Mutations in the Membrane Anchor of Yeast Cytochrome c1 Compensate for the Absence of Oxa1p and Generate Carbonate-Extractable Forms of Cytochrome c1

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ABSTRACT

Oxa1p is a mitochondrial inner membrane protein that is mainly required for the insertion/assembly of complex IV and ATP synthase and is functionally conserved in yeasts, humans, and plants. We have isolated several independent suppressors that compensate for the absence of Oxa1p. Molecular cloning and sequencing reveal that the suppressor mutations (CYT1-1 to -6) correspond to amino acid substitutions that are all located in the membrane anchor of cytochrome c1 and decrease the hydrophobicity of this anchor. Cytochrome c1 is a catalytic subunit of complex III, but the CYT1-1 mutation does not seem to affect the electron transfer activity. The double-mutant cyt1-1,164, which has a drastically reduced electron transfer activity, still retains the suppressor activity. Altogether, these results suggest that the suppressor function of cytochrome c1 is independent of its electron transfer activity. In addition to the membrane-bound cytochrome c1, carbonate-extractable forms accumulate in all the suppressor strains. We propose that these carbonate-extractable forms of cytochrome c1 are responsible for the suppressor function by preventing the degradation of the respiratory complex subunits that occur in the absence of Oxa1p.

In mitochondria, five enzymatic complexes located within the inner membrane catalyze the oxidative phosphorylation. The respiratory complexes I–IV transfer the electrons from NADH and succinate to oxygen; the complex V or ATP synthase utilizes the electrochemical gradient of protons generated by the electron flow to produce ATP. Complexes III (coenzyme QH2-cytochrome c reductase), IV (cytochrome c oxidase), and V each consist of 10 or more nonidentical subunits encoded by the mitochondrial or the nuclear genomes. The assembly of these oligomeric enzymes is an intricate process that also requires the action of assembly-assisting factors, also called chaperones, which are not intrinsic components of the complexes, but are required for their formation (see Grivell 1995 for review). In addition, a proteolytic system able to rapidly degrade the unassembled subunits that may disturb the organization of the inner membrane has been discovered, two of its components exhibiting both the chaperone and the protease functions (Artl et al. 1996; Rep and Grivell 1996b; see Suzuki et al. 1997 and references therein).

Oxa1p, one of the assembly-assisting factors, appears particularly interesting because we have shown that its protein sequence is conserved between prokaryotes and eukaryotes and that it is functionally conserved in yeast, humans, and higher plants (Bonnefoy et al. 1994b; Hamel et al. 1997). Yeast cells carrying an inactivated OXA1 gene are respiratory deficient. They display no cytochrome c oxidase activity, a block in the export and proteolytic maturation of the mitochondrially encoded subunit Cox2p of cytochrome c oxidase, a drastic reduction of oligomycin-sensitive ATPase activity correlated with the low presence of the ATP synthase complex within the membranes, and a slight decrease of cytochrome b content and complex III activity (Bauer et al. 1994; Bonnefoy et al. 1994a; Altamura et al. 1996; He and Fox 1997; Hell et al. 1997). Oxa1p presents five hydrophobic segments and is a polytopic inner membrane protein (Herrmann et al. 1997; Kermorgant et al. 1997; Meyer et al. 1997a,b). The study of its import within mitochondria suggests that, during evolution, its import pathway has also been conserved between prokaryotes and eukaryotes (Herrmann et al. 1997). Thus, Oxa1p is likely to play a crucial role in all respiring cells in the insertion/assembly of oxidase and ATP synthase complexes within the prokaryote plasma membrane or within the mitochondrial inner membrane, and Hell et al. (1998) have recently proposed that Oxa1p could be a component of a general export machinery of the mitochondrial inner membrane.
Interestingly, overexpression of the OXA1 gene suppresses respiratory defects associated with the single or double inactivation of RCA1 and AFG3 (Rep et al. 1996a). Rca1p, Afg3p, as well as Yme1p, belong to the ATP-dependent protease family and constitute the chaperone/protease system specific for inner membrane proteins (Thorner et al. 1993; Tzagoloff et al. 1994; Arti et al. 1996; Rep and Grevill 1996b; see Suzuki et al. 1997 and references therein). Thus, the genetic interaction between Rca1p/Afg3p and Oxa1p suggests that a tight cooperation exists between these chaperones/proteases and the assembly-assisting factors. Although the mechanism of action of the first ones was recently the subject of numerous studies, the function of Oxa1p or other proteins participating in mitochondrial respiratory complex formation is still poorly understood.

In this article, we present the isolation, molecular cloning, and sequencing of five independent nuclear suppressor mutations that restore the insertion/assembly of both cytochrome c oxidase and ATPase in the absence of Oxa1p. The five suppressor mutations are all located in the membrane anchor of cytochrome c1, the catalytic subunit of complex III. However, we show that the suppressor function is independent of the electron transfer activity of cytochrome c1. As new carbonate-extractable forms of cytochrome c1 are detected in all the suppressors, we discuss the possible role of soluble forms of cytochrome c1 in preventing the degradation of subunits that occurs when the insertion/assembly of respiratory complexes is perturbed by the oxa1 inactivation.

**MATERIALS AND METHODS**

**Media, strains, genetic methods and transformation:** Media used for Saccharomyces cerevisiae have been described in Dujardin et al. (1980); YPG: 1% Yeast extract, 1% bactopeptone, 2% galactose, 0.1% glucose, 20 µg/ml adenine. Glucose or galactose were used as fermentable substrates, and glycerol or ethanol as respiratory substrates. Genotype and construction of yeast strains are summarized in Table 1. Yeast genetic methods (crosses, cytoductions, rho0 strain construction, and genetic nature of suppressors) were described in Dujardin et al. (1980) and Bonnefoy et al. (1994a).

**Isolation of genetic suppressors:** Respiratory-competent revertants were selected from strains carrying an oxa1::LEU2 allele and either an intron-containing mitochondrial genome (NBT1) or an intron-free mitochondrial genome (NBT2; Table 1). Yeast cells were grown to late logarithmic phase, plated on glucose medium, and UV-irradiated in the dark for 5–20 sec at 254 nm. Irradiated plates were incubated for 3 days at 25°C, and then replica-plated on glycerol medium. Among 3.1010 UV-mutagenized cells, six independent revertants able to grow on glycerol medium were selected.

**Isolation of mitochondria and respiratory chain activities:** Yeast cells were grown on YPG medium and mitochondria were purified after disrupting cells either by the enzymatic method (Kermorgant et al. 1997) or mechanically (Brasseur et al. 1995). The mitochondrial protein concentration was determined using the Bio-Rad (Hercules, CA) assay. The respiratory activities were measured at 25°C, except ATPase activity performed at 30°C, in EDTA 50 µm, potassium phosphate 50 mm, pH 7.4. The activities of complexes II, III, and II plus III were measured as described by Brassey et al. (1995). Cytochrome c oxidase activity was determined according to Pajot et al. (1976). The ATPase activity was determined according to Pullin et al. (1960), except that 0.1 mm NADH was used, and 4 mm cyanide was added to inhibit respiration. Cytochrome contents of purified mitochondria were determined spectrophotometrically as described in Brassey et al. (1995).

**Western blot of mitochondrial proteins:** Mitochondria were treated with sodium carbonate according to Weiguny et al. (1991). pON163 was digested by Clai and self-ligated. The resulting plasmid was cut by EcoRI, blunt ended with Klenow enzyme, and ligated with a 1.1-kb blunt-ended BglII fragment containing the S. cerevisiae URA3 gene to give pNB30. pNB30 was then digested with Clai and ligated with a 0.5-kb Clai fragment carrying the origin of replication from the 2µ plasmid. CsCl-purified nuclear DNA from R14 was partially digested with HindIII and ligated with the HindIII-digested YEpBN33. The ligated DNA was used to transform electro-competent Escherichia coli cells to both ampicillin and kanamycin resistance. About 260,000 independent transformants were recovered. The library DNA was purified and used to transform the oxa1::LEU2 strain NBT2.

**Cloning and sequencing of the wild-type CYT1 and suppressor alleles:** YEpPH65 (see Figure 3) was cut by Hpal and self-ligated to give YEpPH66, which only carries the CYT1 gene. YEpPH66 was cut by BstEI and Swal, gel purified, and used to transform the strains CW30, NBT2, R101, and R118 to uracil prototrophy. Gap-repaired plasmids were characterized by restriction analysis, and the C-terminal region of the CYT1 gene was sequenced. PCR amplification was also carried out on genomic DNA extracted from the five suppressors, and the amplification products were sequenced on both strands using the sequenase PCR product sequencing kit (United States Biochemical, Cleveland).

**Construction of a CYT1-1 OXA1 strain:** A cyti::LEU2 strain was constructed by introducing the 2.7-kb LEU2 fragment at the NsiI site internal to CYT1 gene. Molecular and genetic analyses were carried out as described in Bonnefoy et al. (1994a) to verify the CYT1 inactivation. As expected the cyti::LEU2 strain (PHT3) is deficient for respiration, and cytochrome spectra show a complete lack of cytochrome c1. The CYT1-1 strain (PHT31) was constructed by transforming PHT3 with YEpPH65 and selecting respiratory-competent transformants that had lost the leucine prototrophy. Sequencing of the 3’ end of the CYT1 gene of these transformants showed that they contain the CYT1-1 mutation.

**Construction of a cytochrome c1 gene carrying both the CYT1-1 and the cyti-164 mutations:** YEpPH219 and YEpPH220 are multicopy plasmids carrying either the CYT1 or the CYT1 cDNA. PCR mutagenesis was carried out using two oligonucleotides O1 (5’ GTCGAATTCTGCGCCT CGT 3’) and the mutagenic oligonucleotide O5 (5’ GCGGGGTTGACCATCCT TCG ACTAACCACATGCTAACAA CACACTTGTGCTTGGCAAT GGAACCC3’) carrying the mutation CAT to CCT (M164 to K164). The PCR product was cut with AgeI and KpnI and cloned in
**TABLE 1**

List of yeast strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Nuclear genotype</th>
<th>Mitochondrial genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW30&lt;sup&gt;+&lt;/sup&gt;</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>Δi</td>
</tr>
<tr>
<td>CW04&lt;sup&gt;+&lt;/sup&gt;</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>Δi</td>
</tr>
<tr>
<td>NBT2</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 oxa1::LEU2</td>
<td>Δi</td>
</tr>
<tr>
<td>NBT1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 oxa1::LEU2</td>
<td>Δi</td>
</tr>
<tr>
<td>R101 (R118, R121)</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 oxa1::LEU2</td>
<td>Δi</td>
</tr>
<tr>
<td>R14 (R18)</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 oxa1::LEU2</td>
<td>Δi</td>
</tr>
<tr>
<td>R14/G481</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 oxa1::LEU2</td>
<td>Δi</td>
</tr>
<tr>
<td>PHT31</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 cyt1-1</td>
<td>Δi</td>
</tr>
<tr>
<td>PHT3</td>
<td>α, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 cyt1-1</td>
<td>Δi</td>
</tr>
</tbody>
</table>

Δi, mitochondrial genome with 13 introns; Δi, mitochondrial genome devoid of all introns. NBT2 was constructed by cytoducting an intron-free mitochondrial genome into the rho<sup>-</sup> derivative (strain devoid of mitochondrial genome) of NBT1. The suppressor strains R14 and R18 were obtained from the strain NBT1, and R101, R118, and R121 from the strain NBT2 (see results). R14/G481 was constructed by cytoducting an intron-containing mitochondrial genome carrying the mitochondrial cox1-G481 mutation into the rho<sup>-</sup> derivative of R14. The construction of the strains PHT31 and PHT3 is described in materials and methods.

<sup>+</sup>See Bonnefoy et al. 1994a.

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**RESULTS**

**Extragenic suppressors can compensate for the respiratory deficiency due to the inactivation of the OXA1 gene:** In the search for genetic interactions involving the OXA1 gene, both the two-hybrid and suppressor strategies could a priori be used. However it is well known that the two-hybrid system is difficult to utilize with highly hydrophobic membrane proteins such as Oxa1p. Thus, we decided to search for suppressor genes that were able to alleviate the respiratory defect of oxa1-null mutants. We were unable to isolate any multicopy suppressors, but six independent genetic suppressor strains were isolated and characterized further (see materials and methods). The six suppressor mutations all correspond to extragenic dominant nuclear mutations. Recombination was never detected between suppressor mutations from R14, R18, R101, R118, and R121, suggesting that they are probably located in the same gene, whereas R102 is not allelic to the other five. The study of the first five suppressor strains was continued further. The suppressor strains R14 to R121 (see Table 1) show different levels of growth on respiratory substrates (Figure 1A and data not shown). All seemed to display a thermosensitive growth at 36°C on nonfermentable medium, suggesting that they cannot replace Oxa1p at high temperatures. Cytoduction of an intron-less mitochondrial genome in the R14 and R18 nuclear backgrounds (and, reciprocally, cytoduction of an intron-containing mitochondrial genome in the R101, R118, and R121 nuclear backgrounds) showed that the compensatory effect is always much stronger in the absence rather than in the presence of mitochondrial introns (Figure 1A). We have previously shown that the major effect of the oxa1 inactivation occurs at a post-translational stage, but that it also leads to pleiotropic secondary defects in the accumulation of intron-containing mitochondrial RNA (Bonnefoy et al. 1994b). The difference in suppression observed between intron-free and intron-containing strains suggests that the suppressors do not accommodate these secondary defects.

**The activities of both the cytochrome c oxidase and ATPase complexes are restored in the suppressor strains:** As we had previously shown that Oxa1p is necessary for the activities of the cytochrome c oxidase and ATPase complexes (Altamura et al. 1996), we have also determined these activities in the suppressor strains. The results are presented for only one or two representative suppressor strains because the data were similar for all five strains.

As shown in Figure 1B, at least 80% of the oligomycin-sensitive ATPase activity and ~30-50% of cytochrome c oxidase activity were restored in the different suppressor strains. Thus, the ATPase activity is restored to nearly wild-type level, whereas cytochrome c oxidase activity is only partially recovered. A partial restoration of the cytochrome aa3 spectrum was also observed in the suppressor strains (Figure 2A and data not shown), indicating that some heme aa3 is correctly assembled within
Figure 1.—Respiratory growth, cytochrome c oxidase, and oligomycin-sensitive ATPase activities of the suppressor strains. 

A) Wild type (CW04); (2) oxa1::LEU2 mutant (NBT1); (3) suppressor strain R14; (4) suppressor strain carrying the cox1-G481 mutation (R14/G481); (5) wild type (CW30); (6) suppressor strain R101. See Table 1 for the complete genotype of strains. R14 and R101 carry the same nuclear suppressor mutation (see Figure 3) and only differ by their mitochondrial genome. Restoration of respiratory growth (A): Strains were grown on ethanol medium as a respiratory substrate and incubated 5 days at 28°C. 

Enzymatic activities (B): Mitochondria were purified and respiratory enzyme activities were measured as described in materials and methods. Activities are the mean values of several independent experiments. The suppressor activities are normalized to the values obtained for the corresponding wild-type strain. Cytochrome c oxidase activity: 540 ± 19 and 547 ± 50 nmol cytochrome c-oxidized/min/mg for CW30 and CW04, respectively. +, wild-type gene with no suppressor activity for the suppressor (SU).

Figure 2.—Cytochrome c oxidase assembly in the suppressor strains. 

A) Cytochrome spectra and mitochondrial translation products from wild-type (CW04 or CW30), oxa1::LEU2 mutant (NBT1 or NBT2), and suppressor (R14 or R101) cells were analyzed. Cytochrome spectra (A): Low-temperature cytochrome absorption spectra of galactose-grown cells were recorded as described in Bonnfoy et al. (1994a). Arrows, absorption maxima of the alpha bands of cytochromes c (546 nm), c1 (552 nm), b (558 nm), and aa3 (602 nm). 

Cox2p maturation (B): Mitochondrial translation products were labeled with 35SO4 in the presence of cycloheximide, as described in Bonnfoy et al. (1994a), and analyzed by a long electrophoresis in a 12.5% SDS acrylamide gel to differentiate precursor (pre-Cox2p) and mature (Cox2p) forms. In these conditions, Cox3p is run out off the gel. Arrowheads, variant 1 ribosomal protein (Var1p), mitochondrial subunits I and II of cytochrome b (Cytbp). 

Immunodetection of Cox2p and Cox6p (C): About 40 μg of mitochondrial membranes were separated by 15–25% SDS acrylamide gradient gels and analyzed by Western blotting with anti-Cox2p and anti-Cox6p. Under these gel electrophoresis conditions, the precursor and mature forms of Cox2p comigrate.
oxa1::LEU2 mutant, is present in lower than wild-type amounts in the suppressor strains (Figure 2C). The same observation was made for Cox1p and Cox3p (data not shown). As a control, the nuclear-encoded subunit Cox6p, which is only slightly affected by the oxa1::LEU2 mutation, was used; Cox6p is present in normal amounts in the suppressor strains. Thus, a fully active cytochrome c oxidase complex, with a correctly matured Cox2p, is present in the suppressors but at lower levels than in the wild type, and the ATPase complex is fully active in the suppressor strains. Thus, the suppressor mutations are able to compensate for both the cytochrome c oxidase and the ATPase activity defects due to the absence of Oxa1p.

Because the suppressors compensate for both the cytochrome c oxidase and the ATPase activity defects, we have asked whether the restoration of ATPase activity could occur independently of that of cytochrome c oxidase. We have constructed a suppressor strain R14/G481 carrying the mitochondrial mutation cox1-G481. This mutation, located in the gene coding for subunit 1 of the cytochrome c oxidase gene, results in a lack of the aa3 and cytochrome c oxidase activity consequent to the absence of the Cox1p subunit, but does not impair oligomycin-sensitive ATPase activity (Altamura et al. 1996). As shown in Figure 1B, the cytochrome c oxidase activity of the strain R14/G481 is nil, and the ATPase activity is reduced to the residual level measured in the oxa1::LEU2 mutant (Figure 1B). Thus, cytochrome c oxidase is required for the restoration of the ATPase activity.

The suppressor mutations are all located in the membrane anchor of cytochrome c1: To identify the suppressor gene, we have constructed a genomic library from the suppressor strain R14 in a shuttle yeast E. coli vector, allowing a positive selection of recombinant plasmids (see materials and methods). The oxa1::LEU2 strain was transformed with this library, and two respiratory-competent transformants were obtained. From these, two different recombinant plasmids, named YEph65 and YEph622, containing overlapping inserts, were isolated. YEph65 contains a 11.2-kb HindIII fragment with one internal HindIII site (Figure 3), while YEph622 carries a larger insert that includes all the YEph65 insert. The cloned fragment was located on the yeast chromosomes by sequencing the two extremities of the YEph65 insert. Comparison to the yeast genome sequence revealed that we had cloned a fragment from chromosome XV, encompassing six open reading frames (ORFs). By deleting either the 9.5-kb Hpal or the 9.8-kb Hpal/Swal restriction fragment from YEph65, we could restrict the region responsible for the suppression to the 1.3-kb HindIII/Swal fragment with an internal HindIII site. This region contains the CYT1 gene that encodes the cytochrome c1, a catalytic subunit of complex III of the respiratory chain. The 1.3-kb fragment includes a truncated form of the CYT1 promoter, the open reading frame, and 23 bases following the stop codon.

The fragment carrying the suppressor function was sequenced and compared to the sequence of the CYT1 gene present in the control wild-type strains. Two nucl-
otide substitutions (TTA to AAA) were found at positions 831 and 832, corresponding to codon 216 of the CYT1 ORF, changing a leucine into a lysine residue (Figure 3). The wild-type sequence was in agreement with the sequence published by Sadler et al. (1984).

We have cloned the other suppressor alleles by gap repair (see materials and methods). Gap-repaired plasmids, as well as PCR amplification of genomic DNA, were used to sequence the suppressor mutations. All of the suppressor mutations occur in the same C-terminal domain of Cyt1p, and the same amino acid substitution is found in R14 and R101 that was isolated from two different oxa1::LEU2 strains (Figure 3). This C-terminal domain is highly hydrophobic, depicted as an -helix in the wild type, and constitutes the anchor of cytochrome c1 in the mitochondrial inner membrane (for review see Trumpower 1990; Xia et al. 1997; Zhang et al. 1998). As a control, we have verified that the cloning on a multicopy plasmid of the wild-type cytochrome c1-encoding gene does not suppress the oxa1 inactivation.

Comparison of the amino acid sequences of cytochrome c1 from yeast and other eukaryotes (Figure 3C) shows that the membrane anchor is conserved and always highly hydrophobic. All of the suppressor mutations appear to decrease the hydrophobicity of the cytochrome c1 anchor. CYT1-1 and CYT1-2 replace a leucine residue with a lysine residue at positions 216 (L216K) and 219 (L219K), respectively. CYT1-3 mutation is due to the substitution of a leucine by an asparagine (L219N) and to the deletion of the adjacent tyrosine, leading to a shorter and less hydrophobic anchor. CYT1-4 carries three substitutions, T212K, V213M, and L216S. Such multiple mutation events are often observed at or near the sites of dimers with UV mutagenesis in the dark, particularly in A/T-rich regions. The three mutations detected in the CYT1-4 allele are located in such an A/T-rich region. The V213M substitution is probably silent because there is a methionine at this position in the yeast Kluyveromyces lactis. The substitution T212K replaces a neutral residue with a hydrophilic one, and substitution L216S replaces a hydrophobic residue with a neutral one. Finally, the CYT1-5 and CYT1-6 mutations were subsequently isolated from an oxa1::URA3 mutant: CYT1-5 replaces the leucine 216 with an arginine (L216R), and CYT1-6 corresponds to the deletion of the isoleucine 215. Both mutations also diminish the hydrophobicity of the anchor.

Carbonate-extractable forms of cytochrome c1 are present in the suppressor strains: The fact that the suppressor activity is due to various amino acid substitutions all leading to a decrease in the hydrophobicity of the anchor domain suggests that these substitutions could modify the binding of cytochrome c1 to the membrane. A priori, two methods of fractionation, osmotic swelling and carbonate extraction, could be used to separate membrane and soluble proteins. However, it is known that upon osmotic swelling, the soluble cytochrome c stays mainly associated with the membrane through electrostatic interactions. In contrast, cytochrome c is well extracted by carbonate treatment, so we used this technique to study the binding of cytochrome c1 to the membrane in the various suppressor strains. As expected from previous work (Hase et al. 1987b), cytochrome c1 is totally resistant to carbonate extraction in CYT1 OXA1 strains (CW04 or CW30) because it is tightly anchored within the inner membrane (see Figure 4). Cytochrome c1 is also completely resistant to carbonate extraction in CYT1 oxa1::LEU2 strains (NB1 or NBT2). In the suppressor strains, membrane-bound form of cytochrome c1 is still present but in addition, minor fast-migrating forms are found in the supernatant. These extractable forms are systematically detected in the different suppressors, which were independently isolated from different oxa1-null mutants (Figure 4 and data not shown). Thus, the suppressor mutations seem to modify the insertion of cytochrome c1 into the mitochondrial inner membrane.

The suppressor mutation CYT1-1 does not affect complexes II and III maximal activities: Cytochrome c1 is a catalytic subunit essential for electron transfer between complex III and cytochrome c. To determine whether the suppressor mutation located in the membrane anchor of cytochrome c1 affects the activity of complex III, we constructed a strain carrying the mutation CYT1-1 in an OXA1 context (PHT31, see Table 1 and materials and methods). This strain is respiratory competent, exhibits a complex III maximal activity, and cytochrome spectra similar to that of wild-type CYT1 strain (see Figure 5, A and B). Measurements of heme b content in mitochondria showed that the turnover of complex III is also unaffected, although a slight modification of the kinetic interaction between cytochrome c and complex III cannot presently be ruled out. Bruel et al. (1996) have described mutants of the complex III subunit 8 which do not affect complex III activity but decrease the complex II activity. Therefore, we have measured the complex II maximal activity in CYT1-1 cells. Figure 5A shows that complex II maximal activity is not significantly decreased by the CYT1-1 mutation. Carbonate extraction of CYT1-1 mitochondria (Figure 5C) reveals that all the cytochrome c1 is found in the pellet, suggesting that the cytochrome c1 insertion is not modified by the CYT1-1 mutation in a wild-type OXA1 genetic background. Thus, the carbonate-extractable forms are only detected when suppressor mutations are associated to the oxa1::LEU2 inactivation.

Suppressor function is independent of the electron transfer activity: To directly test the relationship between the suppressor and the electron transfer activity of cytochrome c1, we have constructed a CYT1 gene carrying both the suppressor mutation CYT1-1 and the mutation cyt1-M164K (see materials and methods). The M164K mutation was chosen because it drastically decreases the complex II plus III activity without
blocking cytochrome c1 assembly in complex III (Nakai et al. 1990). We have determined whether the double-mutant cyt1-1,164 is still able to compensate for the absence of Oxa1p. As expected the plasmid YEpPH220 carrying the CYT1-1 mutation partially restores the respiratory defect due to the oxa1::LEU2 mutation, and YEpPH221, which carries the double-mutant cyt1-1,164, does not complement the respiratory deficiency due to
the cyt1::LEU2 inactivation (see Figure 6); the complex II plus III activity measured in these latter transformants (cyt1::LEU2/YEpPH221) was found to be 10% of the level measured in the cyt1::LEU2 strain transformed by YEpPH219 carrying the CYT1 gene. On the contrary, CYT1 oxal::LEU2 cells transformed by YEpPH221 (cyt1-1,164) are able to grow on respiratory medium (Figure 6). These transformants carry two cytochrome c1 genes, the chromosomal CYT1 and the plasmidic cyt1-1,164 genes. The chromosomal CYT1 gene is able to ensure full electron transfer activity, but does not have the suppressor activity. Thus, the suppressor activity comes from the plasmidic cyt1-1,164 gene, which codes for a protein with drastically reduced electron transfer activity. This result strongly suggests that the suppressor activity of the mutant cytochrome c1 is independent of its catalytic activity in electron transfer.

DISCUSSION

Oxa1p is a mitochondrial inner membrane protein that is required for the insertion/assembly of the respiratory complexes. Oxa1p seems to be present in all aerobic organisms, and we have shown here that in yeast, it is possible to isolate suppressors which compensate for the absence of Oxa1p at 28°C. In the suppressor strains, the oligomycin-sensitive ATPase activity is fully restored and there is a partial recovery of cytochrome c oxidase activity. Turnover measurements and steady-state levels of mitochondrial subunits show that the assembled complex IV is fully active but present in reduced amounts within the membranes. We have cloned the suppressor gene and shown that it corresponds to CYT1 that encodes cytochrome c1.

Cytochrome c1 is a catalytic subunit of complex III, it transfers the electrons from complex III to cytochrome c and is conserved in bacterial and eukaryotic systems. The protein has a bipartite structure with a large N-terminal hydrophilic domain (often called the soluble

![Diagram](image_url)

Figure 7.—Model to explain suppressor activity. Complex III, rectangle including the eight membrane-spanning helices of Cytbp; bold line, cytochrome c1 anchor; gray circle, hydrophilic region of the molecule exposed to the intermembrane space (IMS). Complex IV, rectangle including the 12 membrane-spanning helices of Cox1p and a schematic representation of Cox2p (membrane-spanning helices and a rectangle that represents the hydrophilic part extending in the IMS). The topological arrangement of Oxa1p within the inner membrane is drawn according to Herrmann et al. (1997). Arrows, electron flux (e⁻) from cytochrome c1 to cytochrome c (black circle) and complex IV. OXA1 CYT1: Oxa1p is present in the membrane, the respiratory complexes are correctly assembled, and electron transfer proceeds normally from complex III to complex IV via cytochrome c. oxal::LEU2 CYT1-1: Oxa1p is absent, complex IV cannot assemble, and some subunits are degraded by proteases. Electron flow is blocked between cytochrome c and complex IV. oxal::LEU2 CYT1-1: A mutation in the membrane anchor of Cyt1p modifies its insertion into the inner membrane and generates soluble forms of the molecule that prevent mitochondrial cytochrome c oxidase subunits from degradation.

![Diagram](image_url)

Figure 6.—Compensation for the oxal inactivation by a catalytically inactive cytochrome c1. The cyt1::LEU2 strain (PHT3) and the oxal::LEU2 strain (NBT2) were transformed by plasmids carrying the CYT1 gene, with either the suppressor mutation CYT1-1 (YEpPH220) or the double mutations cyt1-1,164 (YEpPH221). Transformants were streaked on non-fermentable medium and incubated at 28°C.
domain) that is located in the intermembrane space and binds the heme c1 and a short C-terminal domain composed of a stretch of hydrophobic amino acids, responsible for the binding to the mitochondrial inner membrane (Li et al. 1981; for review see Trumpower 1990; Xia et al. 1997). In S. cerevisiae, replacement of a large part of the C-terminal region by a foreign sequence leads to a loss of assembly and activity of the complex III, due to modified binding of cytochrome c1 to the inner membrane (Hase et al. 1987a,b). Similarly, in Rhodobacter sphaeroides, deletion of the C-terminal domain results in a soluble protein located in the periplasmic space, which is no longer catalytically active although heme c1 is correctly bound to the apoprotein (Konishi et al. 1991). All suppressor mutations described here correspond to amino acid substitutions that diminish the hydrophobicity of the C-terminal membrane anchor. These mutations do not seem to affect the electron transfer activity of complex III. Moreover, a cytochrome c1 with drastically reduced electron transfer activity and that carries the suppressor mutation CYT1-1 still retains the suppressor activity. Together, these results show that the suppressor function of cytochrome c1 is independent of its electron transfer activity.

It has been shown that oxal1 mutants in vivo affect the insertion/assembly of complexes IV and V and to a lesser extent of complex III, and that proteolysis occurs leading to the degradation of the mitochondrial subunits of complex IV (Altamura et al. 1996; Meyer et al. 1997b and this study). When the oxal1 mutant is combined with the CYT1 suppressor mutations, faster migrating carbonate-extractable forms of cytochrome c1 are observed, which are probably soluble forms resulting from proteolysis. We suspect that in an oxal1+ context where insertion of respiratory complex subunits is already perturbed (He and Fox 1997; Hell et al. 1997, 1998), the presence of the mutations in the anchor of cytochrome c1 slows down the insertion into the membrane sufficiently to allow proteolysis to occur, producing the carbonate-extractable forms of cytochrome c1 detected in our experiments.

These new carbonate-extractable forms of cytochrome c1 are probably catalytically inactive as the association to the membrane is essential for electron transfer (Konishi et al. 1991), and we propose that these new forms are responsible for the suppressor function (Figure 7). It is unlikely that these carbonate-extractable forms of cytochrome c1 can replace the inner membrane protein Oxal1p. However, as we have shown that in the absence of Oxal1p, proteolysis occurs leading to the degradation of mitochondrial cytochrome c oxidase subunits, we propose that the carbonate-extractable forms of cytochrome c1 prevent the degradation of these subunits. These new forms of cytochrome c1 could interact with complex IV either directly or indirectly via its usual partner for the electron transfer function, cytochrome c. Indeed, cytochrome c1 is protected from degradation by interactions with cytochrome c1 or complex IV (Pearce and Sherman 1995a), and cytochrome c-deficient mutants present a defect in complex IV correlated to the degradation of Cox2p and Cox3p by the protease Yme1p (Pearce and Sherman 1995b). However, the overexpression of iso-1 or iso-2 cytochrome c-encoding genes does not compensate for the absence of Oxal1p, and the inactivation of the gene YME1 in the oxal1-null mutant does not restore the stability of Cox2p (data not shown). Thus, we propose that the carbonate-extractable forms of cytochrome c1 interact directly with complex IV. To our knowledge, no interactions between cytochrome c1 (or complex III) and ATP synthase have been reported. Thus, we postulate two interactions to explain the restoration of assembly of the ATP synthase in the suppressor strains: first, an interaction between cytochrome c1 and complex IV, and then an interaction between complex IV and ATP synthase, which was already reported in bovine mitochondria (Qiu et al. 1992).

Although further biochemical experiments will be necessary to determine if the carbonate-extractable forms of cytochrome c1 are directly responsible for the suppression by interacting with complex IV, several results favor this model. First, in a preliminary experiment, we have detected carbonate-extractable forms of cytochrome c1 in the suppressor R102 that is not allelic to CYT1 (data not shown). Second, we have shown that in the strain R14/G481 carrying the oxal1 null, the suppressor CYT1-1, and the oxal1-G481 mutations, and thus displaying no active complex IV, no restoration of the ATPase activity is observed (Figure 1B), although carbonate-extractable forms of cytochrome c1 are still present (data not shown). This suggests that complex IV is also required for the suppression. Third, Zhang et al. (1998) have very recently shown that the structure of the extrinsic domain of cytochrome c1 shares high similarity to the structure of cytochrome c. It is well known that cytochrome c directly interacts with complex IV, and Pearce and Sherman (1995b) have shown that cytochrome c protects complex IV from degradation. This similarity of structure is consistent with a direct interaction between cytochrome c1 and complex IV.

Finally, both OXA1 and CYT1 are conserved through evolution, and we have cloned and inactivated the Schizosaccharomyces pombe homolog of OXA1 (N. Bonnefoy, unpublished results). Interestingly, the S. cerevisiae CYT1-1 suppressor is also able to suppress the inactivation of the OXA1 homolog in S. pombe. The fact that the suppressor can be exchanged between these two yeasts that are highly diverged suggests that the intricate relationships existing between insertion, assembly, and stability of the respiratory complex subunits are conserved through evolution.

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**LITERATURE CITED**


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