Intrinsic uncoupling of cytochrome *c* oxidase may cause the maternally inherited mitochondrial diseases MELAS and LHON

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Received 14 May 1998

Abstract Mutations in the human mtDNA gene encoding subunit III of cytochrome *c* oxidase (CO) have been reported to cause MELAS and LHON. *Paracoccus denitrificans* cells expressing substitutions homologous to these MELAS- and LHON-causing mutations had lower growth yield than wild type cells and lower efficiency of proton pumping by CO (e.g. lower H^+/e^- ratio and lower $\Delta\Psi$), but had similar CO activity. These results indicate that both substitutions (F263L > A212T) cause intrinsic uncoupling, which may be the direct cause of the diseases. These results also suggest that subunit III is involved in proton pumping.

Key words: Mitochondrial disease; Cytochrome *c* oxidase; Proton pumping; Membrane potential; *Paracoccus denitrificans*

1. Introduction

Mutations in mitochondrial DNA (mtDNA) can lead to a number of maternally transmitted degenerative disorders [1]. In most cases these mutations are associated with a significant reduction of the activity of one or more of the mitochondrially encoded enzyme complexes, which is believed to be the direct cause of the clinical syndromes. Mutations in tRNA-(LEU), most commonly the substitution A3243G, usually result in a severe clinical syndrome – myoclonic encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). However, the same substitution may alternatively result, in other patients, in diabetes mellitus and deafness or in less common syndromes [2]. Additionally, three rare cases of MELAS were described that resulted from point mutations in peptide-encoding genes – NADH dehydrogenase genes ND1 (T3308C) [3], and ND5 (G13513A) [4], and cytochrome *c* oxidase subunit III gene (COIII (T9957C)) [5].

More commonly, point mutations in electron transport complexes result in a much milder syndrome – Leber's hereditary optic neuropathy (LHON). The most common mutations that cause LHON are three point mutations in NADH dehydrogenase peptides: ND1 (G3460A), ND4 (G11778A) and ND6 (T14484C) [1]. However, a few cases of LHON-associated point mutations in the COIII gene (COIII (G9438A) and COIII (G9804A)) were also reported [6]. Evidently, specific substitutions in the same polypeptide-coding gene can result in very different pathologic syndromes. For example, COIII G9804A produces an A200T substitution in subunit III, which can cause LHON, while COIII T9957C produces an F251L substitution, resulting in MELAS. No change in cytochrome *c* oxidase (CO) activity was reported for these patients, leaving open the question of the mechanism by which the latter mutations cause either MELAS or LHON.

CO reduces O_2 to water, in a reaction that is coupled to the generation of the mitochondrial electrochemical proton gradient, $\Delta\mu_H$ [7]. In eukaryotes, the three largest subunits are encoded by the mtDNA, and up to 10 additional subunits by nuclear genes. Similar bacterial oxidases contain homologs of subunits I–III. The recent determination of high resolution structures for the CO from beef heart [8] and that from the bacterium *Paracoccus denitrificans* [9] confirmed the high degree of structural similarity between the core subunits of the mammalian and bacterial enzymes previously predicted from the high degree of sequence similarity. Subunits I and II contain the metal cofactors (Cu, Fe) and catalyze electron transfer (subunit II) and oxygen reduction (subunit I). Subunit I also participates in proton consumption and proton pumping. However, subunit III has no well-established function.

In order to gain further insight into the role of CO subunit III, while at the same time investigating the molecular mechanisms by which MELAS- and LHON-causing mutations produce their pathological effects, we have introduced mutations in COIII from *P. denitrificans* homologous to three mitochondrial mutations proposed to cause LHON or MELAS. We found that the MELAS mutation and one LHON mutation cause intrinsic uncoupling of CO, which may explain their pathological effects and supports the suggestion that subunit III plays an important role in proton pumping.

2. Materials and methods

2.1. Bacterial growth and genetics

P. denitrificans strains were grown aerobically in Ludwig's succinate medium [10]. Mutations were prepared using the Altered Sites in vitro mutagenesis system (Promega) with minor modification. Details and sequences of the mutagenic primers available on request. Mutated COIII genes were subcloned into a derivative of pMS13 [11] and were mobilized into *P. denitrificans* TN-57 [11] for expression by conjugation as described [12], using *Escherichia coli* S17-1 [13] as the donor host.

2.2. Cell starvation

Cells (200 ml) were harvested during exponential growth at an A_{690} of 1.0–1.1, and the protein content was determined [14]. The cells were washed (×2) in one-fourth volume 200 mM KCl, 1.5 mM HEPES, pH 7.4 and collected by centrifugation (10 min, 6000×*g*). Cells to be used in proton pumping experiments were washed a third time, resus-

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Abbreviations: CO, cytochrome *c* oxidase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; LHON, Leber's hereditary optic neuropathy; MELAS, myoclonic encephalomyopathy with lactic acidosis and stroke-like episodes; mt, mitochondrial; PDB, Protein Data Base; TM, transmembrane helix (of a polypeptide); TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; TPP^+, tetraphenyl phosphonium ion; wt, wild type

PII: S0014-5793(98)00891-6

pended in one-half volume of the same solution, and incubated at 30°C for 1 h with moderate shaking. The starved cells were collected and resuspended in 200 mM KCl, 1.5 mM HEPES (pH 7.4), 2.5 mM $MgCl_2$ (weakly buffered KCl medium) at ~16–24 mg protein/ml. Cells to be used in TPP^+ uptake measurements ($\Delta\Psi$ determination) were washed again in 160 mM KCl, 50 mM HEPES (pH 7.4), 10 mM NaCl, 2 mM $MgCl_2$, resuspended in one-half volume 150 mM KCl, 50 mM HEPES (pH 7.4), 5 mM Na_2HPO_4, 1 mM $MgCl_2$ (buffered KCl medium), and incubated at 30°C for 1 h. The starved cells were collected and suspended in buffered KCl medium (~16–24 mg protein/ml).

2.3. Determination of turnover number

Membranes were prepared as described [11,15] and used immediately. The cytochrome content was determined after extraction with 2% dodecyl maltoside, as described [11]. Difference spectra were calculated by digital subtraction, dithionite reduced minus ferricyanide oxidized. Cytochrome concentrations were calculated after the method of Vanneste [16], with extinction coefficients extracted from published spectra of *P. denitrificans* cytochromes [15,17]. Oxygen consumption was measured with a Clark-type oxygen electrode in a 1.5 ml stirred cell; each membrane sample was incubated at 25°C in buffered KCl medium containing 10 μM myxothiazol for 10 min prior to addition of 10 mM ascorbate+0.2 mM TMPD.

2.4. Determination of proton translocation (H^+/O ratio)

The H^+/O ratios were determined using the oxygen pulse method [18]. Starved cells were incubated anaerobically under Ar gas for 1 h in a special stirring cuvette in a reaction medium of 100 mM KCl, 100 mM KSCN, 0.5 mM HEPES (pH 7.4), 0.5 mM $MgCl_2$, 60 μM phenol red, 20 μM myxothiazol and 30 μM rotenone. As required, 0.2N KOH was added to maintain the pH near 7.4. Proton pumping was initiated by injection of 15 μl low CO_2 air-saturated 150 mM KCl solution (14.1 nmol oxidizing equivalents), and observed as a decrease in the absorbance of phenol red [19], calibrated by injection of 15 μl anaerobic 1 mM HCl in 150 mM KCl.

2.5. Measurement of TPP^+ uptake and $\Delta\Psi$

$\Delta\Psi$ was estimated from the uptake of tetraphenyl phosphonium (TPP^+) [20], using a TPP^+-sensitive electrode [21]. The assays were conducted in 4 ml oxygen-saturated buffered KCl medium containing 4 μM TPP^+. 200 μl of concentrated starved cell suspension (~3.6 mg protein) was added, followed by myxothiazol to 20 μM, and the assay mixture incubated 20 min with stirring. Ascorbate and TMPD were then added to 10 mM and 0.2 mM (see Fig. 4A). The TPP^+ ratio (15 min after substrate addition) was calculated using 4.78 μl/mg protein as the internal volume of *P. denitrificans* cells [22], and was corrected for non-specific binding of TPP^+ to NaN_3-treated cells (0.6 nmol TPP^+/mg protein). The ascorbate plus TMPD oxidase activity of starved cells was measured with the oxygen electrode in buffered KCl medium with 20 μM myxothiazol and 30 μM rotenone, but without TPP^+.

3. Results

COIII variants with mutations equivalent to those proposed to cause LHON (A212T and G85S) or MELAS (F263L) were expressed in a *P. denitrificans* host strain that has a chromosomal deletion removing the gene coding for COIII. During exponential growth, the growth rates of the *P. denitrificans* COIII mutant strains were virtually the same as that of the complemented strain expressing wild type (wt) COIII. However, the growth of the F263L strain, and to a lesser extent, that of the A212T strain, slowed during early stationary phase, and the final cell density of F263L cultures is considerably lower than that of the complement, suggesting less efficient metabolism resulting in a lower growth yield (Fig. 1).

P. denitrificans strains expressing the A212T, F263L, and G85S COIII variants contain similar amounts of cytochromes in their cell membranes as does the complemented strain expressing the wt subunit III, when measured in situ in cell

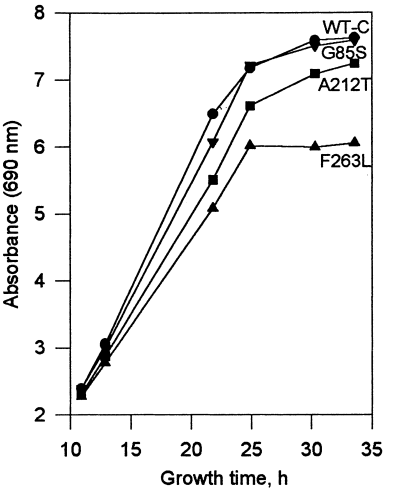

Fig. 1. Representative stationary phase growth curves of COIII mutant and wt complemented *P. denitrificans* cultures. Exponentially growing bacterial cells were inoculated into fresh, pre-warmed media, each at the same initial density.

suspensions (not shown) or in the membrane fraction isolated from lysed cells (Fig. 2A). The cellular oxidase activities of the cells expressing the COIII mutations were not significantly different than the wt activity (347 ± 7, 328 ± 24, 315 ± 36, and 294 ± 18 ng AO/min/mg protein for the A212T, F263L, G85S, and wt strains, respectively). Similarly, the turnover numbers measured in cell membranes were not significantly different from that of the complement (Fig. 2B).

The proton-pumping stoichiometry of CO containing each mutated COIII subunit was assessed by the oxygen pulse method, following a short starvation step to reduce endogenous electron transport activity, as described in Section 2. It was observed that the H^+/e^- stoichiometry of the A212T and of the F263L cells was significantly less than that of the wt cells, while the G85S stoichiometry was about the same (Fig. 3A). The average measured stoichiometries of the F263L and the A212T cells, 0.5 and 0.6 H^+/e^- respectively (corrected for the chemical release of 0.5 H^+ upon oxidation of ascorbate; Fig. 3B), are significantly lower than that of the wt ($P < 0.001$ for F263L, and $P < 0.01$ for A212T). The average H^+/e^- stoichiometry of the G85S strain was somewhat lower than the wt, but the difference was not statistically significant. The stoichiometry of 0.8 H^+/e^- measured for the wt strain using ascorbate as reductant is essentially the same as that previously reported for *P. denitrificans* [23].

We measured $\Delta\Psi$ from the uptake of TPP^+ in starved *P. denitrificans* cell suspensions at pH 7.4 (where the $\Delta pH \approx 0$). Starved cells maintain a reduced $\Delta\Psi$, which is largely reversed upon the addition of cyanide and azide (not shown). Upon the addition of ascorbate and TMPD, CO pumps and consumes protons, thus increasing the membrane potential (Fig. 4A). The average total potential generated upon ascorbate addition in suspensions of wt cells was 146 ± 2 mV, similar to values previously determined for these cells [22]. The uptake of TPP^+ by F263L cells and A212T cells was always less than that of wt complemented cells (Fig. 4A). The average $\Delta\Psi$ of F263L and A212T cells was significantly less than that produced by wt cells ($P < 0.00001$) (Fig. 4B). There was no significant difference in the $\Delta\Psi$ generated by G85S and wt

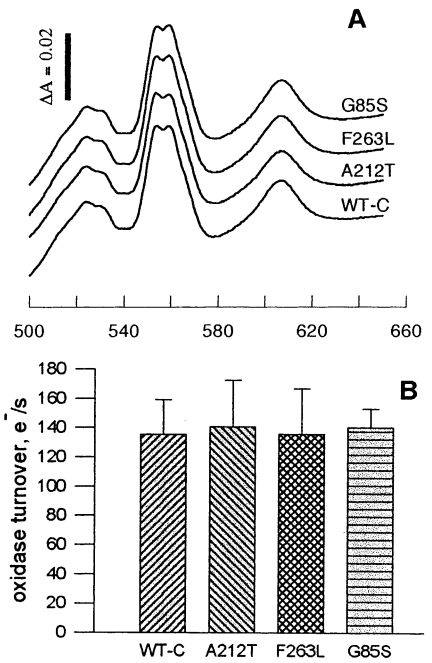

Fig. 2. Cytochrome content and oxidase activity of membranes from *P. denitrificans* strains. A: Reduced minus oxidized absorbance difference spectra of detergent extracted membranes from cells expressing mutated and wt COIII. B: Ascorbate+TMPD oxidase specific activity (turnover number) of membranes prepared from *P. denitrificans* cells (average of three preparations each).

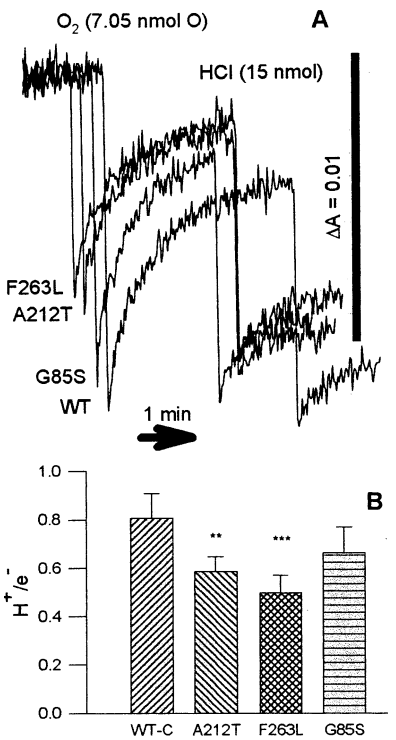

Fig. 3. Proton translocation by starved *P. denitrificans* cells. A: Representative proton pumping assays. B: Average H^+/e^- values from batches (n) of wt ($n=8$), A212T ($n=3$), F263L ($n=3$), and G85S ($n=3$) cells (≥ 3 measurements with each batch). $^{**}P<0.01$ and $^{***}P<0.001$, H^+/e^- values significantly different from the ratio of wt cells.

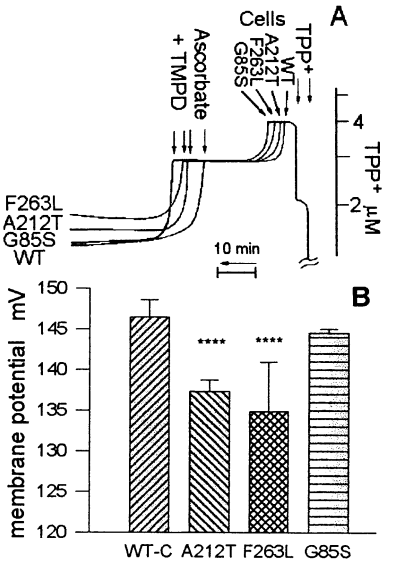

Fig. 4. Membrane potential generated by starved *P. denitrificans* cells. A: Uptake of TPP^+ by starved cells before and after addition of ascorbate+TMPD (oxidase substrate). Two aliquots of TPP^+ (8 nmol) were added before the cells to charge the solution and calibrate the electrodes. Downward deflection of the trace indicates TPP^+ uptake (lower external concentration). B: Average $\Delta\Psi$ calculated from TPP^+ uptake measurements with batches (n) of wt ($n=6$), A212T ($n=3$), F263L ($n=5$), and G85S ($n=3$) cells. $^{****}P<0.00001$, $\Delta\Psi$ values significantly different from the value of wt cells.

cells. The uptake of TPP^+ by F263L cells reached a maximum after 15–20 min, and then declined slowly, while wt cells (and G85S cells) maintained the accumulated TPP^+ for at least 30 min. Thus, it appears that F263L cells not only generate lower $\Delta\Psi$ by CO, but cannot maintain the generated $\Delta\Psi$ over time.

4. Discussion

4.1. The etiology of mitochondrial diseases caused by mutations in COIII

We have constructed mutations in COIII of *P. denitrificans* homologous to mutations in human COIII that were reported to cause MELAS (F263L) or LHON (A212T and G85S). More recent reports indicate that the G85S is not a pathologic mutation, but a natural polymorphism that was accidentally present in the disease cases [24]. This conclusion is in agreement with our findings that this mutation does not affect the growth yield of the mutant cells, and has no effect on the turnover number, H^+/e^- ratio or the $\Delta\Psi$ generated by CO. However, both the LHON-associated (A212T) mutation and the MELAS-associated (F263L) mutation reduced the growth yield of the mutant strain, reduced the proton pumping stoichiometry of CO and reduced the magnitude of membrane potential generated by CO. In all three effects, the enzyme carrying the MELAS-associated mutation was considerably more strongly affected than the enzyme carrying the LHON-associated mutation. Thus the degree of reduction in energy coupling caused by the mutations in *P. denitrificans* COIII parallels the severity of the symptoms caused by the corresponding mutations in human CO. Most previous studies assumed that the mitochondrial dysfunction in the various mtDNA diseases resulted from a reduction in activity of electron transport complexes or ATP synthase [1,2]. However, it

has been pointed out frequently (cf. [25]) that there is a large reserve capacity for most of these complexes, and that it is necessary to inhibit complex activity by at least 80–90% to affect oxidative phosphorylation. In many cases, the inhibition that is observed is modest and is not sufficient to account for the mitochondrial dysfunction. Our data suggest that intrinsic uncoupling of the mitochondrial complexes (i.e. reduced efficiency of $\Delta\mu_H$ generation) may be a direct cause of mitochondrial dysfunction in many cases of MELAS and LHON, and possibly in other mtDNA diseases. Although the magnitude of these effects in the *P. denitrificans* enzymes is not large, it is possible that the effects on the human enzyme are larger. Moreover, a 10–20 mV reduction in maximal $\Delta\Psi$ could greatly reduce the rate of ATP synthesis, and perhaps more importantly, the magnitude of the phosphate potential, (ATP/(ADP$\times$Pi)), that can be generated in the cell. It should be pointed out that all the peptides coded by mtDNA are essential membrane-embedded subunits of proton pumping complexes, and in most cases are involved directly or indirectly in proton pumping. It is therefore likely that many mutations in these peptides would result in proton leaks.

4.2. The role of subunit III in proton pumping and energy conversion

The crystal structures of CO (PDB structure #10CC [8,9]) show that subunit III is composed of seven transmembrane helices (TM). The first two (TMI–TMII), which are less conserved, form one domain apart from a five helix bundle, which is highly conserved. From the five helix bundle (TMIII–TMVII) only TMIII interacts with subunit I next to what appears to be a pore (now called the D channel), which is believed to be the pumped protons pathway from the mitochondrial matrix into the core of subunit I [26]. The other four helices, TMIV–TMVII, form a four helix bundle of tilted helices that appears to form a pore that is open to the cytoplasmic surface, resembling the structures of four helix ion channels (cf. [27,28]).

The subunit III-less enzyme can still couple O_2 reduction to proton pumping, but reportedly with a decreased extent [29], much reduced rate [30], and lower thermodynamic efficiency [31]. The subunit III-deleted enzyme also inactivates rapidly during continuous turnover [32]. The fact that subunit III in human CO is the site of several disease-causing mutations suggests that the subunit plays some role in mitochondrial energy metabolism. Substitution of neutral amino acids for two conserved, membrane-embedded, acidic residues in subunit III of *P. denitrificans* CO (E97A and D258A), did not significantly affect proton pumping (i.e. H^+/e^- ratios) [30]. However, we have found that these mutations inhibited CO-driven ATP synthesis, and one mutation (E97A), also inhibited the generation of $\Delta\mu_H$ [22]. All the subunit III point mutations that have been found to affect proton pumping and/or energy conversion are in the same peptide domain, either in the core of this bundle or just above the core, closer to the periplasmic surface. The MELAS-associated F263L mutation described here is on TMVII one and a half turns above (closer to the periplasmic surface) the D258 residue, which was shown previously to play a role in energy conversion [22]. The A212T mutation is on the adjacent TMVI one turn above a highly conserved histidine, H216, which is in the center of the four helix bundle. A conserved serine residue in yeast COIII (S203), the mutation of which was reported to inhibit aerobic growth, is also on TMVI, closer to the periplasmic surface [33]. Finally, the DCCD-reactive glutamate E97 (TMIII), which was previously thought to be involved in proton translocation [34], and was recently shown to be important for optimal energy conversion and the generation of $\Delta\Psi$ [22], is connected by a hydrogen bond chain to another conserved histidine on TMVI, H219, through a conserved tyrosine on TMVII (Y253) (see PDB structure #10CC).

These observations suggest that the periplasmic side of the four helix bundle of subunit III, and particularly TMVI and TMVII, play an important role in proton pumping and energy conversion. While the mechanism of proton pumping by CO is still largely unknown, it should be noted that to date all members of the Fe-Cu oxidase superfamily that maintain the conserved residues in subunit I thought to be important for the D channel proton pathway also contain a subunit III, and those oxidases that lack most of these residues also lack subunit III [35].

We suggest that a four helix bundle of subunit III (TMIV–TMVII) constitutes an exit channel for pumped protons in CO. There is evidence that protons are taken up by CO into subunit I through the D channel up to a conserved glutamate residue at the core of the membrane (E278 on helix VI of subunit I) [36]. However, the proton pathways across the membrane into the periplasmic space are not yet known. It is possible that a proton pathway exits, most likely composed of water molecules, from E278 on subunit I to E97 on subunit III. It was previously suggested, on the basis of the crystal structure, that a pathway for molecular oxygen exists along this route [37]. It is possible that this is a water filled channel that can conduct protons. Because E97 is hydrogen bonded to H219 on TMVI, a proton that reaches E97 could reach the core of the four helix bundle and from there diffuse to the periplasmic surface.

4.3. Subunit VIa and the control of human CO function

Another observation that is compatible with our explanation and may be relevant to the pathological effects of these mutations comes from studies of the role of subunit VIa. In the bovine heart enzyme (which is very similar to the human enzyme), subunit VIa interacts only with subunit III. The outer compartment tail of this subunit is situated on top of the outer compartment loops (periplasmic in the bacterial enzyme) of the four helix bundle blocking the hypothetical proton exit channel [8]. The role of subunit VIa in CO function is largely unknown. However, in the heart isoform, ATP binds to subunit VIa, and causes a reduction of the H^+/e^- ratio of the enzyme [38]. The behavior of the ATP-reacted heart enzyme, is similar to the behavior of the subunit III mutants described in this study. It is thus not unlikely that the disease-causing human mutants are not only intrinsically uncoupled but are also defective in the ability of subunit VIa to modulate their function.

Acknowledgements: We thank Dr. T. Haltia for providing *P. denitrificans* strains, as well as timely advice and encouragement. Supported by PHS Grant AG13779.

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