The *Saccharomyces cerevisiae OXA1* gene is required for the correct assembly of cytochrome *c* oxidase and oligomycin-sensitive ATP synthase

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Abstract The nuclear gene *OXA1* was first isolated in *Saccharomyces cerevisiae* and found to be required at a post-translational step in cytochrome *c* oxidase biogenesis, probably at the level of assembly. Mutations in *OXA1* lead to a complete respiratory deficiency. The protein Oxa1p is conserved through evolution and a human homolog has been isolated by functional complementation of a yeast *oxa1*⁻ mutant. In order to further our understanding of the role of Oxa1p, we have constructed two yeast strains in which the *OXA1* open reading frame was almost totally deleted. Cytochrome spectra and enzymatic activity measurements show the absence of heme *aa*$_3$ and of a cytochrome *c* oxido-reductase activity and dramatic decrease of the oligomycin sensitive ATPase activity. Analysis of the respiratory complexes in non-denaturing gels reveals that Oxa1p is necessary for the correct assembly of the cytochrome *c* oxidase and the ATP synthase complex.

Key words: Respiratory complex assembly; Cytochrome *c* oxidase; ATP synthase; *OXA1*; *Saccharomyces cerevisiae*

1. Introduction

In mitochondria, oxidative phosphorylation is catalyzed by five enzyme complexes located in the inner membrane. The complexes I–IV are respiratory complexes which generate the proton gradient which drives ATP synthesis at the fifth complex, the oligomycin-sensitive ATP synthase. The complexes III (coenzyme QH_2-cytochrome *c* reductase), IV (cytochrome *c* oxidase) and V each consist of ten or more non-identical subunits encoded by the mitochondrial and the nuclear genomes. The biogenesis of these three complexes is also dependent on several nuclear-encoded proteins which are not components of the complexes but control the expression of the mitochondrially encoded subunits or the final assembly of the complexes within the inner mitochondrial membrane [1].

In a search for nuclear genes involved in the biogenesis of cytochrome *c* oxidase in yeast, Bonnefoy et al. [2] isolated the nuclear gene *OXA1* and showed that this gene is primarily required at a post-translational step in cytochrome *c* oxidase biogenesis, probably at the level of assembly of the complex. However, the *oxa1-79* mutation leads to some pleiotropic secondary defects in earlier steps of mitochondrial gene expression, particularly in a strain whose mitochondrial genome contains introns. It has also been shown that the Oxa1p sequence is conserved from prokaryotes to eukaryotes and the human *OXA1*-like cDNA has been cloned by Bonnefoy et al. [3] who have shown that the human protein can functionally replace the yeast Oxa1p protein.

In order to further our understanding of the role of this evolutionary conserved protein in cytochrome oxidase biogenesis, we have constructed two yeast strains in which the *OXA1* open reading frame has been almost totally deleted and which carry either an intron-containing or an intron-free mitochondrial genome. Cytochrome spectra, enzymatic activities and analysis of the respiratory complexes in non-denaturing gels have been performed in these two strains and compared to the corresponding wild type and to two other cytochrome *c* oxidase deficient mitochondrial mutants. The results presented in this paper show that the *OXA1* gene product is necessary not only for the correct assembly of the cytochrome *c* oxidase complex but also for the assembly or the stability of the ATP synthase complex.

2. Materials and methods

2.1. Media, strains and genetic methods

Wild type and mutant yeast strains were routinely grown on complete medium containing 1% yeast extract, 2% peptone and 2% galactose (YPGal). Wild type CW04 and CW30 are isonuclear strains (*alpha, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112*), carrying either an intron-containing mitochondrial genome (*rho*$^+$ *mit*$^+$ *[Σi]*) or an intron-free mitochondrial genome (*rho*$^+$ *mit*$^+$ *[Δi]*), respectively. Cytochrome *c* oxidase deficient strains CG481 (*alpha, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112, rho*$^+$ *cox1-G481[Σi]*) and CV45 (*alpha, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112, rho*$^+$ *cox2-V45[Σi]*) were constructed by cytoduction [4]. They are both isonuclear to CW04. CG481 is isomitochondrial to 777-3A/G481 [5] and CV45 is isomitochondrial to AB1-4D/V45 [6]. NBT3 (*alpha, ade2-1 oxa1::URA3 his3-11,15 trp1-1 leu2-3,112, rho*$^+$ *mit*$^+$*[Σi]*) and NBT4 (*alpha, ade2-1 oxa1::URA3 his3-11,15 trp1-1 leu2-3,112, rho*$^+$ *mit*$^+$*[Δi]*) carry the *oxa1::URA3* deleted allele (see contruction below).

2.2. Construction and analysis of a deleted oxa1::URA3 allele

The plasmid pNB15 was constructed by subcloning the 4.4 kb *Kpn*I genomic fragment encompassing the *OXA1* gene into a pBluescript KS-vector (Stratagene), whose *Hin*dIII and *Cla*I sites have been removed by a small deletion in the polylinker. pNB15 was digested at its unique *Cla*I and *Hin*dIII sites, located at both extremities of the *OXA1* ORF [2], and the generated 950 bp *OXA1 Cla*I-*Hin*dIII fragment was replaced by the 1.4 kb *Cla*I-*Hin*dIII *URA3* fragment from vector pFL44L. To further delete 126 bp of the 5′ end of the *OXA1* ORF, the recombinant plasmid was cut at its unique *Cla*I and *Msc*I sites, blunt-ended and religated. Only the first 58 bp and the last 76 bp of *OXA1* ORF remain in the final construct. The recombinant 4.7 kb *Kpn*I fragment containing the disrupted *OXA1* gene was used for transformation of the CW04 and CW30 strains by the one-step method [7] to give NBT3 and NBT4, respectively. Molecular and genetic analyses were carried out as described in [2] to verify *oxa1* inactivation.

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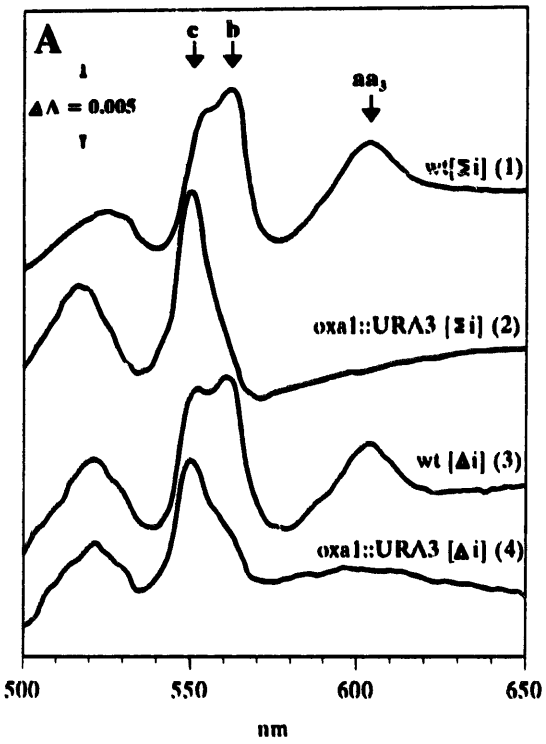
2.3. Preparation of mitochondria and enzyme assays

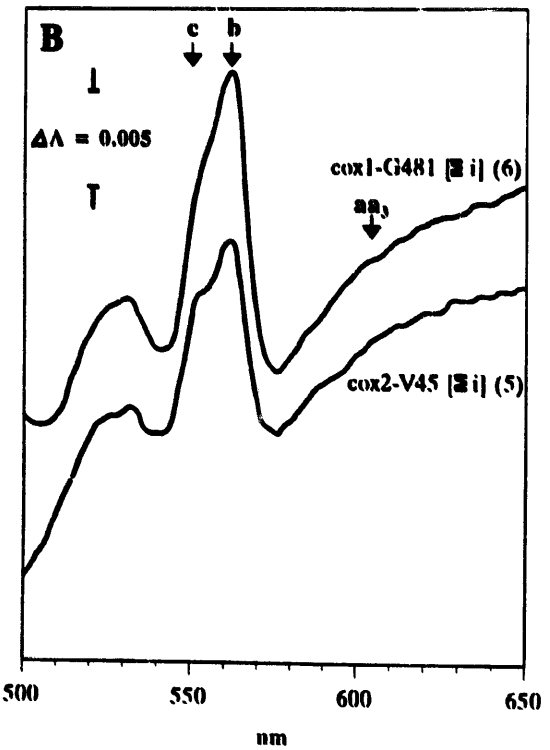
Cells were grown in liquid YPGal medium, at 29°C, until an OD of 1.3. Mitochondria were isolated essentially as described in [8] except that lyticase instead of glucusulase was used for the preparation of the spheroplasts. Alternatively, the glass bead step was omitted. Protein concentration was determined by the method of Bradford. Succinate-cytochrome *c* reductase activity was measured spectrophotometrically by monitoring the rate of ferricytochrome *c* reduction at 550–540 nm. Mitochondria were suspended in a 75 mM sucrose, 30 mM Tris-HCl, 50 mM KCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 1 mM potassium phosphate buffer, pH 7.4, supplemented with 2 mM of potassium cyanide and 10 μM ferricytochrome *c*. The reaction was started by the addition of 10 mM succinate. The rate of cytochrome *c* reduction was corrected for non-specific reaction by measurement in the presence of 1 μg antimycin A/ml. Cytochrome *c* oxidase activity was measured by following the initial rate of ferrocytochrome c oxidation in the presence of antimycin A. Heme content (*b* and $a + a_3$) was estimated as described in [9,10].

ATPase activity was measured following ATP hydrolysis with an ATP-regenerating system coupled to NADH oxidation [11]. Freeze-thawed mitochondria were suspended in 375 mM sucrose, 75 mM KCl, 30 mM Tris-HCl, 3 mM $MgCl_2$, pH 7.4, supplemented with 1.5 mM phospho*enol*pyruvate and 2 μl of the commercially available stock mixture of pyruvate plus lactate dehydrogenase. Oxidation of 50 μM of NADH was started by addition of 350 μM ATP and corrected by a parallel measurement in the presence of 1.5 μg/ml oligomycin.

2.4. Electrophoresis and immunoblot

Blue Native Gel electrophoresis of mitochondrial complexes was performed as described by Shagger and Von Jagow [12] After electrophoresis the gel was either stained with coomassie R250 or electroblotted onto nitrocellulose (Schleicher and Schuell). ATPase and cytochrome *c* oxidase complexes were revealed with rabbit antisera raised against the holoenzymes, using the Protoblot system (Promega). The cytochrome *c* oxidase antiserum was a gift from Prof. G. Schatz. The ATP synthase antiserum was a gift from M. Kermorgant [13].





3. Results

To further understand the role of the nuclear gene *OXA1*, we have compared the activity and the assembly of the various respiratory complexes in *oxa1* inactivated and wild type strains. In the previous study, the construction of the *oxa1* inactivated strain was achieved by interrupting the *OXA1* sequence with the insertion of the *LEU2* gene [2]. In order to obtain a strain lacking the *OXA1* sequence, we constructed two new *oxa1::URA3* deleted strains, NBT3 and NBT4, in which the *OXA1* gene was almost totally deleted (see section 2). NBT3 possesses an intron-containing mitochondrial genome and NBT4 an intron-free mitochondrial genome. As expected, both strains are respiratory deficient. These two *oxa1::URA3* inactivated strains have been used for all the following studies.

3.1. Pleiotropic deficiency of mitochondrial respiratory complexes in oxa1 inactivated strains

Whole cell absorption spectra (not shown) of both NBT3 and NBT4 strains carrying the *oxa1::URA3* deletion are identical to the spectrum of the NBT1 strain carrying the

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Fig. 1. Cytochrome spectra of mitochondria. Mitochondria isolated from the wild type and various cytochrome *c* oxidase deficient strains were purified as described in section 2 and resuspended in 100 mM Hepes, 1.5% sodium cholate, pH 7.4, at a protein concentration of 2 mg/ml. Differential spectra (dithionite-reduced minus ferricyanide-oxidized) were recorded by split beam or diode array spectrophotometry. [Σi] and [Δi] indicate the presence or absence of introns in the mitochondrial genome, respectively. Absorption maxima for the alpha bands of cytochrome *c*, *b* and aa_3 are indicated by arrows. The absorbance scale is indicated in each panel. (A) Cytochrome spectra of *oxa1* inactivated and wild type mitochondria. 1, CWO4; 2, NBT3; 3, CW30; 4, NBT4. (B) Cytochrome spectra of *cox1* and *cox2* mutated mitochondria 5, CV45; 6, CG481. See section 2 for the complete genotype of each strain.

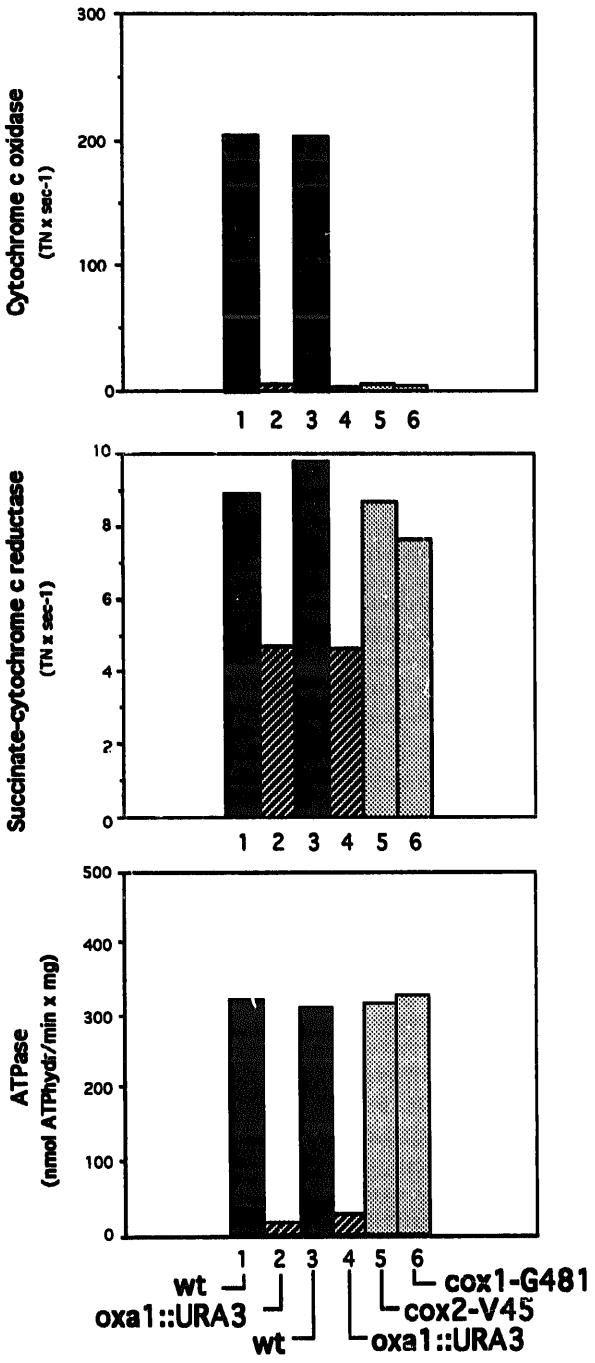

Fig. 2. Cytochrome *c* oxidase, succinate-cytochrome *c* oxido-reductase and ATPase activities in wild type and cytochrome *c* oxidase deficient mitochondria. Cytochrome *c* oxidase, succinate-cytochrome *c* oxido-reductase and ATPase activities were measured at 25°C as described in section 2. Activities are expressed as indicated. Turnover (TN) refers to μmol of substrate oxidized or reduced/heme content per mg mitochondrial protein. Strains are as in Fig. 1.

oxa1::LEU2 disruption [2]. Cytochrome aa_3 is undetectable and cytochrome *b* is decreased. Mitochondria from both NBT3 and NBT4 were purified and differential cytochrome spectra were recorded. As shown in Fig. 1A, both the NBT3 and NBT4 mitochondria totally lack cytocrome aa_3 and exhibit a drastic decrease of cytochrome *b* in comparison with the corresponding wild type mitochondria.

In order to analyse the consequence of these cytochrome defects on the oxidative phosphorylation activities, we have measured the succinate-cytochrome *c* reductase, cytochrome *c* oxidase and oligomycin-sensitive ATPase activities in the *oxa1*

Fig. 3. Blue Native PAGE profile of respiratory complexes in wild type and *oxa1* inactivated mitochondria. Mitochondria were prepared as described in section 2. Inner membrane complexes were extracted from mitochondria in 0.75 M aminocaproic acid, 50 mM Bistris/HCl, pH 7.0 and 1% laurylmaltoside. 100 μg of proteins were loaded on a 1% step gradient of acrylamide gel, from 5% to 10%. Lane a, CW04; lane b, NBT3; lane c, NBT4.

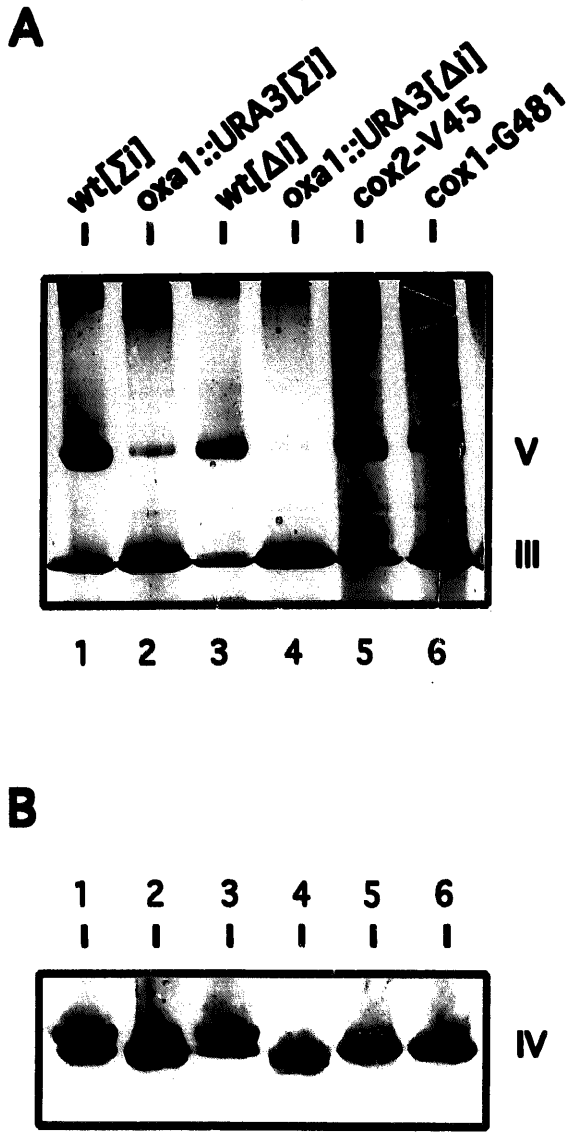

Fig. 4. Comparison of Blue Native PAGE profile of respiratory complexes of various cytochrome *c* oxidase deficient mitochondria. BN-PAGE was performed as in Fig. 3 except that a 5–15% acrylamide gradient was used. (A) Coomassie blue staining of the gel. (B) Immunoblot revealed with the cytochrome *c* oxidase holoenzyme antibody (see section 2). Strains are as in Fig. 1.

inactivated strains. Fig. 2 shows that in both *oxa1::URA3* mutants, the cytochrome *c* oxidase activity is abolished, the succinate-cytochrome *c* reductase activity is diminished and the oligomycin-sensitive ATPase activity is dramatically decreased.

A priori, the diminution of cytochrome *b* and complex III activity as well as the strong decrease of ATPase activity could be either the direct effect of the lack of Oxa1p or the consequence of the total absence of cytochrome aa_3 and cytochrome *c* oxidase activity. In order to see if a cytochrome oxidase deficient mutant would lead to such pleiotropic defects, we have constructed two other cytochrome *c* oxidase deficient strains isonuclear to NBT3 and NBT4 (see section 2). The strain CG481 carries the mutation *cox1-G481* in the mitochondrial gene encoding the subunit I (COXI) of cytochrome oxidase [5]. The strain CV45 carries the mutation *cox2-V45* in the mitochondrial gene encoding the subunit II (COXII) of cytochrome oxidase [6]. Cytochrome spectra of purified mitochondria were recorded and the three activities measured. As expected, the two mitochondrial mutants totally lack cytochrome aa_3 (Fig. 1B) but both possess an almost normal cytochrome *b* content and succinate-cytochrome *c* reductase activity (Fig. 2). In addition the ATP synthase activity is not affected in the two mitochondrial mutants. Thus, the ATPase activity decrease observed in *oxa1* inactivated strains should not be a secondary consequence of the lack of cytochrome *c* oxidase activity but is probably a direct consequence of the *oxa1* inactivation.

3.2. *Alteration of the native cytochrome c oxidase complex is associated with a deficiency in the ATP synthase complex in the oxa1 inactivated strains*

In a previous study [2], we have proposed that the *OXA1* gene is involved in the cytochrome *c* oxidase assembly. In order to study directly the native state of the different complexes of the oxidative phosphorylation pathway, we have used the Blue Native electrophoresis (BN-PAGE) technique described by Schagger and Von Jagow [12]. This 'charge shift' method allows a one-step separation of the four respiratory chain complexes as well as the ATP synthase complex. A typical BN-PAGE experiment is shown in the Fig. 3 in which mitochondria prepared from the two *oxa1*-inactivated strains, NBT3 and NBT4, are compared to the wild type CW04.

Under our conditions the complexes separate in the same order, complex V, III, IV and II, as for mammalian mitochondria [12] except that the complex I is absent in yeast. Interestingly, two main differences can be detected in both *oxa1* inactivated strains (NBT3 and NBT4) in comparison to the wild type. First, the amount of complex V is severely diminished in NBT3 and almost absent in NBT4. Second, the complex IV displays a mobility shift in NBT3 and NBT4. This difference in mobility is reproducibly observed and under some electrophoresis conditions, cytochrome *c* oxidase can even be separated into several subcomplexes (Fig. 4). In contrast, no effect on complex III assembly was observed although the cytochrome *b* appears decreased in the spectra.

The assignment of the complexes IV and V was verified by immunoblot analysis in native gels using antisera raised against the two holoenzymes. As shown in Fig. 4B, the antiserum recognizing the cytochrome *c* oxidase holoenzyme reveals the various subcomplexes IV.

We have also examined the assembly of the four complexes in the two mitochondrial mutants CG481 and CV45. As shown in Fig. 4, a mobility shift of complex IV migration is also observed in these mutants indicating that mutations in either *cox1* or *cox2* result in an altered complex IV. Interestingly, the complex V is unaffected in these latter mutants. Taken together, our results indicate that the *OXA1* gene is necessary for the assembly of a correct cytochrome *c* oxidase complex as well as for the assembly or the stability of the ATP synthase complex. These assembly defects lead to a strong decrease in the corresponding activities of the oxidative phosphorylation pathway.

4. Discussion

In this study, we show that the absence of Oxa1p leads to a

defect in the assembly of cytochrome *c* oxidase and of ATP synthase independent of the intron content of the mitochondrial genome. A priori, three hypotheses can explain this dual deficiency. (i) The lack of Oxa1p could primarily lead to cytochrome *c* oxidase deficiency resulting in an ATP synthase defect. (ii) The lack of Oxa1p could primarily lead to the ATP synthase deficiency resulting in the oxidase defect. (iii) Both defects could directly result from the lack of Oxa1p. We have ruled out the first hypothesis because we have shown that two mitochondrial mutations leading to a complete cytochrome *c* oxidase deficiency and to a mis-assembled complex IV have no effect on activity and assembly of ATP synthase. Concerning the second hypothesis, it has been shown that mutations affecting the formation of ATPase induce pleiotropic phenotype with lowered activities for both cytochrome *c* oxidase and coenzyme QH_2-cytochrome *c* reductase ([14] and references therein). However, in the *oxa1* inactivated mutants, the cytochrome *c* oxidase activity is nil and cytochrome aa_3 is undetectable. Thus the *oxa1*$^-$ phenotype clearly differs from the phenotype of the ATPase mutants and we favour the third hypothesis that both the cytochrome *c* oxidase and the ATPase deficiency are a direct consequence of the *oxa1* inactivation. Bauer et al. [15] have shown that the *oxa1* inactivation also blocks the proteolytic processing of the cox2 presequence. Such accumulation of unprocessed cox2 subunit is also observed under conditions that prevent the formation of the normal electrochemical gradient [16] and these authors have proposed that an energized inner membrane may be required for proper folding or insertion of the cox2 subunit into the membrane. Thus, the cox2 processing defect observed in the *oxa1* inactivated strains could be a secondary effect of the ATPase defect.

Several genes are already known to govern specifically the assembly of cytochrome *c* oxidase or ATPase [14,17–20]. Recently two genes, *RCA1* and *AFG3*, were shown to control the assembly of more than one respiratory complex [21]. These genes belong to the AAA protein family and would code for ATP-dependent metallo-proteases required for the degradation of incompletely synthesized or misfolded polypeptides of the mitochondrial inner membrane [22–28]. The inactivation of *RCA1* or *AFG3* was shown to diminish the NADH cytochrome *c* oxido-reductase, the cytochrome *c* oxidase and the ATPase activities, as well as to cause an arrest in assembly of the complexes III and IV and to delay the assembly of the F_1-ATPase complex components [21,29]. In this study, we show that the inactivation of the *OXA1* gene, which does not belong to the AAA protein family, decreases the assembly or the stability of only two complexes, the ATPase and the cytochrome *c* oxidase complexes. Thus, we believe that *OXA1* codes for a chaperon-like protein involved in the assembly of the cytochrome *c* oxidase and the ATP synthase. However, Wagner et al. [30] have shown that molecular chaperons such as mt-hps70 cooperate with PIM1 protease in the degradation of misfolded chimeric proteins in mitochondria. Similarly, it is likely that the assembly of respiratory complexes would imply a tight cooperation between chaperons such as Oxa1p and proteases belonging to the AAA family.

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