Involvement of Cytochrome *c* Oxidase Subunit III in Energy Coupling†

Shaolong Wu, Rafael Moreno-Sanchez,‡ and Hagai Rottenberg*

Department of Pathology, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19102

Received March 16, 1995; Revised Manuscript Received October 17, 1995®

ABSTRACT: The role of the conserved acidic residues of subunit III of cytochrome *c* oxidase (COIII) in energy transduction was investigated. Using a COIII deletion mutant of *Paracoccus denitrificans*, complemented with a plasmid expressing either the wild type (wt) COIII gene or site-directed mutants of the COIII gene, we measured cytochrome *c* oxidase−dependent ATP synthesis, respiration, and membrane potential. Cytochrome *c* oxidase−dependent ATP synthesis was attenuated in nonacidic mutants of either Glu^{98} (E98A and E98Q), or Asp^{259} (D259A) but not in the acidic mutant E98D. The rates of respiration in the energy conversion−defective mutants were as high as or higher than that in the wt. The cytochrome *c* oxidase−induced increment of membrane potential in the nonacidic mutants was similar to or higher than that in the wt. In contrast, when succinate-driven ATP synthesis was mediated solely by ubiquinol oxidase (e.g., in the presence of myxothiazol), the rates of ATP synthesis in the nonacidic mutants were higher than that in the wt. Moreover, myxothiazol, which inhibited succinate respiration as well as ATP synthesis in wt and E98D, stimulated ATP synthesis, while inhibiting succinate respiration, in the nonacidic mutants. These results indicate that the attenuation of energy conversion in these mutants is limited to cytochrome *c* oxidase and thus suggest that subunit III plays a role in energy conversion by cytochrome *c* oxidase.

Cytochrome *c* oxidase (cyt aa_3; COX),[1] the terminal oxidase of the mitochondrial electron transport chain, oxidizes cytochrome *c* and reduces O_2 to water, a reaction that is coupled to proton pumping (Wikstrom, Krab, & Saraste 1981; Haltia & Wikstrom, 1992). In prokaryotes several homologous, proton pumping, terminal oxidases, all belonging to the heme−copper respiratory oxidases superfamily, often coexist in the plasma membrane (Gracia-Horsman et al., 1994). The complexes of the bacterial oxidases are much smaller than the 13-subunit mitochondrial COX, sharing only three conserved peptides: subunits I, II, and III.

Subunit I contains a low-spin heme (cyt *a*), and a high-spin heme (cyt a_3) which forms a Fe−Cu binuclear center (with Cu_B). The binuclear center binds O_2, catalyzes the four-electron reduction of O_2 to water, and couples this process to proton pumping. Subunit II (in COX) contains two other Cu atoms (Cu_A), binds and oxidizes cytochrome *c*, and transfers the electron to cyt *a* on subunit I. Subunit II of the quinol oxidases, which have no Cu_A, binds and oxidizes ubiquinol (Hosler et al., 1993).

Subunit III is highly conserved in most of the heme−copper respiratory oxidase superfamily, and in some enzymes it is partially or completely fused with subunit I (Garcia-Horsman et al., 1994). However, its function is still unknown. The deletion of subunit III of the *P. denitrificans* enzyme, genetically or physically, resulted in inhibition and inactivation of electron transport (Haltia et al., 1991, 1994) and in modulation of the dielectric environment of heme a_3 (Heibel et al., 1993). These observations suggest a role for subunit III in assembly and stabilization of the entire complex.

It was also suggested, previously, that subunit III of the mitochondrial COX participates in proton pumping because removal or proteolytic digestion of subunit III of this enzyme greatly inhibits proton pumping (and to a lesser extent electron transport) and because DCCD, which reacts specifically with a conserved acidic residue on subunit III (homologous to Glu^{98} in *P. denitrificans*), also inhibits proton pumping (Casey, Thelen, & Azzi, 1980; Prochaska et al., 1981; Prochaska & Fink, 1987).

Haltia et al. (1991) constructed several site-directed mutants of two highly conserved acidic residues of subunit III of *P. denitrificans* (Glu^{98}, Asp^{259}) by complementing a COIII deletion mutant with a plasmid expressing wt or mutagenized COIII gene. These conserved acidic residues are predicted to reside in the hydrophobic core of the membrane, as expected from residues that participate in proton pumping. However, Haltia et al. (1991) found that these mutations had no effect on the rate of electron transport by the enzyme or the stoichiometry of proton pumping (H^+/e^-). Moreover, the subunit III deletion mutant, while exhibiting lower electron and proton transport rates, nevertheless pumped protons with the same stoichiometry as the three subunits enzyme. It was also reported that the reconstituted subunit I and II complex from *P. denitrificans* pumps protons as well as the reconstituted three-subunit complex (Hendler et al., 1991). These findings were taken as evidence that subunit III (and particularly Glu^{98} and Asp^{259}) plays no role in proton pumping by cytochrome *c* oxidase (Haltia et al., 1991, Hendler et al., 1991).

† This work was supported by USPHS Grant GM 28173.

* Author to whom all correspondence should be addressed. Pathology, M.S 435, Hahnemann University, Broad and Vine, Philadelphia, PA 19102. Tel: 215-762-7988. Fax: 215-246-5918. E-Mail: ROTTENBERGH@HAL.HAHNEMANN.EDU.

‡ Present address: Departamento Bioquimica, Instituto Nacional De Cardiologia, Juan Badiano #1, Mexico D.F., Mexico 14080.

® Abstract published in *Advance ACS Abstracts,* December 1, 1995.

[1] Abbreviations: COX, cytochrome *c* oxidase; CCCP, carbonylcyanide *m*-chlorophnylhydrazone; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TMPD, *N,N,N*′*,N*′*′*-tetramethyl-*p*-phenylenediamine; TPP^+, tetraphenylphosphonium; wt, wild type.

0006-2960/95/0434-16298$09.00/0

Table 1: List of Mutants of *P. denitrificans* Used in This Study[a]

strain	relevant characteristics
TN-57	Rif^R, Spc^R, Km^R, $COIII^-$, mod^+, enhanced conjugation frequency
plasmids	
pMMB67EH	Ap^R, $lacI^Q$, tacP, rrnB, bla, multilinker mp18
pMS13	pMMB67EH, Sm^R, COIII as a *KpnI*−*BamHI* fragment
pTH2B	pMMB67EH, Sm^R, COIII(E98Q)
pTH5B	pMMB67EH, Sm^R, COIII(E98A)
pTH7	pMMB67EH, Sm^R, COIII(E98D)
pTH11	pMMB67EH, Sm^R, COIII(D259A)

[a] All the mutants were obtained from T. Haltia. The mutants were constructed from a COIII deletion strain (TN-57) complemented with a plasmid (pMMB67EH) ligated with a fragment (*KpnI*−*BamHI*) containing mutagenized COIII. A detailed description of the construction of these mutants is given in Haltia et al. (1991).

Nevertheless, it was also reported recently that proton slippage may be induced in bovine COX at high $\Delta\Psi$ (Steverding & Kadenbach, 1991) and that the reconstituted two-subunit enzyme (I + II) of *P. denitrificans* exhibits more slippage than the three-subunit enzyme (Steverding et al., 1993). A proton pump that is not completely coupled may exhibit normal stoichiometry under level flow conditions (i.e., oxygen pulse assay) but reduced coupling under static head conditions (i.e., steady-state $\Delta\mu_H$) (Pietrobon & Caplan, 1985).

In this study, we utilized the Glu^{98} and Asp^{259} mutants of COIII, which were constructed by Haltia et al. (1991), to investigate the role of these residues in energy coupling, as manifested by the generation of $\Delta\mu_H$ and the efficiency of oxidative phosphorylation. Our results show that the Glu^{98} mutants E98A and E98Q and the Asp^{259} mutant D259A are less efficient than is the wt in COX-dependent oxidative phosphorylation and thus indicate that subunit III plays a role in energy conversion by COX.

EXPERIMENTAL PROCEDURES

Paracoccus denitrificans Mutants. All the mutants used in this study were obtained from T. Haltia. A detailed description of the construction of these mutants is given by Haltia et al. (1991). Briefly, these mutants were constructed from a *P. denitrificans* mutant in which the COIII gene was deleted (TN-57) and was complemented with a plasmid containing the wt, Asp^{259} mutagenized, or Glu^{98} mutagenized COIII gene. Table 1 lists all the mutants used in this study. All the original stock suspensions of these mutants were stored in glycerol at −78 °C.

Cells Cultures. Cultures were started from the deep-frozen stock suspension. A small frozen sample was added to 2 mL of Luria Broth medium containing 50 μg/mL of streptomycin and 25 μg/mL of rifampicin and incubated in a shaker at 37 °C for about 24 h. The cells were inoculated on a plate of agar medium (50 mM KH_2PO_4, 10 g/L of succinic acid, 0.1 g/L of yeast extract, 75 mM NH_4Cl, 11.5 mM Na_2SO_4, 1 mM citric acid, 1.25 mM MgCl, 0.1% salts stock, 1.5% agar, 50 μg/mL of streptomycin, and 25 μg/mL of rifampicin, pH 7.0) and incubated at 37 °C for about 24 h and then stored in the refrigerator. This procedure was repeated weekly. Daily cultures were started by inoculating 2 mL of Luria Broth (+streptomycin and rifampicin) with cells from the agar plate, placed in a shaker at 37 °C for approximately 18 h. One milliliter of this suspension was diluted with 10 mL of succinate medium (as above but without the agar) and incubated in a shaker at 37 °C for about 8 h. This suspension was diluted 1:1000 in succinate medium (+streptomycin) and incubated in a shaker with strong aeration (200 rpm) at 30 °C for up to 18 h (Ludwig, 1986). The cells were collected in the logarithmic growth phase ($OD_{690} = 1.5 \pm 0.1$).

Cell Starvation. The cell suspension, usually in 200 mL volume, was centrifuged (Beckman J21, rotor JA-10, 4 °C) for 10 min at 4400*g*. The pellet was resuspended in the same volume of NaCl buffer (50 mM NaCl, 10 mM Tris-Cl, pH 7.3), centrifuged again (6400*g*, 10 min, 4 °C), and resuspended again in the same volume of NaCl buffer. This suspension was incubated for 1 h at room temperature, and centrifuged again (8700*g*, 10 min, 4 °C). The final pellet was weighed carefully and resuspended in a small volume of NaCl buffer (approximately 250 mg pellet in 1 mL). This stock suspension was kept on ice in the refrigerator for 24 h and then used for the experiments with starved cells. In all the experiments we used measured volumes of the stock suspension to obtain the desired cell content. There was no diference in protein content between the mutants, and therefore, the activities are expressed per milligram of protein. One milligram of cell pellet contained about 100 μg of protein.

Measurement of ATP Content. A 2 mL sample of cell suspension (containing protein at 0.2 or 0.6 mg/mL) was mixed with 0.4 mL of 30% TCA, vortexed quickly, and centrifuged for 5 min at 16000*g*. Two milliliters of the supernatant cell extract was neutralized with 64 μL of 10 N NaOH (adjusted pH was 7.3−7.5). A small sample of the neutralized extract (5 or 10 μL, calculated to obtain approximately 1−3 nM ATP, which is within the linear response range) was added to 3 mL of the assay medium which contained 30 μL of a Luciferase−Luciferine suspension (40 mg/mL, Sigma), 20 mM Na-HEPES buffer (pH 7.5), and 5 mM $MgSO_4$. The luminescence was counted in a scintillation counter for 0.5 min; then 6 μL of 0.5 μM ATP (final concentration 1 nM) was added immediately and the luminescence counted again for 0.5 min for internal calibration of the signal.

Measurement of $\Delta\Psi$. $\Delta\Psi$ was estimated from the distribution of ^3H-TPP^+ (Rottenberg, 1989). First, 1.4 mL of cell suspension (protein at 0.6 mg/mL) was preincubated for 10 min at room temperature with 0.3 μCi of ^3H-TPP^+ (3 μM) and other reagents as required (e.g., uncouplers, inhibitors, etc., except for respiration substrates). Binding was corrected for by substraction of the TPP^+ accumulated by cells incubated without added substrate and in the presence of 10 μM CCCP. Respiration substrates were added after 10 min of preincubation. To determine the probe distribution the suspension was divided into three microtubes (0.4 mL each) and centrifuged at 16000*g* for 3 min. Immediately after centrifugation 30 μL of the supernatants were taken from each tube and mixed with 1 mL of 1% SDS. The pellets were cut quantitatively from the bottom of the tube, wiped clean of adhering supernatant, and mixed with 1 mL of 1% SDS. The samples were vortexed several times until the pellet was completely dissolved. Then 0.5 mL of the solubilized pellet and 0.5 mL of the supernatant were mixed with 5 mL each of scintillation liquid and counted for 5 min in a scintillation counter. $\Delta\Psi$ was calculated from the distribution of TPP^+ between the pellet and supernatant (Rottenberg, 1989). The counts from each sample were first

corrected for background. Then the counts were pooled from the triplicate samples of supernatants and pellets. The pooled pellet counts were corrected for TPP^+ binding by substraction of the pooled pellet counts of the CCCP sample. $\Delta\Psi$ was calculated according to the formula $\Delta\Psi = 59 \log ([TPP]_{cell}/[TPP]_{medium})$. To calculate cell concentration of TPP from the pellet content, we measured, with ^{14}C-inulin and ^3H_2O, the cell water volume which was 3.35 μL/mg of protein and comprised 40% of the pellet water.

RESULTS

Under optimal conditions (i.e., fresh cells incubated with high concentrations of succinate under aerobic conditions) there was no significant difference between wt and COIII mutants in growth rates, respiration rates, and ATP content. To test the coupling efficiency of cytochrome *c* oxidase we inhibited electron transport at the bc_1 complex by myxothiazol and added ascorbate and TMPD. The inhibition of respiration by myxothiazol caused only a 15−25% reduction in cellular ATP content, and the addition of ascorbate/TMPD quickly restored the ATP content. However, when cell respiration was inhibited by starvation, prior to the addition of myxothiazol, the ATP content was decreased by 50−80%, which allowed more accurate determination of the effect of ascorbate/TMPD oxidation on ATP synthesis. The endogenous respiration was inhibited by more than 90% by starvation alone, and the addition of myxothiazol further inhibited endogenous respiration (>95%). Addition of ascorbate/TMPD to starved cells, in the presence of myxothiazol, enhanced respiration 5−10-fold and restored, within a minute, normal levels of ATP content. Venturicidin, a specific inhibitor of ATP synthase, and cyanide, an inhibitor of terminal oxidases, further reduced the ATP content of starved cells and completely inhibited ascorbate/TMPD- or succinate-driven ATP synthesis, indicating that the substrate-induced rise in ATP content depends entirely on oxidative phosphorylation (results not shown).

Figure 1 (top panel) shows the time course of the increment of ATP content in starved cells after the addition of ascorbate/TMPD to wt, to each of the three Glu^{98} mutants (E98A, E98Q, E98D), and to D259A. The rise of cellular ATP content, after addition of ascorbate/TMPD, was fast in the first 30 s but proceeded at a much slower rate afterward. The initial rate of increase of cell ATP was slightly higher in the acidic E98D mutant than in the wt but was significantly inhibited in all the nonacidic mutants. The increment of ATP content 10 min after the addition of ascorbate/TMPD in wt and E98D was almost twice as large as the increment in the nonacidic mutants. The increments shown in Figure 1 are the averages of experiments with many cell batches, and the differences between wt and the nonacidic mutants were highly significant statistically. The bottom panel of Figure 1 shows that the rates of ascorbate/TMPD respiration in the mutants were as high as that of wt; in fact, the respiration rates of E98Q and E98D were significantly higher than that of the wt. Comparison of the rates of increment of ATP with the rates of respiration suggests that the P/O ratio of COX-dependent oxidative phosphorylation is reduced in all the nonacidic COIII mutants but not in the acidic mutant E98D, which exhibited higher rates of both respiration and ATP synthesis.

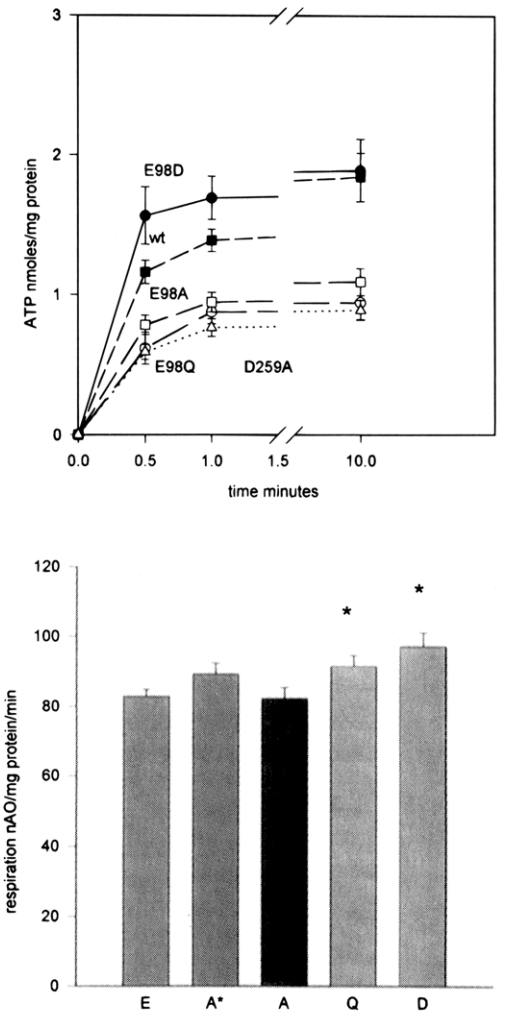

FIGURE 1: The increment of ATP content and respiration in starved cells of the COIII mutants after addition of ascorbate/TMPD. The starved cells (protein at 0.2 mg/mL) were preincubated for 10 min at room temperature in a medium containing 50 mM Na-HEPES, 50 mM NaCl, 5 mM KH_2PO_4, and 1 mM $MgCl_2$ pH 7.5, with 10 μM myxothiazol. Ascorbate (0.5 mM) was added, and the respiration was activated by addition of the redox mediator TMPD (250 μM). Samples for ATP determinations were taken just before and 0.5, 1.0, and 10 min after addition of TMPD. The averaged increment in ATP content (±SEM) is shown in the top panel for each of the mutants. Each experiment was repeated twice for each batch, with many batches of starved cells, as indicated below. Filled squares (■): average of 20 batches of wt cells (E98E, D259D). Filled circles (●): average of nine batches of E98D cells. Empty squares (□): average of nine batches of E98A cells. Empty circles (○): average of nine batches of E98Q cells. Empty triangles (△): average of 21 batches of D259A cells. All the increments in the nonacidic mutants were significantly lower than the corresponding wt increments (p <0.001). The E98D increments at 0.5 and 1 min were significantly higher than wt increments (p <0.05, and p <0.06, respectively), but the increment at 10 min was the same. The bottom panel shows the average rates of respiration (+SEM) of the same batches of cells 1 min after addition of ascorbate/TMPD. [E = wt (E98E, D259D); A* = D259A; A = E98A; Q = E98Q; D = E98D]. The rates of the E98Q and E98D mutants were significantly higher than wt rates (p <0.03 and p < 0.001, respectively).

To find out whether the difference in coupling efficiency between wt and the nonacidic mutants is the results of proton slippage within COX or a nonspecific increase in the membrane proton permeability, we measured the membrane potential ($\Delta\Psi$) in parallel with the ATP content before and after addition of ascorbate/TMPD. These measurements

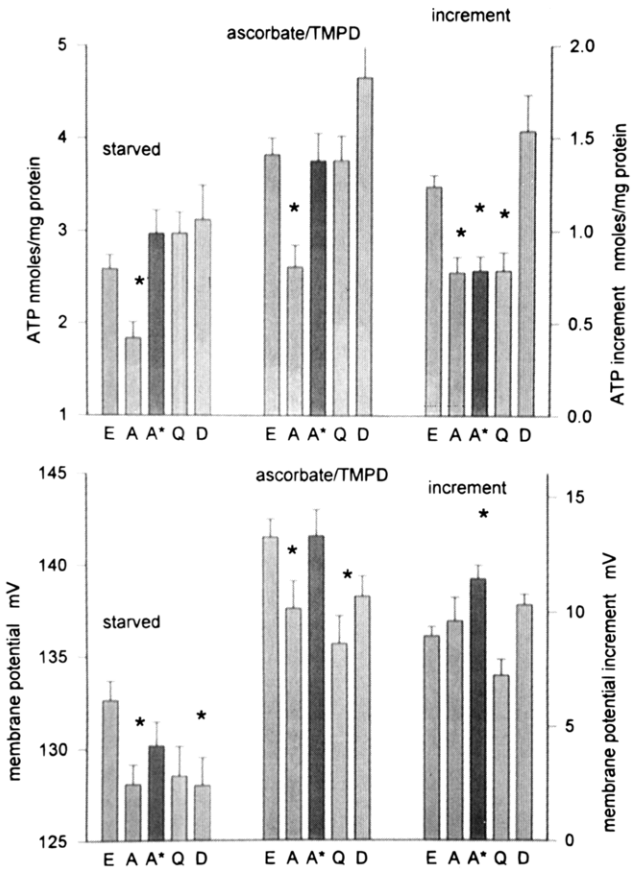

FIGURE 2: The effect of the addition of ascorbate/TMPD on ATP content and $\Delta\Psi$ in starved cells of COIII mutants. ATP content and $\Delta\Psi$ were measured as described in Experimental Procedures. The protocol was the same as in Figure 1 except that protein concentration was 0.6 mg/mL. ATP and $\Delta\Psi$ were measured just before and 1 min after the addition of the ascorbate/TMPD. The determinations were repeated twice with each batch of cells. The results shown are the averages (+SEM) of 27 batches of wt (E), 12 batches of E98A (A), 18 batches of D259A (A*), 9 batches of E98Q, and 9 batches of E98D. In the top panel (ATP content) all the values that are significantly different at $p < 0.01$ from the corresponding wt values are marked (*). In the bottom panel ($\Delta\Psi$) all the values that are significantly different at $p \leq 0.06$ from the corresponding wt values are marked (*).

were conducted at pH 7.5, an external pH at which the magnitude of ΔpH is negligible in these cells and $\Delta\Psi$ is essentially identical to $\Delta\mu_H$ (results not shown).

The top panel of Figure 2 shows a summary of the measurements of ATP content, while the bottom panel shows a summary of the measurements of $\Delta\Psi$. The group of columns on the left of each panel shows the average values of initial ATP content (top) and $\Delta\Psi$ (bottom) without substrate (with myxothiazol) for each of the mutants. The initial ATP content of E98A was significantly lower than that of the wt but not that of the other mutants, which were slightly higher. The initial $\Delta\Psi$, without substrate, was slightly lower in all the mutants, particularly in E98A and E98D. The middle group of columns shows these parameters 1 min after the addition of ascorbate/TMPD. The ATP content of the E98A mutant was significantly lower than that of the wt, but the ATP content of the other mutants was equal to or higher than that of the wt. The magnitude of $\Delta\Psi$ of all the Glu^{98} mutants was slightly lower than that of the wt (3−5 mV), but this difference was significant only with the nonacidic mutants E98A and E98Q. The group of columns on the right shows the increment in ATP content (top) and the increment in $\Delta\Psi$ (bottom) 1 min after the addition of ascorbate/TMPD (scales are on the right). As already shown in Figure 1, the increment of ATP in the nonacidic mutants after ascorbate/TMPD addition is only half as large as that of the wt (and E98D). However, the increment in $\Delta\Psi$, measured in parallel, is significantly higher in the D259A mutant and approximately the same as that of the wt in the other mutants. Thus, the attenuation of the increment in ATP content in the nonacidic mutants is not correlated with a parllel attenuation of the $\Delta\Psi$ increment. Moreover, the cell content of ATP, after the addition of ascorbate/TMPD, is not generally correlated with the magnitude of $\Delta\Psi$ (e.g., both E98Q and E98D showed a lower $\Delta\Psi$ than did the wt, but an ATP content equal to or higher than the wt).

These results are not compatible with the suggestion that the inhibition of energy conversion by COX, in the nonacidic mutants, results from an attenuation of their ability to increase $\Delta\mu_H$ (see Discussion). Nevertheless, it is possible that the expression of the mutated COIII gene somehow resulted in a nonspecific uncoupling effect, at the membrane level, or in a specific inhibition of ATP synthase. To test this possibility we examined oxidative phosphorylation with succinate as a substrate.

Paracoccus denitrificans cells contain several alternative terminal oxidases (de Gier et al., 1994). Under aerobic conditions ubiquinol (which is reduced by succinate dehydrogenase) is oxidized mainly by ubiquinol oxidase/cytochrome *c* reductase (cyt bc_1 complex), and reduced cytochrome *c* is then oxidized by COX. Although two redox proton pumps participate in this pathway (with an overall pathway stoichiometry of $3H^+/e^-$), a reduced efficiency of coupling in COX could also result in a reduction of the overall efficiency of oxidative phosphorylation. However, when this pathway is inhibited by a specific cyt bc_1 inhibitor (e.g., myxothiazol), ubiquinol is oxidized exclusively by the alternative, proton pumping, terminal oxidase, ubiquinol oxidase (cyt bb_3). The overall stoichiometry of this pathway is $2H^+/e^-$ (Puustinen et al., 1989), and this pathway should not be affected by a coupling defect in COX. Succinate-driven ATP synthesis by the ubiquinol oxidase pathway is much slower in wt cells because the rate of succinate oxidation is inhibited by myxothiazol by up to 60% (see Figure 4), and because of the lower overall H^+/e^- stoichiometry (2 instead of 3). Therefore, if the coupling defect of the nonacidic mutants is in ATP synthase, or at the membrane level, it is expected that myxothiazol will inhibit succinate-driven ATP synthesis in the mutants to the same extent as in the wt. However, if the defect is specific to COX, succinate-driven ATP synthesis in the nonacidic mutants should be less inhibited by myxothiazol than in the wt and perhaps even stimulated.

The rise of ATP content after succinate addition to starved cells (without myxothiazol) is faster than with ascorbate/TMPD, and the ATP level quickly reaches a new steady state, most likely because succinate is a carbon source for these cells and the anabolic reactions consume large amounts of ATP. To slow down the rise of cellular ATP we conducted a series of kinetic experiments with starved cells at 10 °C.

Figure 3 shows a summary of the results of these experiments with the five mutants. Each panel shows the time course of the averaged increment of ATP content, after succinate addition, in control experiments (COX pathway,

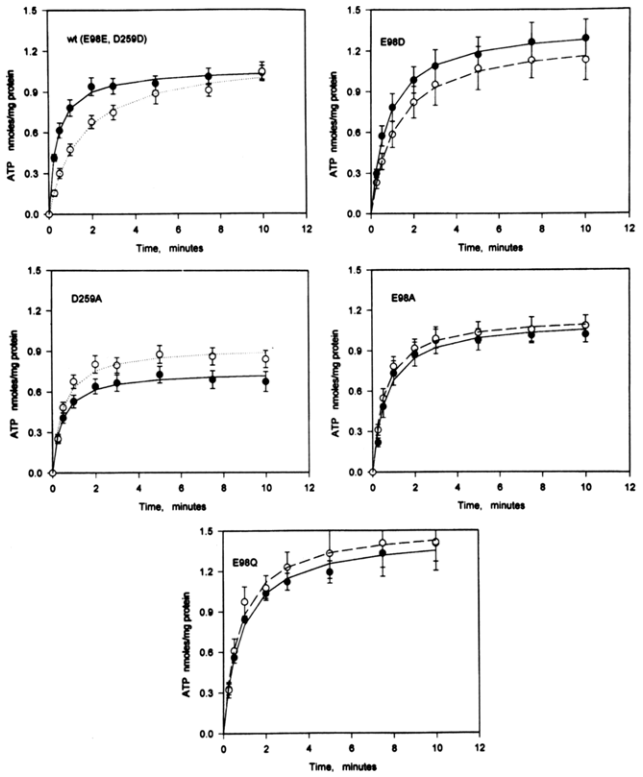

FIGURE 3: The kinetic of the rise of ATP after addition of succinate to starved cells of COIII mutants. Starved cells (protein 0.2 mg/mL) were incubated for 30 min, at 10 °C in 20 mL of a reaction medium composed of 50 mM Tris-Cl, 5 mM Tris-phosphate, 50 mM NaCl, and 1 mM $MgCl_2$ pH 7.3 with or without 10 μM myxothiazol. A 2 mL sample was taken for ATP determination immediately before the addition of succinate, and additional samples were taken at the indicated time after succinate addition (10 mM). ATP was determined as described under Experimental Procedures. Each experiment was repeated twice with each batch of starved cells. Each panel shows the averaged ATP increments (±SEM), from a number of batches (n). The average increment in cells incubated without myxothiazol are represented by filled circles while the increments in cells incubated with myxothiazol are represented by empty circles. The data were fitted to the hyperbolic function $\Delta ATP\ (t) = \Delta ATP_\infty * t/(T_{0.5} + t)$, where t is time (in minutes), ΔATP_∞ is maximal ΔATP (at very large t), and $T_{0.5}$ is the half-time of the maximal ΔATP. The shown curves represent the fitted functions. *Top-left panel*: wt cells (E98E, D259D, n = 12); *top-right panel*: E98D cells (n = 6); *middle-left panel*: D259A cells (n = 12); *middle-right panel*: E98A cells (n = 8); *bottom panel*: E98Q cells (n = 6).

filled circles) and in cells preincubated with myxothiazol (ubiquinol oxidase pathway, empty circles). The averaged data points were fitted to a hyperbolic function. The kinetic analysis of these experiments is summarized in Table 2.

In wt cells (Figure 3, top left; Table 2, first row), the initial rate of ATP increment supported by the ubiquinol oxidase

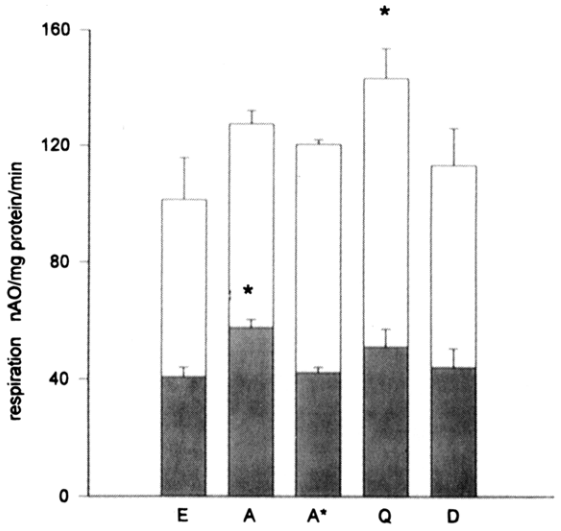

FIGURE 4: The effect of myxothiazol on succinate respiration in the COIII mutants. Starved cells were incubated (protein at 0.2 mg/mL) in the reaction medium (same as in Figure 3) for 10 min, at 20 °C with or without myxothiazol (10 μM). Respiration was started by the addition of 10 mM succinate. The results shown are the averages (+SEM) of duplicate experiments with seven batches of wt (E), and five batches of each of the mutants. Without myxothiazol (empty bars), only E98Q was significantly higher than wt (p <0.05); with myxothiazol (hatched bars), only E98A was significantly higher than wt (p <0.01).

pathway was about a third of the rate of increment supported by the COX pathway, and the half-time of the approach to steady state was increased almost fourfold. This magnitude of the inhibition by myxothiazol of the rate of ATP increment is compatible with the 60% inhibition of the rate of respiration (Figure 4) and the lower stoichiometry of the ubiquinol oxidase pathway. The magnitude of the maximal ATP increment was about the same for both pathways.

In the acidic mutant E98D (Figure 3, top right; Table 2, second row) the rate of the ATP increment supported by the COX pathway was somewhat lower than wt, but the maximal increment was considerably larger. More significantly, the initial rate of ATP increment supported by the ubiquinol oxidase pathway was also significantly slower than that of the COX pathway (34%), and the half-time was also much longer, while the maximal increment of ATP content was reduced. In sharp contrast to the effect of myxothiazol on the ATP increment in the wt and the acidic mutant E98D, all the nonacidic mutants were not inhibited by myxothiazol.

In the nonacidic mutant D259A (Figure 3, middle left; Table 2, third row) the initial rate of ATP increment supported by the COX pathway was nearly half that of the wt. The maximal increment was greatly attenuated compared

Table 2: The Kinetics of the Succinate-Driven ATP Increment[a]

	cytochrome c oxidase (succinate)			ubiquinol oxidase (succinate + myxothiazol)		
mutant	rate (nmoles/min)	half-time (min)	ΔATP_∞ (nmoles)	rate (nmoles/min)	half-time (min)	ΔATP_∞ (nmoles)
wt	1.68 ± .10	0.37 ± .02	1.07 ± .01	0.62 ± .08***	1.46 ± .13	1.15 ± .03
E98D	1.24 ± .14	0.78 ± .04	1.38 ± .02	0.82 ± .13***	1.30 ± .02	1.17 ± .05
D259A	0.92 ± .09	0.42 ± .05	0.75 ± .02	1.02 ± .04**	0.47 ± .07	0.93 ± .03
E98Q	1.20 ± .10	0.81 ± .07	1.46 ± .03	1.30 ± .18 (NS)	0.73 ± .08	1.53 ± .04
E98A	0.88 ± .12	0.65 ± .09	1.12 ± .04	1.24 ± .12***	0.55 ± .04	1.15 ± .02

[a] Results are from the experiments described in Figure 3. The rates are averaged initial rates (±SEM). The half-times (±SEM) and the ΔATP_∞ (±SEM) are the results of the curve-fitting described in the legend of Figure 3. The statistical significance of the difference (paired student t-test) between the initial rate of ubiquinol oxidase- and COX-driven ATP synthesis is indicated: NS = not significant; **p < 0.02; ***P < 0.005.

to the wt and E98D, but the half-time was comparable. More significantly, in this nonacidic mutant the initial rate of ATP increment supported by the ubiquinol oxidase pathway was significantly faster, and the magnitude of the maximal increment was considerably larger, than that of the COX pathway.

In the nonacidic mutant E98A (Figure 3, middle right; Table 2, last row) the initial rate of ATP increment supported by the COX pathway was also half that of wt, and the half-time was significantly longer. Moreover, in this mutant too the initial rate of ATP increment supported by the ubiquinol oxidase pathway was significantly faster than that of the COX pathway, and the half-time of the increment was shorter.

In the nonacidic mutant E98Q (Figure 3, bottom; Table 2, fourth row) the rate of ATP increment supported by the COX pathway was only slightly slower than that of the wt, but the half-time was more than twice as long. In addition, the magnitude of the increment was also much larger than that of the wt. More significantly, both the rate of ATP increment and the magnitude of the maximal increment supported by the ubiquinol oxidase pathway were slightly larger than that of the COX pathway.

Thus, myxothiazol, which inhibited the rate of succinate-induced ATP increment, in both wt and E98D, had the opposite effect on all the nonacidic mutants despite a strong inhibition of respiration (see Figure 4). It is also interesting to note that in all the nonacidic mutants, in the ubiquinol oxidase pathway the initial rate of the increment of ATP was faster than that of the wt and the half-time of approach to steady-state was shorter than that of the wt (see Dicussion).

Figure 4 shows that the rate of succinate respiration in the mutants was generally higher than that of the wt, particularly in E98Q, and that myxothiazol inhibited the mutants respiration approximately to the same extent as in the wt, about 60% inhibition by 10 μM (I_{50} = 1 μM). The rate of succinate respiration of the mutants, in the presence of myxothiazol, was about the same as that of the wt, except for E98A which was significantly higher.

The results of the experiments with succinate indicate that in the nonacidic mutants the ubiquinol oxidase pathway is coupled to ATP synthesis more efficiently than is the COX pathway. This suggests that the reduced efficiency of oxidative phosphorylation in the nonacidic mutants, when respiring on ascorbate/TMPD, is due to a specific defect in COX. The fact that the rate of ubiquinol oxidase-driven increment of ATP (i.e., succinate in the presence of myxothiazol), in the nonacidic mutants, is higher than in wt suggests that these mutants adapt their metabolism to accommodate the defect in COX (see Discussion).

DISCUSSION

The results of the investigation of the coupling efficiency of COX in COIII mutants of *P. denitrificans* indicate that the conserved acidic residues of COIII are important for efficient coupling of COX electron transport to ATP synthesis and thus suggest a role for subunit III in energy conversion.

It should be emphasized that our results do not contradict the results of Haltia et al. (1991), who did not find any difference in COX turnover number or the stoichiometry of proton pumping, H^+/e^-, between the mutants and wt. The slightly higher rates of ascorbate/TMPD and succinate oxidation, which we observed in some of the mutants, in comparison to the rates of wt, may result from a slightly higher content of respiratory enzymes or from subtle modulation of the control of respiration. The equal or slightly higher magnitude of ascorbate/TMPD-induced increment of $\Delta\Psi$, observed here, is also compatible with the results of Haltia et al. (1991). Also in agreement with Haltia et al. (1991), we did not find significant differences between the mutants in cytochrome aa_3 content, as measured from the difference absorption spectra (results not shown). However, we did observe significant differences between the nonacidic mutants and wt in the rate and/or extent of ascorbate/TMPD- and succinate-driven increment of ATP content.

It is the interpretation of these findings which present us with a dilemma. It is generally expected that the rate of COX-driven ATP synthesis would depend on the rates of proton pumping by COX and the membrane proton leak, which together determine the increment of $\Delta\mu_H$. Yet, while ascorbate/TMPD-induced ATP increment was strongly inhibited in all the nonacidic mutants, the ascorbate/TMPD-induced increment of $\Delta\mu_H$ ($\Delta\mu_H = \Delta\Psi$) was larger than or equal to that of wt.

There is a considerable uncertainty in the calculation of the absolute magnitude of $\Delta\Psi$ from the uptake of lipophilic cations, mostly because of the uncertainty in the proper correction for binding (Rottenberg, 1989). However, there is much less uncertainty in comparing the magnitude of the $\Delta\Psi$ increment between the mutants. Whatever systematic errors may exist in these calculations, there is no reason to believe that they affect the calculations in the mutants differnetially. If the ascorbate/TMPD-induced increment of $\Delta\Psi$ was actually attenuated in the nonacidic mutants, it is extremely difficult to explain why the ascorbate/TMPD-induced uptake of TPP^+ was not attenuated at all. There is little doubt that the differences in the rates of ATP increment were real because the experimental errors in these determinations were small, the number of the experiments was very large, and the statistical significance of these differences was very high. Therefore, we cannot explain the decrease in the efficiency of oxidative phosphorylation in all the nonacidic mutants as resulting from increase proton slippage in these mutants.

We note, however, that the absolute value of $\Delta\Psi$ was slightly, but significantly, lower in the nonacidic Glu^{98} mutants but not in the Asp^{259} mutant (Figure 2). Although these values are less certain than the calculated increments, the $\Delta\Psi$ value in the nonacidic Glu^{98} mutants may suggest that these mutants are somewhat deficient in the maintenance of high $\Delta\mu_H$ (see below).

We have also tested the alternative explanation that these defects in oxidative phosphorylation are due to a nonspecific, membrane-dependent uncoupling, or a specific uncoupling of ATP synthase, perhaps as a result of the expression of the mutated COIII gene. However, we found that the rates of succinate-induced increment of ATP in the presence of myxothiazol, i.e., when coupling depended entirely on ubiquinol oxidase, were actually higher in the nonacidic mutants than in the wt. The qualitative difference in the effect of myxothiazol on the rate of ATP synthesis in the nonacidic mutants (i.e., stimulation instead of inhibition, Figure 3) is very strong evidence that these mutants are defective only in COX-driven ATP synthesis. This evidence

is particularly convincing because it does not depend on the comparison of the rate of ATP increment between different cell batches. In essence the coupling of ubiquinol oxidase served as an internal control for the coupling of COX. The enhancement of succinate-driven ATP synthesis by myxothiazol was observed in each batch of cells of the nonacidic mutants. Therefore, it is not possible to explain the defects in COX-dependent oxidative phosphorylation by proposing that the expression of the mutated COIII gene somehow uncouples or inhibits the ATP synthase.

Thus, regardless of the difficulty in finding a simple explanation for the defect, it appears that the conserved acidic residues of subunit III do have an effect on the efficiency of energy conversion by COX.

A possible, but controversial, solution of the dilemma posed by these results can be derived from the hypothesis that there is also a direct coupling pathway between ATP synthase and COX whereby protons can move directly from COX to ATP synthase without equilibrating with the bulk. It has been suggested previously that, in addition to the established role of the bulk proton electrochemical gradient ($\Delta\mu_H$) in oxidative phosphorylation (Mitchell 1979), a direct proton transfer may occur between the redox pumps and the F0F1 complex (cf. Rottenberg, 1985, 1990; Krulwich & Guffanti, 1989, Slater et al., 1984; Westerhoff et al., 1984; Dilly et al., 1987). Our results are compatible with the suggestion that the conserved acidic residues of subunit III mediate the direct proton transfer between COX and ATP synthase (Rottenberg, 1990).

The crystal structure of COX from *P. denitrificans* has been determined recently (Iwata et al., 1995). Subunit III consists of seven transmembrane helices, five of which (SU III-TM III to TM VII) form a flat bundle that interacts with transmembrane helices of subunit I on one side and is exposed to the membrane lipids, where it may interact with another membrane protein on the other side. The residues of subunit III which we have studied here, Glu^{98} and Asp^{259}, are in the middle of the membrane on TM III and TM VII, respectively, in close proximity to each other. TM III (E98) is also in close proximity to pore A of subunit I, which was suggested by Iwata et al. (1995) to be a part of the pumped (vectorial) protons pathway in COX.

It should be noted that although both the Glu^{98} and the Asp^{259} nonacidic mutants appear to be defective in energy conversion, there seem to be significant differences between them. The ascorbate/TMPD-induced increment of $\Delta\Psi$ of the D259A mutant appears to be significantly larger than that of the wt while the absolute value of $\Delta\Psi$ is the same. In contrast, the increment of $\Delta\Psi$ in E98A and E98Q is equal to or lower than that of the wt and the absolute values of $\Delta\Psi$ are significantly lower than that of the wt (Figure 2). We also examined the correlation between the magnitude of $\Delta\Psi$ and ATP synthesis in wt, E98A, and D259A by modulating $\Delta\Psi$ with valinomycin. Each of the mutants exhibited significantly different relationships between $\Delta\Psi$ and ATP content (results not shown). These findings may indicate that Glu^{98} contributes to the maintenance of high $\Delta\mu_H$ while Asp^{259} does not.

The maximal magnitude of the succinate-driven, COX-dependent, increment of ATP in D259A is smaller than that of wt, but the half-time is similar. In contrast, in E98A the maximal succinate-induced ATP increment is the same and that of E98Q is even higher than that of the wt, but the half-times are much longer than that of the wt. This may be due to different mechanisms of adaptation to the defect, as discussed below, but also suggest a difference in the nature of the defect.

It may appear somewhat surprising that a defect in oxidative phosphorylation does not affect the aerobic growth rate of the mutants. However, *P. denitrificans* have at least three other coupling sites for oxidative phosphorylation, e.g., NADH dehydrogenase, cyt bc_1, ubiquinol oxidase, in addition to cytochrome *c* oxidase, which is still partially functional in the nonacidic mutants. The overall reduction of the efficiency of oxidative phosphorylation in the nonacidic mutants should not exceed 10−20%. Therefore, it is not unreasonable that the mutants appear to be able to adapt to this mild defect in the overall efficiency of oxidative phosphorylation. This adaptation is evident from the fact that the rates of the ubiquinol oxidase−driven increment of ATP content is significantly higher in the nonacidic mutants than in the wt and that the maximal magnitude of the succinate-induced increment in ATP content is different from that of wt in some of the mutants (Figure 3). This may be associated with modulation of the rate of utilization of ATP, the activity of ATP synthase, and other adaptive changes. Moreover, the rates of respiration also tend to be 10−30% higher in the mutants (Figures 1, 4), possibly as a compensation for the reduced efficiency of COX. The fact that each of the Glu^{98} mutants behaves somewhat differently when respiring on succinate (Figure 3) suggests that there are subtle differences even between E98A and E98Q and between the wt and E98D.

It has been suggested that the proton pathways in COX are restricted to subunit I and that proton pumping is closely associated with the heme−Cu_B center (Wikstrom et al., 1994; Fetter et al., 1995; Iwata et al., 1995). Our findings suggest that the conserved acidic residues of subunit III are also necessary for efficient energy conversion although the precise mechanism of their contribution remains to be elucidated.

ACKNOWLEDGMENT

We thank Drs. Marten Wikstrom and Tuomas Haltia for providing us with the COIII mutants and for their generous advice, encouragement, and criticism during the course of this work. We thank Dr. Akhil Vaidya for a critical reading of our manuscript.

REFERENCES

Casey, R. P., Thelen, M., & Azzi, A. (1980) *J. Biol. Chem. 255*, 3994−4000.

de Gier, J.-W. L., Lubben, M., Reijnders, W. N. M., Tipkar, C. A., Slotboom, D.-J., van Spanning, R. J. M., Stouthamer, A. H., & van der Oost, J. (1994) *Mol. Microbiol. 13*, 183−196.

Dilley, R. A., Theg, S. M., & Beard, W. A. (1987) *Annu. Rev. Plant Physiol. 38*, 347−389.

Fetter, J. R., Qian, J., Shapleigh, J., et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1604−1608.

Garcia-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J., & Gennis, R. B. (1994) *J. Bacteriol. 176*, 5587−5600.

Haltia, T., & Wikstrom, M. (1992) in *Molecular Mechanism in Bioenergetics* (Ernster L., Ed.) pp 217−239, Elsevier, New York.

Haltia, T., Saraste, M., & Wikstrom, M. (1991) *EMBO J. 10*, 2015−2021.

Haltia, T., Semo, N., Arrondo, J. L. R., Goni, F. M., & Freire, E. (1994) *Biochemistry 33*, 9731−9740.

Heibel, G. E., Hildebrandt, P., Ludwig, B., Stenrucke, P., Souli-

mane, T., & Buse, G. (1993) *Biochemistry 32*, 10866−10877.
Hendler, R. W., Pardhasaradhi, K., Reynafarje, B., & Ludwig, B. (1991) *Biophys. J. 60*, 415−423.
Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., et al. (1993) *J. Bioenerg. Biomembr. 25*, 121−136.
Iwata, S., Ostermeier, C., Ludwig, B., & Michel, H. (1995) *Nature 376*, 660−669.
Krulwich, T. A., & Guffanti, A. A. (1989) *Annu. Rev. Microbiol. 43*, 435−463.
Ludwig, B. (1986) *Methods Enzymol. 126*, 153−159.
Mitchell, P. (1979) *Science 206*, 1148−1154.
Pietrobon, D., & Caplan, S. R. (1985) *Biochemistry 24*, 5764−5776.
Prochaska, L. J., & Fink, P. S. (1987) *J. Bioenerg. Biomembr. 19*, 143−166.
Prochaska, L. J., Bisson, R., Capaldi, R. A., Steffens, G. C. M., & Buse, G. (1981) *Biochim. Biophys. Acta 637*, 3571−3579.
Puustinen, A., Finel, M., Virkki, M., & Wikstrom, M. (1989) *FEBS Lett. 249*, 163−167.
Rottenberg, H. (1985) in *Modern Cell Biology* (Satir, B. H., Ed.) Vol. 4, pp 47−83, Alan R. Liss, New York.
Rottenberg, H. (1989) *Methods Enzymol. 172*, 63−84.
Rottenberg, H. (1990) *Biochim. Biophys. Acta 1018*, 1−17.
Slater, E. C., Berden, M. A., & Herwijer, M. A. (1984) *Biochim. Biophys. Acta 811*, 217−231.
Steverding, D., & Kadenbach, B. (1991) *J. Biol. Chem. 266*, 8097−8101.
Steverding, D., Kohnke, D., Ludwig, B., & Kadenbach, B. (1993) *Eur. J. Biochem. 212*, 827−831.
Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., & Kell, D. B. (1984) *Biochim. Biophys. Acta 768*, 257−292.
Wilkstrom, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase—A Synthesis*, Academic Press, New York.
Wilkstrom, M., Bogachev, A., Finel, M., Morgan, J. E., Puustinen, A., Raitio, M., Verkhovskaya, M., & Verkhovsky, M. I. (1994) *Biochim. Biophys. Acta 1187*, 106−111.

BI950590R