Mutation in the Hydrophobic Domain of ATP Synthase Subunit 4 (Subunit b) of Yeast Mitochondria Disturbs Coupling between Proton Translocation and Catalysis†

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Received March 9, 1994; Revised Manuscript Received May 24, 1994®

ABSTRACT: We introduced mutations to test the function of the hydrophobic sector of subunit 4 from *Saccharomyces cerevisiae* ATP synthase. Mutations were introduced at the chromosomic locus by homologous transformation of a strain disrupted in the ATP4 gene. The strain carrying the replacement Leu68-Val69→Arg-Glu did not grow at 37 °C owing to a lack of assembly of F_1 and F_o sectors at this temperature. The mutant strain grew slowly by oxidative phosphorylation at 28 °C with a growth yield 30% lower than the wild type. Analysis of the mutant strain showed a homogeneous population of altered ATP synthase with an energy coupling impairment. The mutant strain was oligomycin-resistant since the I_{50} value of oligomycin inhibition of ATPase and ATP synthase activities was 2–3-fold higher than that of the wild type, thus showing an alteration of the target to oligomycin. The level of phosphorylation or ATP induced a proton-dissipating pathway through F_o, which was insensitive to oligomycin but was sensitive to dicyclohexylcarbodiimide, thus suggesting an alteration in the regulation of ATP synthase proton permeability by the catalytic sector. From these results, we propose that the dicyclohexylcarbodiimide inhibition site is located upstream of the oligomycin inhibition site when considering the proton flux occurring during ATP synthesis.

F_1F_o-ATP synthase is responsible for the synthesis of ATP from ADP and P_i at the expense of the proton chemical gradient generated by the respiratory chain. The enzyme is composed of two sectors: the catalytic sector F_1 is a hydrophilic portion of the enzyme retaining the ability to hydrolyze ATP when in the soluble form; the F_o sector is embedded in the membrane and is composed of hydrophobic subunits forming a specific proton-conducting pathway. When the two sectors are coupled, the enzyme functions as a reversible H^+-transporting ATPase or ATP synthase (Senior, 1988; Fillingame, 1990). The F_o sector of eukaryotes seems more complicated than that of the prokaryotes, since it contains not only three subunits equivalent to that of *Escherichia coli* F_o (a, b, c) and called subunits 6, b (F_oI or 4), and 9 (dicyclohexylcarbodiimide-binding protein), respectively, but also subunits d, OSCP,[1] A6L (or 8), and F_6 (Senior, 1988). The F_o part of *Saccharomyces cerevisiae* contains at least six different subunits. Three of them are mitochondrially encoded (subunits 6, 8, and 9) (Nagley, 1988). The other three are nuclear encoded and termed subunit 4 (Velours et al., 1987), OSCP (Uh et al., 1990), and subunit d (Norais et al., 1991). Subunit 4 is an amphiphilic protein which contains 209 amino acid residues with a predicted molecular mass of 23 250 Da. It is homologous to the *E. coli* b subunit (Velours et al., 1988) and to the b subunit of beef heart mitochondria (Walker et al., 1987). The primary structure of the b subunit of eukaryotes consists of two distinct domains: the N-terminal part is predominantly hydrophobic, and the C-terminal part is charged and hydrophilic. Houstek et al. (1988) and Zanotti et al. (1988) have shown by proteolytic cleavages and reconstitution experiments that subunit b is involved in proton conduction through F_o and in the sensitivity to oligomycin. From the analysis of yeast mutants showing shorter versions of subunit 4, Paul et al. (1992) have shown that, as in *E. coli*, the C-terminal part of the subunit is involved in the assembly of the whole complex.

The experiments described in this paper focus on the hydrophobic domain of subunit 4. Our aim was to modify slightly the structure of the F_o domain in order to detect alterations in the function of the whole complex and, thus, to study the mechanism of energy transduction. By site-directed mutagenesis of the DNA region of the ATP4 gene encoding the postulated transmembrane segment, we obtained a mutant strain showing a defect in enzyme energy coupling and in assembly.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes and DNA-modifying enzymes were purchased from Bethesda Research Laboratories. The pSELECT-1 phagemid was provided by Promega Corp. Radionucleotides and [^{32}P]phosphate was obtained from Amersham International (Amersham, England). Oligomycin and DCCD were purchased from Sigma. All other chemicals were of reagent-grade quality.

Bacterial Strains and Plasmids. The bacterial strain JM 109 was grown in L medium (1.6% tryptone, 1% yeast extract, 1% NaCl pH 7.4) containing, when appropriate, 50 μg of ampicillin/mL or 15 μg of tetracycline/mL. The *E. coli* strain BMH 71–18 mutS was used for mutagenesis procedures. Bacterial strains were transformed using the $CaCl_2$ proce-

† This research was supported by the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de l'Enseignement Supérieur, the Université de Bordeaux II, and the Etablissement Public Régional d'Aquitaine. D.R.-J. was supported by a student's grant from the Ministère de la Recherche et de l'Enseignement Supérieur.

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® Abstract published in *Advance ACS Abstracts*, July 15, 1994.

[1] Abbreviations: bp, base pair(s); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; OSCP, oligomycin sensitivity-conferring protein; SDS, sodium dodecyl sulfate.

0006-2960/94/0433-9684$04.50/0

Table 1: Oligonucleotide-Directed Mutagenesis of ATP4 Gene and Growth of Mutants with Lactate as Carbon Source[a]

strain	synthetic oligonucleotides	generation time (min) 28 °C	37 °C
PVY162, wild type		188	380
PVY175, Y50→D	5′-ATGAATTG(G)ACGTTATC-3′	220	380
PVY176, V51→G	5′-ATTGTACG(G)TATCAACG-3′	200	350
PVY177, V51→D	5′-ATTGTACG(A)TATCAACG-3′	210	350
PVY179, L60-T61→P-P	5′-ATTTTATTGC(C)G(C)CTTTTTTGGG-3′	180	370
PVY184, G64-F65-T66→E-F-H	5′-ACTTTTTTGGAGTTTCAC GGTTTAGT-3′	190	nd[b]
PVY185, V69→E	5′-TGGTTTAG(A)GGCAAAGT-3′	190	300
PVY187, L68→R	5′-TTCACTGGT(AG)AGTGGCAAAG-3′	200	360
PVY190, L68-V69→R-E	5′-TTCACTGGT(AG)AG(A)GGCAAAGTA-3′	370	ng[c]

[a] Parenthese in the oligonucleotides indicate mutation(s). The underlined base and the hole in the sequence correspond to an insertion and a deletion, respectively. Yeast strains were PVY10 strain complemented with the shuttle vector pRS313 harboring the wild-type ATP4 gene (PVY 162) or the mutated form of atp4 gene. [b] nd, not determined. [c] ng, no growth.

dure described in Maniatis et al. (1982). The shuttle vector pRS313 (Sikorski & Hieter, 1989) was used for transformation of the yeast strain PVY10.

Yeast Strains and Procedures. The *S. cerevisiae* strains used were as follows: D273-10B/A/U/H: MATα, met, ura3, his3 (wild type) (Paul et al., 1992); PVY6: MATα, met, ura3, ATP3::URA3 (disrupted) (Paul et al., 1989); PVY10: MATα, met, ura3, his3, ATP4::URA3 (deleted) (Paul et al., 1992); and PVY188: MATα, met, ura3, atp4 Leu68-Val69→Arg-Glu (this study).

The LiCl method (Ito et al., 1983) was used to transform the PVY10 and PVY6 strains with the pRS313 vector bearing the 1770-bp *Bam*H1–*Sal*1 fragment or with a linear piece of DNA 1580-bp *Eco*R1–*Sal*1 containing the altered ATP4 gene, respectively. Cells were grown aerobically at 28 or 37 °C in a complete liquid medium: 1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate (pH 4.5) and 2% sodium lactate (lactate medium) or 2% galactose as the carbon source. Cells were harvested in the logarithmic growth phase.

Nucleic Acid Techniques. Commercially available restriction enzymes and other DNA-modifying enzymes were used as advised by the suppliers. Plasmid DNA was prepared by the alkaline lysis method. The 1580-bp *Eco*R1–*Sal*1 fragment containing the wild-type ATP4 gene (Paul et al., 1992) was inserted into the polylinker of the phagemid pSELECT-1 for site-directed mutagenesis. Single-strand DNA was prepared from JM109 cultures containing the recombinant phagemid and the helper phage R408. This served as a template for mutagenesis by using the phosphorylated mutagenic oligonucleotide (see Table 1), the phosphorylated AMP 5′-GCCATTGCTGCAGGCATCGT-3′ oligonucleotide and T7 DNA polymerase. Repair of mutation was prevented by using the *E. coli* BMH 71–18 mutS. Mutants were identified by sequencing (Sanger et al., 1977). The entire gene was sequenced with the use of four primers: 5′-AAAGACT-GACGAGAATT-3′ (–46 to –30), 5′-CTCTATCATCAAT-GCCA-3′ (153–169), 5′-GCCGATGCAAGAATGAA-3′ (346–362), and 5′-AATTAGCTCACGAAGCA-3′ (539–555). The mutated 1580-bp *Eco*R1–*Sal*1 fragment was ligated within a linear piece of DNA consisting in the shuttle vector pRS313, which contained the 1770-bp *Bam*H1–*Sal*1 fragment of wild-type ATP4 gene from which the 1580-bp *Eco*R1–*Sal*1 was removed. The resulting plasmid bearing the yeast marker HIS3 was used to transform the PVY10 strain. Transformants were selected and subcloned on minimal medium containing methionine and glucose as the carbon source.

Biochemical Preparations and Analyses. Mitochondria were prepared from protoplasts according to Guérin et al. (1979). Protein amounts were determined according to Lowry et al. (1951) in the presence of 5% SDS. Bovine serum albumin was used as a standard protein. The ATP synthase was immunoprecipitated with F_1 antiserum as described by Todd et al. (1980). Extraction of F_0 subunits was performed as in Paul et al. (1989). SDS–polyacrylamide gel electrophoresis was performed as previously described (Velours et al., 1987).

Cellular respirations were performed at 28 °C in 1.5 mL of 1% yeast extract, 0.1% KH_2PO_4, and 0.12% $(NH_4)_2SO_4$, pH 5.8, containing 1.5 mg dry weight of cells.

Mitochondrial ATP synthesis and oxygen consumption rates were measured as described by Rigoulet and Guérin (1979). Rates were expressed by cytochrome oxidase activity. The latter was measured in the presence of 1.4 mM *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine, 15 mM ascorbate, and 3 μM CCCP. Respiration rate data [obtained as μatom of oxygen min^{-1} (mg of protein)$^{-1}$] were divided by the cytochrome oxidase activity of the preparation. One cytochrome oxidase unit equals 1 μmol of oxygen min^{-1} (mg of protein)$^{-1}$. Thus, 1 unit of respiration rate equals 1 μatom of oxygen/unit of cytochrome oxidase. ATP synthesis rates [obtained as μmol of ATP min^{-1} (mg of protein)$^{-1}$] were also divided by the cytochrome oxidase activity (1 unit = 1 μmol of ATP/unit of cytochrome oxidase. [In our experimental conditions, the mean value of specific cytochrome oxidase activity was 1.1 μmol of oxygen min^{-1} (mg of protein)$^{-1}$.] $\Delta\psi$ was measured as in Ouhabi et al. (1991). Mitochondrial volume was measured as described by Rottenberg (1979). Yeast mitochondria were submitted to hypotonic treatment and sonication as in Paul et al. (1992). ATPase activity was measured at pH 8.4 according to Somlo and Krupa (1974).

RESULTS

Eight mutations were constructed in the N-terminal part of subunit 4 (Table 1). Three of them (Y50→D, V51→D, V51→G) were substitutions of amino acid residues located near the hydrophilic segment separating the two hydrophobic domains. These replacements had no effect on growth at 28 and 37 °C in complete medium containing lactate as the carbon source. Another target was the second hydrophobic segment. G64-F65-T66→E-F-H and L60-T61→P-P mutations also had no effect. Substitution of the two consecutive amino acid residues L68-V69 for R-E increased 2-fold the generation time of the yeast strain PVY190 at permissive temperature, and no growth at 37 °C was detected. On the other hand, the replacement of either L68→R or V69→E did not significantly increase the generation time. The effect of the double mutation L68-V69→R-E was studied. To avoid problems of expression of the mutated ATP4 gene borne by the shuttle vector, the altered ATP4 gene was integrated at its locus by using the

Table 2: Growth of Wild-Type and PVY188 Strains at 28 and 37 °C[a]

strain	generation time (min) 28 °C	generation time (min) 37 °C	stationary phase ($A_{600\ nm}$) 28 °C
D273-10B/A/U/H, wild type	160	300	8.5
PVY188	325		6

[a] Cells of wild-type (D273-10B/A/U/H) and PVY188 strains were grown on a complete medium containing 2% lactate. Turbidimetry was measured at 600 nm for the estimation of cell concentrations after appropriate dilutions. The ratios of dry mass/$A_{600\ nm}$ in logarithmic and stationary growth phases were the same whatever the strain studied.

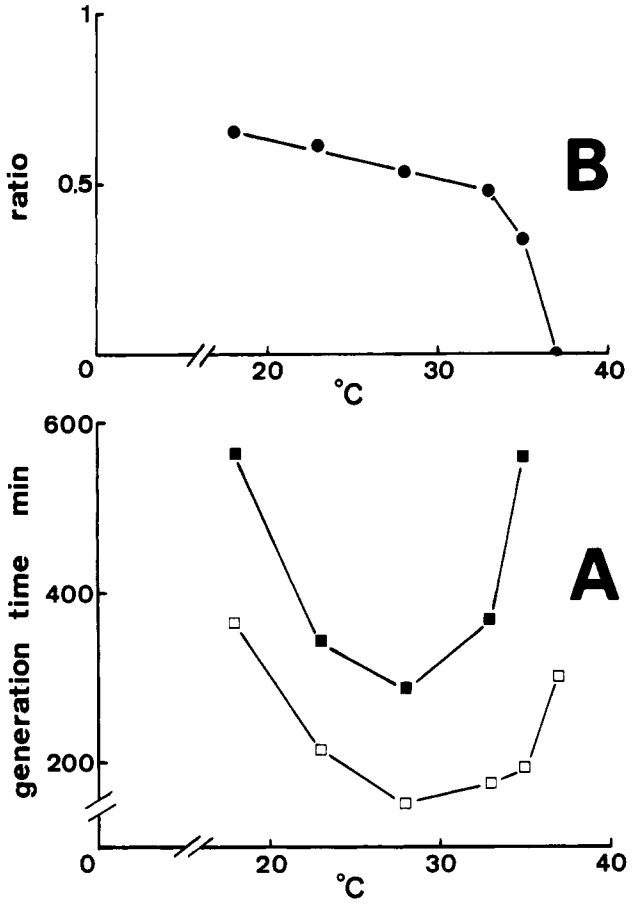

FIGURE 1: Doubling times of the wild-type and PVY188 strains as a function of temperature. Yeast were grown at various temperatures in a complete medium containing 2% lactate as the carbon source (A). Panel B shows the wild-type/PVY188 strains doubling time ratio. (□) Wild-type strain; (■) PVY188 strain.

homologous transformation method, giving rise to strain PVY188. Integration was confirmed by Southern blot analysis of the genomic DNA of PVY188 strain and by the recovery of uracil auxotrophy (not shown).

Phenotypic Analysis of PVY188 Strain at the Cellular Level. At permissive temperature, both the PVY188 and PVY190 strains displayed a 2-fold higher generation time and a final growth 30% lower than that of the wild type, probably reflecting a defect in the oxidative metabolism of these mutant strains. At 37 °C, only the wild type grew. With a similar ratio dry mass/$A_{600\ nm}$, a lower amount of matter was formed at 28 °C by the mutant strain (Table 2). This was confirmed with cultures containing limiting amounts of lactate (not shown). The doubling time of the wild-type and PVY188 strains was calculated as a function of temperature (Figure 1). A temperature higher than 33 °C dramatically increased the doubling time of PVY188 strain.

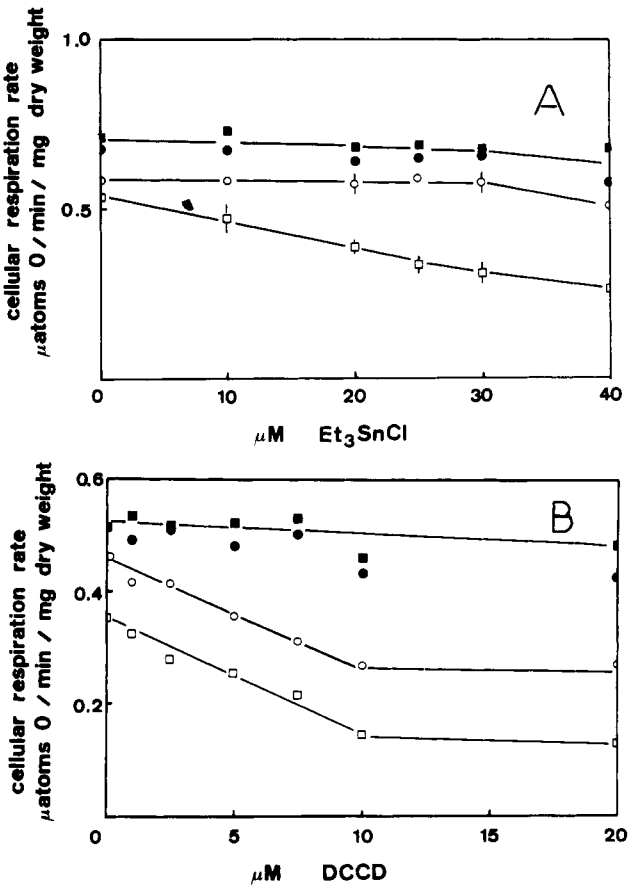

FIGURE 2: Respiration of yeast cells. Cells were grown in a lactate medium, harvested in exponential growth phase, and assayed in a complete medium devoid of carbon source in the presence or absence of Et_3SnCl (A) or DCCD (B). Ethanol was added as a substrate at a final concentration of 0.6%. At the end of the experiment, CCCP was added at the final concentration of 4 µM (●, ■): (□, ■) wild-type cells; (○, ●) PVY188 cells.

The wild type/PVY188 doubling time ratio as a function of temperature indicated two temperature-dependent phenomena: first, a slight decrease in the ratio as temperature increased from 18 to 33 °C, and then a sharp decrease from 33 to 37 °C, at which point the growth of the PVY188 strain was no longer detected. Further experiments were performed with yeast grown at 28 °C. The respiration rate of yeast cells was measured in the presence of increasing amounts of Et_3SnCl and DCCD, two inhibitors of ATP synthase (Cain & Griffith, 1977; Beechey et al., 1966; Kovak et al., 1968) used at the cellular level.

(1) PVY188 strain always displayed a higher respiration rate than the wild type in the absence of inhibitor and CCCP (Figure 2A,B).

(2) Only the wild type was inhibited by Et_3SnCl (Figure 2A). This inhibition reflected a decrease in proton translocation as a consequence of F_o inhibition. The lack of respiration rate inhibition of the PVY188 strain pointed either to a lack of sensitivity to this inhibitor or to the existence of an alternative proton pathway.

(3) CCCP addition stimulated the respiration rate of both strains to the same degree, indicating the same respiratory capacity for both strains and that the PVY188 strain was not fully uncoupled.

Respiration of the two types of cells was inhibited by DCCD with a similar I_{50} value of 5 µM DCCD, thus showing that the proton pathway through F_o was inhibited in the same way. This inhibition was completely released by the uncoupler for

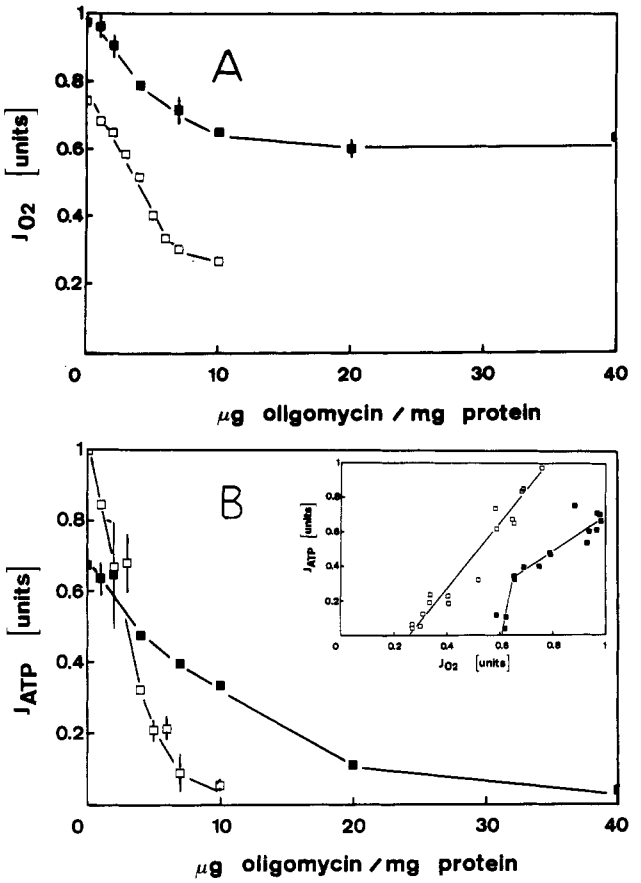

FIGURE 3: Oxygen consumption rate and ATP synthesis rate versus oligomycin concentration. Mitochondria (0.3 mg/mL) were suspended in 2.2 mL of a respiration medium containing 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris/maleate, pH 6.8, supplemented with 3 mM Tris/[^{32}P]P_i (6.18 MBq/mmol). Oligomycin was added 90 s before starting the respiration with 4 mM NADH. ATP synthesis was initiated 30 s later by the addition of 1.2 mM ADP. Mitochondrial oxygen consumption was measured simultaneously with a Clark oxygen electrode (Gilson). Respiration rate during state 3 (A). The ATP formation rate was estimated by [^{32}P]P_i incorporation into adenine nucleotides after acid extraction of 200-μL aliquots withdrawn at different times. Measurements were performed in duplicate (B). The insert shows ATP synthesis rate as a function of oxygen consumption rate. Each point characterized by an ATP synthesis rate value and the related oxygen consumption rate value are shown in the insert. The units are defined in the Experimental Procedures: (□) wild-type mitochondria; (■) PVY188 mitochondria.

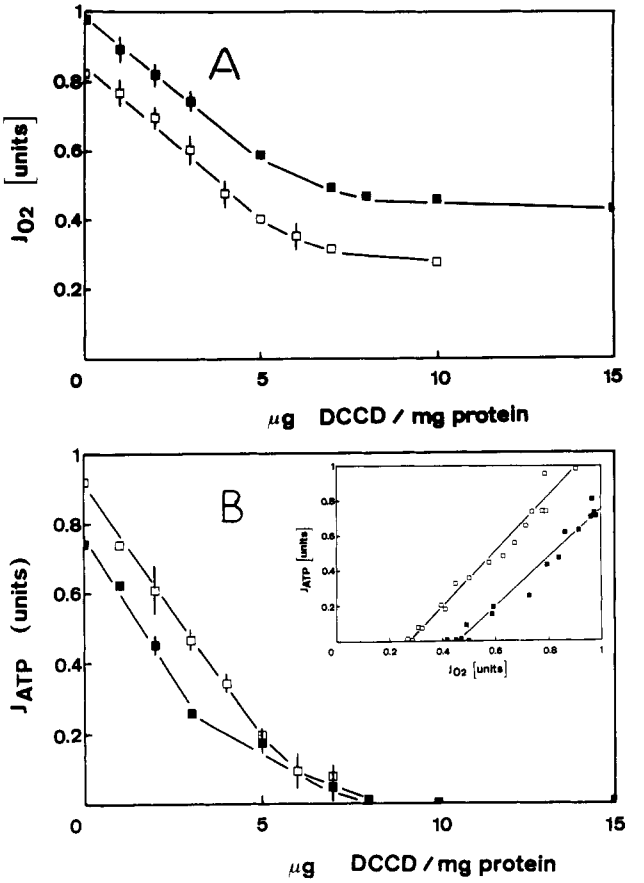

FIGURE 4: Oxygen consumption rate and ATP synthesis rate versus DCCD concentration. For experimental details see the legend of Figure 3.

concentrations lower than 20 μM (Figure 2B). Higher concentrations of DCCD inhibit other metabolic processes in yeast cells which cannot be released upon the addition of CCCP (Kovac et al., 1968). Most importantly, the DCCD-insensitive respiration rate was increased 2-fold for the PVY188 cell with 20 μM DCCD. This result reflected a higher uncoupling of the mutant cell. This respiration rate increase, which was insensitive to F_o inhibitors, correspond to the difference between the respiration rates measured in the absence of inhibitors.

Phenotypic Analysis of Mutation at the Mitochondrial Level. Respiration rates, phosphorylation rates, and ATPase activities were expressed as a function of cytochrome oxidase activity measured in the presence of CCCP. This was because (i) at the cellular level, the same respiratory capacity was measured in the presence of the uncoupler for both strains; and (ii) spectrophotometrically, the two strains displayed similar cytochrome oxidase contents of 17 and 19 pmol of cytochrome *a*+*a*3/mg of dry weight of PVY188 and wild-type cells, respectively. In this way, heterogeneity in the mitochondria yield was avoided, and it was thus possible to compare all the experiments performed during this study.

The oxygen consumption rates of the first and second state 4 of PVY188 mitochondria were higher than those of the wild type (Table 3). ATP synthesis rates and ATP/O ratios of PVY188 mitochondria were 35% lower than those of the wild type. These results confirm the presence of the partial uncoupling of the PVY188 strain observed at the cellular level.

Figures 3 and 4 also show a lower ATP synthase activity and a higher respiration rate of PVY188 mitochondria than the wild type in the absence of an inhibitor. The ADP-stimulated respiration rate (state 3) was inhibited by oligomycin to a maximum value of 60% and 40% for the wild-type and PVY188 mitochondria, respectively (Figure 3A). Oligomycin inhibited ATP synthesis with I_{50} values of 3.5 and 10 μg of oligomycin/mg of protein for wild-type and PVY188 mitochondria, respectively (Figure 3B), thus showing a higher resistance of the mutant mitochondria toward the inhibitor. Titration of the ATP synthase by oligomycin resulted in a linear relationship between the ATP synthesis rate and the oxygen consumption rate for the wild-type mitochondria with a slope value of 1.9 (insert of Figure 3B). The mutant mitochondria clearly displayed two linear relationships with slope values of 1 at high oxygen consumption–high ATP synthesis rates and a slope value varying from 4 to 10 according to the experiments at low oxygen consumption–low phosphorylation rates. The latter value was obtained for oligomycin amounts varying from 10 to 40 μg of oligomycin/mg of protein. These high concentrations of oligomycin titrated the remaining phosphorylation rate without decreasing the respiration rate. These results pointed to a deviation of the proton pathway at high oligomycin concentrations in PVY188 mitochondria. At

Table 3: Respiration Rates and Phosphorylation Rates of Wild-Type and PVY188 Mitochondria[a]

strains	respiration rates (JO2 units)			phosphorylation rates (JATP units)	ATP/O
	1st state 4	2nd state 4	state 3		
D273-10B/A/U/H, wild type	0.33 ± 0.06 (6)	0.36 ± 0.08 (6)	0.81 ± 0.08 (6)	0.88 ± 0.12 (4)	1.09 ± 0.21 (4)
PVY188	0.46 ± 0.04 (7)	0.59 ± 0.06 (7)	0.86 ± 0.05 (7)	0.57 ± 0.1 (3)	0.69 ± 0.06 (3)

[a] Mitochondria were isolated from yeast cells grown at 28 °C with lactate as the carbon source. Assays were performed as described in the method section. Each experiment was performed in triplicate. The first state 4 corresponded to the respiration rate after NADH and before ADP addition. The second state 4 corresponded to the respiration rate after consumption of 0.2 mM ADP. The respiration rate during state 3 was obtained by the addition of 1.2 mM ADP. Numbers in parentheses represent the number of different mitochondrial preparations. The units are defined in the Experimental Procedures. (JO2: 1 unit = 1 μatom of oxygen/unit of cytochrome oxidase; JATP: 1 unit = 1 μmol of ATP/unit of cytochrome oxidase).

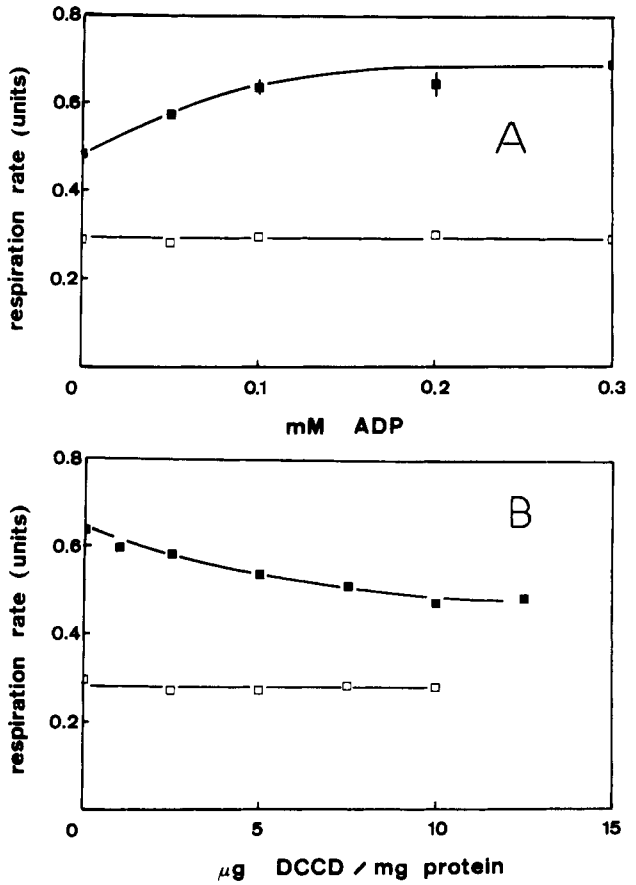

FIGURE 5: Stimulation of DCCD-sensitive state 4 upon increasing amounts of nucleotides in PVY188 mitochondria. Mitochondria (0.3 mg/mL) were incubated at 28 °C, in 1.5 mL of a respiration medium containing 3 mM P_i/Tris and 4 mM NADH. Oxygen consumption rate was measured during the second state 4 after consumption of various concentrations of ADP (A). State 3 was induced by 0.1 mM ADP; increasing concentrations of DCCD were added 30 s after the end of phosphorylation, and measurement of the respiration rate was performed 1 min later (B). The units are defined in the Experimental Procedures: (□) wild-type mitochondria; (■) PVY188 mitochondria.

null ATP synthesis, the two lines intercept the x-axis at values of 0.26 and 0.6 μatom of oxygen/unit of cytochrome oxidase for the wild-type and PVY188 mitochondria, respectively. The latter value was close to that of the second state 4 (Table 3). On the other hand, Et_3SnCl inhibited state 3 as did oligomycin, leading also to a high residual respiration rate in the mutant mitochondria, but without significant modification of the I_{50} value (not shown).

The same amount of DCCD inhibited both the ADP-stimulated respiration rate (state 3) and the ATP synthesis rate of wild-type and PVY188 mitochondria, with I_{50} values of 3 μg of DCCD/mg of protein (Figure 4). The Figure 4B insert shows two linear relationships with slopes of 1.6 and 1.4 for wild-type and mutant mitochondria, respectively. In this experiment, the two lines intercept the x-axis at values of 0.28

Table 4: Effect of ATP Addition on Respiration Rate of Yeast Mitochondria[a]

adducts	strain (respiration rate units)	
	PVY188	D273-10B/A/U/H, wild type
no addition	0.504	0.285
ATP	0.727	0.294
ATP + DCCD	0.509	nd[b]

[a] Mitochondria (0.3 mg/mL) were suspended in 1.5 mL of respiration medium containing 3 mM tris-phosphate, pH 6.8. Respiration was initiated with 4 mM NADH in the presence or absence of 10 μg of DCCD/mg of protein. ATP concentration was 0.2 mM. The units were defined in Experimental Procedures. [b] nd, not determined.

and 0.46 μatom of oxygen/unit of cytochrome oxidase for the wild-type and PVY188 mitochondria, respectively. These values corresponded to the respiration rates measured during the first state 4 (Table 3). The difference between these two values was the consequence of the proton influx insensitive to the F_o inhibitors observed at the cellular level.

The oxygen consumption rate measured during the second state 4 of the mutant mitochondria was dependent on the ADP concentration used during state 3 (Figure 5A). This stimulation of the respiration rate was oligomycin- and Et_3-SnCl-insensitive but was DCCD-sensitive with a I_{50} value of 3.5 μg of DCCD/mg of protein (a similar value to that used to inhibit ATP synthesis). At 10 μg of DCCD/mg of protein, the respiration rate returned to 0.48 μatom of oxygen/unit of cytochrome oxidase, a value found in the absence of ADP (Table 3) or after full inhibition of state 3 by DCCD (Figure 4A and insert of Figure 4B). Neither ADP nor P_i alone was responsible for the stimulation of the respiration rate of the second state 4 (data not shown), but rather ADP and P_i together. Table 4 shows that ATP addition also increased the mitochondrial respiration rate in a DCCD-sensitive manner. In this preliminary experiment, it was found that this stimulation was 50% sensitive to carboxyatractyloside (not shown).

ATPase Activity. At basis pH (8.4), PVY188 mitochondria had an ATPase activity 1.5-fold higher than the wild type. The hydrolytic activity was 40% oligomycin, DCCD, and Et_3-SnCl insensitive (Table 5). The presence of free F_1 was excluded since catalytically active ATP synthases extracted by Triton X-100 from the two types of mitochondria had the same sedimentation property with superposed oligomycin-insensitive ATPase activity on a continuous sucrose gradient performed at 4 °C (not shown). In our experimental conditions, oligomycin inhibited ATP hydrolysis with I_{50} values of 1.5 and 3 μg of oligomycin/mg of protein for wild type-and PVY188 mitochondria, respectively, whereas, a similar amount of DCCD (I_{50} = 1.4 μg of DCCD/mg of protein) inhibited ATP hydrolysis for both types of mitochondria.

ATPase Assembly. When mitochondria were prepared from cells grown with 2% galactose at 37 °C and extracted with Triton X-100, the wild-type extract contained a whole

Table 5: Sensitivity of Mitochondrial ATPase Activities toward F_0 Inhibitors[a]

adducts	temp (°C)	strain PVY188	strain D273-10B/A/H/U
no addition	30	1.55[b] ± 0.38 (5) (100%)	1.05[b] ± 0.42 (4) (100%)
oligomycin		44% ± 4 (3)	13% ± 1 (2)
DCCD		42% ± 5 (3)	9% ● 4 (2)
Et_3SnCl		33% ± 3 (2)	12% (1)
no addition	37	2.25[b] ± 0.43 (4) (100%)	1.52[b] ± 0.29 (3) (100%)
oligomycin		40% ± 1 (3)	12% ± 1 (2)
DCCD		34% ± 6 (3)	7% ± 1 (2)
Et_3SnCl		32% (1)	7% (1)

[a] Mitochondria were isolated from cells grown at 28 °C on a complete medium containing 2% lactate as the carbon source. Assays were performed at 30 and 37 °C with the addition of 20 μg, 10 μg, and 20 μM of oligomycin, DCCD, and Et_3SnCl, respectively. [b] Results are expressed as μmol of P_i/unit of cytochrome oxidase and activities in the presence of inhibitor as percentage of the control. The numbers in parentheses indicate the number of experiments.

Table 6: ATPase Activity of Mitochondria and Mitochondrial Membranes[a]

sample	(μmol of P_i min^{-1} (mg of protein)$^{-1}$) no addition	(μmol of P_i min^{-1} (mg of protein)$^{-1}$) oligomycin	% remaining activity
PVY188 mitochondria	2.25 ± 0.16	1.10 ± 0.02	49
PVY188 membrane pellet	2.03 ± 0.13	0.78 ± 0.05	38
wild-type mitochondria	2.47 ± 0.10	0.44 ± 0.02	18
wild-type membrane pellet	2.86 ± 0.06	0.24 ± 0.02	8.4

[a] Mitochondria were submitted to a hypotonic treatment followed by sonication. After centrifugation, the membrane pellet was assayed at 30 °C with the addition of 20 μg of oligomycin/mg of protein where indicated. Measurement was performed in triplicate. In this experiment, sonication removed 32 and 46% of the ATPase activity of the wild-type and PVY188 mitochondria, respectively.

complex that was immunoprecipitable by antibody anti-F_1 (Paul et al., 1992). This polyclonal antibody was not able to coprecipitate F_o subunits from the mitochondrial Triton X-100 extract of PVY188 cells grown at 37 °C (not shown). Furthermore, PVY188 extracts were devoid of subunits 4 and 6 only when the cells were grown at 37 °C, resulting in an impairment in the assembly of the two sectors as shown previously by Paul et al. (1992). As a result, experiments were performed to determine the stability of the complex of PVY188 cells grown at 28 °C. In these conditions, antibody anti-F_1 coprecipitated F_1 and F_o subunits (not shown). Furthermore, as indicated in Table 5, once assembled with the F_o sector at 28 °C, the catalytic sector did not dissociate from the F_o at 37 °C during the course of the experiment. We have already reported that a hypotonic treatment followed by sonication released 30% of the wild-type F_1-ATPase activity in the supernatant (Paul et al., 1992). With this treatment, 32% and 46% of the ATPase activity was released from the wild-type and mutant mitochondria, respectively, indicating a lower stability of the mutant ATPase complex. The oligomycin sensitivity of the remaining ATPase activity of the treated membranes is reported in Table 6. It clearly shows that hypotonic treatment and sonication removed 10% of an oligomycin-insensitive ATPase activity for both mitochondria. This probably was due to contaminating ATPases. This experiment shows that although 46% of soluble ATPase activity was removed from PVY188 mitochondrial membranes, the remaining ATPase complexes displayed the same oligomycin sensitivity as before the treatment.

DISCUSSION

The hydrophobic profile of yeast subunit 4 contains two hydrophobic domains from T31 to I45 and from I57 to A74 separated by a short hydrophilic segment (Velours et al., 1988). The two hydrophobic domains are found in most b subunits (Walker et al., 1987; Tsurumi et al., 1990; Higuti et al., 1991; Rasmussen et al., 1992), except in the *E. coli* b subunit which contains only the second hydrophobic region (Kumamoto & Simoni, 1986). The eukaryotic b subunit might consist of a hairpin-like structure, with the two hydrophobic segments crossing the membrane and the N-terminus and the polar segment of the C-terminus exposed on the matrix side.

To gain information on the location and the role of the membranous part of the eukaryotic b subunit, we have produced yeast mutants by site-directed mutagenesis. Introduction of two consecutive proline residues at the beginning of the second postulated transmembranous domain had no effect on cell growth, thus showing that breaks in the predicted α-helix region did not significantly disturb the structure of subunit 4 or the activity of the enzyme.

The substitutions of the two consecutive hydrophobic Leu68-Val69 for hydrophilic and charged Arg-Glu residues produced a strain that was able to grow with a low yield at permissive temperature with lactate as the carbon source. The PVY188 strain was fully deficient in oxidative metabolism at 37 °C. Therefore, this strain is a useful tool for a bioenergetic study.

PVY188 Mitochondria Contained a Homogeneous Population of Altered ATP Synthase. The PVY188 strain was completely deficient in ATP synthase assembly at 37 °C. An important question is to know if at permissive temperature there is a homogeneous population of altered enzymes, or if the mutation causes misassembly of some complexes, resulting in two classes of enzymes. A misassembly of the complex might be temperature-sensitive. It probably occurred from 33 to 37 °C, whereas the slight decrease in doubling time ratio between 18 and 33 °C could be attributed to an increase in the proton-dissipating pathways of the mutant enzyme (see Figure 1). Other points are in favor of a homogeneous population of altered enzymes. (i) In the presence of both intact and defective enzymes, sonication might mainly disrupt the latter. We clearly observed that the remaining ATP synthase complexes had the same oligomycin sensitivity as before treatment. (ii) No free F_1 was found on the sucrose gradients. (iii) The existence of an altered I_{50} value for oligomycin is not in agreement with the simultaneous presence of defective enzymes and properly assembled ATP synthase complexes. (iv) Moreover, the presence of free F_o was unlikely; in a previous paper, we showed that the F_o part of a yeast ε-deficient ATPase mutant, showing functional impairment between the two sectors, was titrated by oligomycin amounts 10-fold lower than the wild type (Guélin et al., 1993). From these observations, we propose that PVY188 mitochondria contain a homogeneous population of altered enzymes at permissive temperature.

PVY188 Mitochondria Are Partially Resistant to Oligomycin. High levels of oligomycin were necessary to inhibit ATP synthesis fully, whereas similar amounts of DCCD inhibited mutant and wild-type mitochondria. Oligomycin-resistant mutants are mutated in the yeast mitochondrial genes Oli1 and Oli2, encoding for subunits 9 and 6, respectively. The oligomycin resistance found here has a nuclear origin, indicating an interaction between subunit 4 and the two mitochondrially encoded proteins. Schneider and Altendorf (1987) and Cox et al. (1992) have proposed different models of the F_o sector showing a direct contact of the two *E. coli*

b subunits with subunits a and c. It is conceivable that replacement of hydrophobic for hydrophilic and charged amino acid residues in the hydrophobic domain of the subunit 4 could produce a defect in the interaction of F_o subunits and lead to a decrease in the accessibility or the contact of oligomycin with its target. Nevertheless, this would not modify the accessibility of a small hydrophobic molecule like DCCD, which binds covalently to the Glu58 of subunit 9.

ATP hydrolysis was partially inhibited by F_o inhibitors, since 40% of the activity was insensitive to oligomycin, DCCD, and Et_3SnCl. This result was different from ATP synthesis inhibition data, since full inhibition of the proton pumping involved in the phosphorylation process led to full inhibition of ATP synthase activity. The high residual ATPase activity of PVY188 mitochondria in the presence of F_o inhibitors pointed to an alteration in the coupling between F_1 and the membranous factor, which led to a proton dissipation between F_1 and F_o (see below).

Existence of Different Proton-Dissipating Pathways in PVY188 Mitochondria. In addition to the main proton influx involved in oxidative phosphorylation, a DCCD-, Et_3SnCl-, and oligomycin-insensitive proton pathway exists in PVY188 mitochondria. This energy-dissipating pathway is a discrete uncoupling, since the $\Delta\psi$ component of the protonmotive force was decreased only by 12 mV (not shown). Greater impairment of mutant mitochondria due to the isolation procedure was unlikely, since the same residual DCCD-insensitive respiration rate was found at the cellular level. We cannot fully exclude the presence of a small proportion of defective F_o contributing to this DCCD-insensitive proton leak. However, we have reported that the respiration rate of a strain devoid of subunit 4 was still stimulated by CCCP, thus showing that although this strain contained the subunits 8 and 9, the resulting F_o (if any) was not leaky (Paul et al., 1989).

Most interesting was the fact that PVY188 mitochondria displayed another proton-dissipating pathway which was DCCD-sensitive but Et_3SnCl- and oligomycin-insensitive. This energy dissipation was revealed by the 40% residual ATPase activity, which was insensitive to F_o inhibitors, and by the increase in the respiration rate (i) after phosphorylation (the second state 4 displayed a $\Delta\psi$ decrease of 13 mV after phosphorylation of 0.5 mM ADP); (ii) in the absence of phosphorylation but in the presence of ADP, P_i, and high oligomycin concentrations; and (iii) in the presence of ATP.

The increase in the oxygen consumption rate during the second state 4 could be the result of the balance between ATP synthesis and hydrolysis during this state. A leaky F_o could result in an increase in oxygen consumption to reach an equilibrium between synthesis and hydrolysis. However, in this hypothesis, the proton influx might be inhibited by the high concentrations of oligomycin as is ATP synthesis (Figure 3). This was not the case since this proton influx was inhibited only by DCCD.

The second hypothesis concerns the opening of a proton channel downstream from the DCCD-binding site and occuring upon the binding of nucleotides on the mutant enzyme in the absence of phosphorylation. In this eventuality, the low respiration rate of the first state 4 could be explained by the loss of nucleotides from yeast mitochondria during their isolation.[2] The lack of Et_3SnCl inhibition of the cellular respiration rate would indicate that the cells in culture are in a physiological state near the second state 4, conditions leaving open the DCCD-sensitive but Et_3SnCl- and oligomycin-insensitive proton channel.

From the analysis of the differential sensitivity of the proton-dissipating pathway toward inhibitors and considering that PVY188 mitochondria contained a homogeneous population of altered ATP synthase, we propose that the DCCD binding site might be located upstream of the oligomycin inhibition site when considering the proton influx during ATP synthesis.

Regarding the above hypothesis, an important point will be to provide evidence that the binding of ADP and P_i or ATP to a nucleotide binding site promotes the switching on of a proton-dissipating pathway in the mutant mitochondria. Our observation could be reminiscent of conformational communications between the binding of ADP and P_i at catalytic sites and the structures in F_o responsible for proton translocation (Matsuno-Yagi et al., 1985; Penefsky, 1985; Wagner et al., 1986; Zhou & Boyer, 1993).

CONCLUSION

The relacement L68-V69→R-E modifies the hydrophobic character of the postulated transmembranous domain of subunit 4, leading to a lack of assembly of F_1 and F_o sectors at nonpermissive temperatures. At 28 °C, the mutant mitochondria was characterized by proton-dissipating pathways that were differentiated by their sensitivity to F_o inhibitors and nucleotides. Since the mutation does not abolish proton conduction and coupling, the two amino acids are not essential and are not involved in these functions. We hypothesize that the α-helix region containing the amino acid residues L68-V69 is in contact with subunits 6 or 9 (two components of the proton channel). Introduction of two hydrophilic and charged residues in this environment might alter this contact and destabilize the structure of the proton channel, leading to deflections of the proton influx. As a consequence, a part of the proton influx could be lost to phosphorylation, thus leading to a 35% decrease in the phosphorylation capacity of the organelle, and in vivo to a 30% decrease in growth yield.

ACKNOWLEDGMENT

We are grateful to Dr. P. V. Graves for stimulating discussions and Dr. Cooke for linguistic help.

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