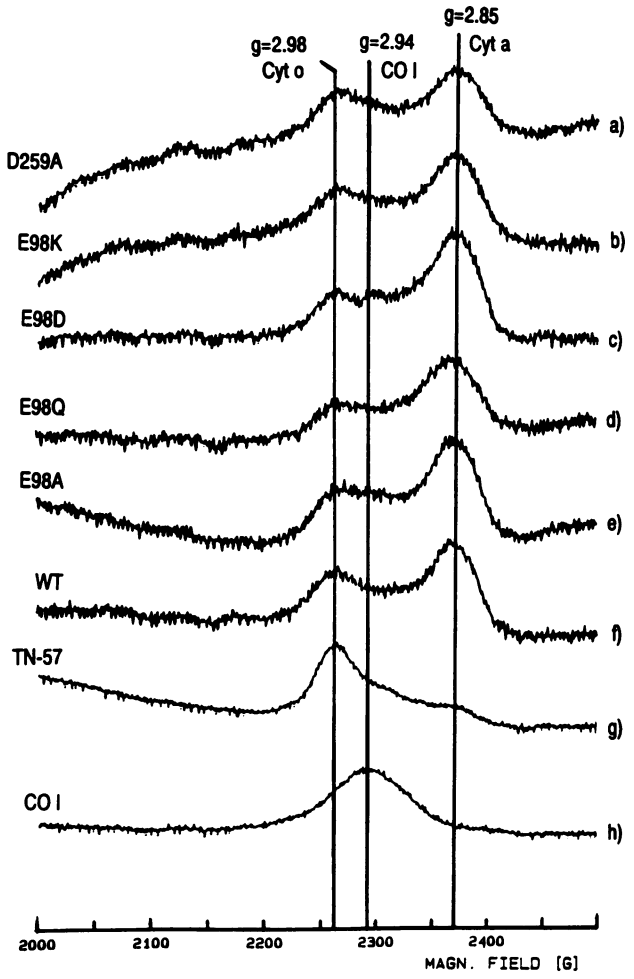

Fig. 3. g = 3 region EPR spectra of isolated COI and membrane particles from the mutant and wild type strains. Cytochrome aa_3 has a signal at g = 2.85. Uncomplexed COI isolated from the membranes of the COIII$^-$ mutant is shown to have a broad resonance at g = 2.94. The signal at g = 2.98 is due to an alternative oxidase, cytochrome *o*. Spectra **a**–**e** and **f** are from the membranes of the site-specific mutants and of the wild type, respectively. Spectrum **g** from the deletion strain membranes should be compared with spectrum **h** of isolated COI (11 μM cytochrome *a*) and with the spectra of the specific mutants. Conditions for EPR spectroscopy: modulation frequency, 100 kHz; modulation amplitude, 20 G; microwave frequency, 9.44 GHz; microwave power, 5 mW; sample temperature, 12 K; time constant, 41 ms; scanning rate, 3.0 G/s; each spectrum is a sum of four scans. Spectra g and h are plotted on a scale two times less sensitive than the other spectra. Protein concentrations range from 37 to 42 mg/ml (8–10 μM oxidase).

(Puustinen *et al.*, 1989) and is identical to results with mitochondria. As shown previously, the electron transfer pathway from ubiquinol via the bc_1 and aa_3 complexes is normally preferred over the cytochrome *o* pathway (see Figure 4A). This may be due to a lower affinity of cytochrome *o* for ubiquinol. When ferricyanide, which accepts electrons from cytochrome *c*, is used in place of O_2, the H^+/e^- ratio drops to a value near 2 (trace B). This is the expected result with the proton-motive cytochrome bc_1 complex. These data thus strongly suggest that cytochrome aa_3 is indeed proton pumping in the mutants. The cytochrome *o* pathway is recruited when the bc_1 complex is inhibited by myxothiazol (MX) or when cytochrome aa_3 is totally absent as in *P.denitrificans* NS-3. In both cases, the H^+/e^- ratio is found to be just below 2 (see trace C and also Puustinen *et al.*, 1989).

To activate the cytochrome *c* oxidase complex alone, we used ferrocyanide as a redox mediator to reduce cytochrome *c*, together with ascorbate as the ultimate electron donor (see Figure 4A). Figure 4B (trace D) shows that the H^+/e^- ratio approaches 1.5 under these conditions. The control in trace E shows that 0.5 H^+/e^- is due to the expected proton release on oxidation of ascorbate by ferricyanide. These results confirm that the mutant cytochrome oxidases indeed translocate close to 1 H^+/e^-, as deduced from the succinate oxidation data above (see also Table I).

The cytochrome *c* oxidase activity in the COIII$^-$ mutant is low but clearly detectable in the bacterial membranes (Haltia *et al.*, 1989; Table I). However, it is sufficient in the intact spheroplasts to effect normal proton translocation (Figure 4B), which by the oxygen pulse method yields results indistinguishable from the wild type. This supports the earlier proposal (Haltia *et al.*, 1989) that a small but significant fraction of the enzyme is assembled normally even in the absence of the COIII gene.

We conclude that COIII is not involved at all in the molecular mechanism of proton translocation. The total (Penttilä, 1983) or partial (see Solioz *et al.*, 1982; Brunori *et al.*, 1987; Prochaska and Fink, 1987) loss of proton pumping activity upon removal of COIII from the isolated enzyme must be ascribed to a secondary structural perturbation of the COI–COII complex. The inhibition of proton pumping by DCCD (Casey *et al.*, 1980; Prochaska *et al.*, 1981) may, analogously, be due to a perturbation resulting from the introduction of a bulky dicyclohexyl residue to a membrane-spanning segment of COIII. Alternatively, the correlation between the loss of proton pumping and the binding of DCCD may be coincidental, the real reason for the loss of proton translocation being an intramolecular cross-link in COI or COII produced by DCCD (Nalecz *et al.*, 1986). Since such a cross-link does not incorporate the isotopic label of [^{14}C]DCCD, it can remain undetected.

On the role of COIII

Calorimetric and thermal denaturation studies have shown that COIII unfolds as a separate entity with a transition temperature clearly lower than the rest of the enzyme (Rigell *et al.*, 1985; Rigell and Freire, 1987). Phosholipids influence most the transition temperature of COIII (Morin *et al.*, 1990). COIII is much more sensitive to proteolysis than COI and COII (Carroll and Racker, 1977; Malatesta *et al.*, 1983). These data as well as the relative ease with which COIII can be removed indicate that it may form a separate domain in the complex.

Biosynthesis of COI and COII and the formation of their redox centres appears to take place independently of COIII during the assembly of the cytochrome aa_3 complex (Haltia *et al.*, 1989). However, the COI–COII complexes formed are largely inactive in electron transfer; there appears to be a block of electron transfer between haem *a* and the binuclear Fe–Cu centre. Yet, a small fraction of these complexes appears to have a relatively high electron transfer activity, and as shown here, this is normally coupled to proton translocation. These results can be explained by assuming that COIII is required to assist a final step in the folding of COI, which may include structural fine-tuning of the haem centres. Only a minor fraction of the COI–COII complexes cross the activation barrier to yield functional enzyme in the

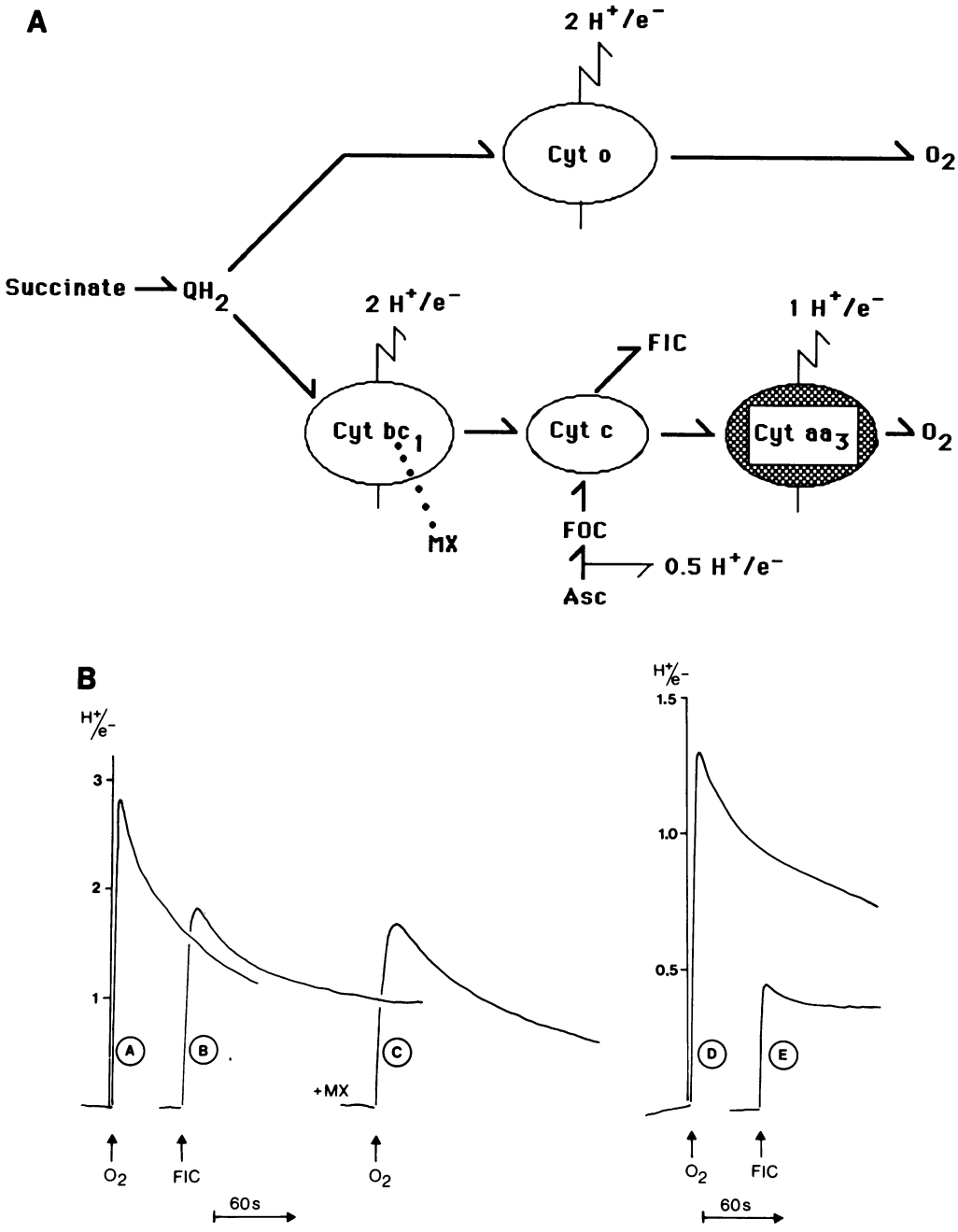

Fig. 4. (**A**). A scheme describing the proton translocation experiments. Ideal H^+/e^- ratios of proton extrusion are shown. Abbreviations: ascorbate, Asc; ferricyanide, FIC; ferrocyanide, FOC; myxothiazol, MX. (**B**). Proton translocation in *P.denitrificans* spheroplasts containing either a wild type COIII, mutagenized COIII, or no COIII. The electrode traces shown are from a set of experiments with the $COIII^-$ mutant but the other mutants and the wild type gave identical results. The ordinate was calibrated by additions of a HCl standard (5–10 μM), and by the amount of added O_2 (1–3 μM) or ferricyanide (FIC, 5 μM). The medium contained 100 mM KCl, 100 mM KSCN, 0.5 mM HEPES (pH 7.4), and 5 μM rotenone. In traces A–C, 2.5 mM potassium succinate was also present. In trace C, 5 μM myxothiazol (MX) was also added. In traces D and E, 10 mM potassium ascorbate, 5 mM potassium ferrocyanide (FOC) and 5 μM MX were present. Traces A and B show proton translocation linked to succinate oxidation by oxygen and ferricyanide, respectively. When MX is present, the pathway through cytochrome bc_1 is blocked, and succinate oxidation by O_2 is catalysed by cytochrome *o* (trace C). Trace D shows proton translocation coupled to oxidation of ferrocyanide by O_2.

absence of COIII. Such control may be necessary to keep nascent COI–COII complexes with their redox-active metal centres and the associated proton pump inactive until the assembly is completed. Otherwise, proton leaks and uncontrolled electron transfer might lead to collapse of the proton-motive force across the cell membrane, or to generation of oxygen radicals.

COIII remains a part of the enzyme after the assembly is completed. A reason for this may be that COIII stabilizes the active state of the enzyme. In the presence of COIII, conformational 'breathing', which may be necessary during catalysis (see Malmström, 1989), cannot cause the enzyme to jump out of the potential well that characterizes the optimal catalytic conformation. Enzyme from which COIII has been removed was reported to be less stable than enzyme containing this subunit. For example, the Cu_A site in COII becomes more prone to a structural perturbation (Li *et al.*, 1988). In addition, since mutagenesis of the DCCD-binding glutamic acid does not bring about any change in the proton pump, but modification with a bulky reagent does, COIII

might influence proton translocation indirectly by affecting the conformation of the rest of the enzyme. Removal of COIII from the assembled enzyme may, on this basis, be thought to destabilize the COI–COII complex, the first sign of which may be a slipping proton pump with a lower H^+/e^- ratio (Brunori *et al.*, 1987; Prochaska and Fink, 1987; Prochaska and Wilson, 1990). Finally, careful inspection of Figure 3 reveals that the E98 and D259 mutants may have slightly elevated levels of free COI (cf. signals at $g = 2.94$), as compared with the wild type. This suggests that the mutations may influence the ability of COIII to assist the folding of the two other subunits, although the effect is very small in comparison with that in the deletion mutant.

In summary, it is very unlikely that COIII is an intrinsic part of the proton pump in cytochrome oxidase (see also Finel and Wikström, 1986; Gregory and Ferguson-Miller, 1988; Finel, 1989). Our results imply that the search for protein structures involved in this activity should focus on COI and COII, and that the more than ten years of speculation about a role of COIII in proton pumping (Casey *et al.*, 1980; Prochaska *et al.*, 1981; Wikström and Casey, 1985; Capitanio *et al.*, 1990; Prochaska and Wilson, 1990; for reviews, see Azzi *et al.*, 1984; Brunori *et al.*, 1987; Prochaska and Fink, 1987; Chan and Li, 1990) should come to an end. COIII has a distinct role in the assembly of the functional oxidase (for other assembly factors needed at earlier stages of oxidase biosynthesis, see Nobrega *et al.*, 1990; Tzagoloff *et al.*, 1990; Steinrücke *et al.*, 1991). In addition, COIII may be a structural stabilizer of the mature oxidase complex.

Materials and methods

Materials

Strains and plasmids used and constructed in this study are found in Table II. Antiserum was a gift of Dr B.Ludwig (Lübeck, FRG) or was raised in a rabbit using cytochrome oxidase purified as described by Haltia *et al.* (1988) as the antigen.

Table II. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
E.coli		
SM10	Km^R, *thi*, *thr*, *leu*, *lacY*, *tonA*, *phx*, *supE*, RP4–2 integrated (Tc::Mu)	Simon *et al.*, 1983
P.denitrificans		
TN-57	Rif^R, Spc^R, Km^R, $COIII^-$, mod^+, enhanced conjugation frequency	Haltia *et al.*, 1989
NS-3	cytochrome aa_3^-	Willison *et al.*, 1981
Plasmids		
pMMB67EH	Ap^R, $lacI^Q$, *tacP*, *rrnB*, *bla*, multilinker mp18	Fürste *et al.*, 1986
pMS13	pMMB67EH, Sm^R, COIII as a *KpnI–BamHI* fragment	Haltia *et al.*, 1989
pTH2B	pMMB67EH, Sm^R, COIII(E98Q)	This study
pTH5B	pMMB67EH, Sm^R, COIII(E98A)	This study
pTH7	pMMB67EH, Sm^R, COIII(E98D)	This study
pTH11	pMMB67EH, Sm^R, COIII(D259A)	This study
pTH12	pMMB67EH, Sm^R, COIII(E98K)	This study

Strategy of mutagenesis

The oligonucleotides were synthesized on an Applied Biosystems 381A synthesizer. General DNA and cloning methods are described by Sambrook *et al.* (1989). The site-directed mutagenesis was carried out by the method of Eckstein (Sayers *et al.*, 1988), using a kit made by Amersham. A 1.4 kb *PstI–BamHI* fragment in M13mp18 containing the whole COIII gene (Raitio *et al.*, 1987) was used as the template. The mutants were screened by dideoxy nucleotide sequencing using specific primers and Sequenase 2.0 (USB); 10–30 plaques had to be sequenced to find at least one mutant. A 1.2 kb *KpnI–BamHI* fragment (Haltia *et al.*, 1989) containing a mutagenized COIII gene was ligated to the mp18 multilinker of pMMB67EH, to which a streptomycin marker (from Tn1831; Harms, 1988) was also cloned as an EcoRI fragment. *E.coli* SM10 transformed with this construct was allowed to conjugate with *P.denitrificans* TN-57. Rifampicin and streptomycin resistant colonies were selected for further studies. In order to check the presence of the desired mutation on the final construct after conjugation, the plasmid was isolated from the transformed *P.denitrificans* TN-57, single stranded DNA was generated by asymmetric PCR (Gyllensten and Erlich, 1988; White *et al.*, 1990) and the mutation was verified by dideoxy nucleotide sequencing.

Activity measurement

Cytochrome oxidase activity was measured polarographically in 50 mM K-HEPES, 50 mM KCl, 1 mM EDTA, pH 7.2, containing 11 mM ascorbate and 1.1 mM TMPD. When measuring the activity of the isolated oxidase from the $COIII^-$ strain, 24 μM cytochrome *c* (from horse heart, type VI, Sigma) was included in assay medium. The numbers were corrected for background auto-oxidation by subtracting the activity observed after addition of 10 μl cytochrome aa_3 antiserum.

Miscellaneous

Cultivation of cells, membrane preparations and recording of optical spectra were done as described previously (Haltia *et al.* 1989). Proton translocation was measured as described earlier (Puustinen *et al.*, 1989). EPR spectroscopy was carried out with a Bruker ESP-300 X-band spectrometer equipped with a liquid helium cooled ESR-900 cryostat (Oxford Instruments). Bruker 1600 software version 2.2 was used in data handling. The subunit I preparation used in Figure 3 was purified from *P.denitrificans* TN-57 membranes with two successive DEAE–Sepharose columns essentially as described earlier (Haltia *et al.*, 1989). Cytochrome *a* of this preparation stays reduced even in air-saturated medium, and therefore has no EPR signal. To render cytochrome *a* EPR-visible, the sample was oxidized with ferricyanide, which was subsequently removed by passing it through a Sephadex G-50 centricolumn.

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