Molecular and biochemical analysis of Saccharomyces cerevisiae *cox*1 mutants

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Abstract We report on the molecular and biochemical analysis of a set of 13 respiratory deficient mutants of *Saccharomyces cerevisiae* which are specifically altered in *COX1*, the gene encoding the subunit Cox1p of cytochrome *c* oxidase. DNA sequence analysis shows that three are due to frameshift mutations, two to nonsense mutations, and eight to missense mutations. All, except the missense mutant S157L, have impaired electron transfer and respiratory activity. Analysis of the mitochondrial translation products shows that when Cox1p is absent, Cox2p and Cox3p are still synthesized. In the missense mutants, the steady state levels in the mitochondrial membranes of the three mitochondrially encoded subunits Cox1p, Cox2p and Cox3p and the nuclear-encoded subunit Cox4p are reduced. In the frameshift and nonsense mutants, Cox1p is absent and Cox2p, Cox3p and Cox4p are considerably decreased or undetectable. A comparison of the steady state levels of Cox1p through Cox4p in the *COX1, COX2, COX3* and *COX4* mutants shows the interdependance of the accumulation of these four subunits in the mitochondrial membranes.

Key words Cytochrome *c* oxidase · *Saccharomyces cerevisiae* · Complex assembly

Introduction

Cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, catalyzes oxygen reduction and connects this reaction to the generation of a proton gradient across the inner mitochondrial membrane. Recently, two different three-dimensional structures of heme copper oxidases have been reported almost simultaneously: the bacterial cytochrome *aa3* of *Paracoccus denitrificans* which consists of four subunits (Iwata et al. 1995) and the eucaryotic cytochrome *aa3* of beef heart mitochondria which contains 13 subunits (Tsukihara et al. 1995, 1996). These structures present the detailed organization of the metal centers. In the case of the beef heart enzyme, involvement of some amino acids in proton-, water- and bimolecular oxygen-channels has been discussed (Tsukihara et al. 1996). In *P. denitrificans*, the structure has led the authors to propose a mechanism of proton pumping that can be tested by site-directed mutagenesis (Iwata et al. 1995).

In eucaryotes, the possibility of isolating mutants is limited and is more laborious than in bacteria. However, the facultative aerobe *Saccharomyces cerevisiae* is a powerful tool for isolating respiratory deficient mutants which would be lethal in strictly aerobic eucaryotes. In *S. cerevisiae*, cytochrome *c* oxidase consists of 11 subunits (Geier et al. 1995). Cox1p, Cox2p and Cox3p are encoded in the mitochondrial DNA (Rubin and Tzagoloff 1973) while the other subunits are of nuclear origin (for review, see Poyton and McEwen 1996). The gene coding for Cox1p is split and contains eight exons (A1 to A8) and seven introns (aI1 to aI7) (Bonitz et al. 1980; Hensgens et al. 1983). *S. cerevisiae* Cox1p is 50% identical (and 69% similar) to that of *P. denitrificans*, and 58% identical (and 77% similar) to that of beef heart mitochondria. Studies of missense mutants of *S. cerevisiae* could be useful in elucidating the reaction mechanism of the cytochrome *c* oxidase complex. On the other hand, analysis of cytochrome *c* oxidase subunits in strains carrying nonsense mutations may give information on the mode of assembly of the complex (for a review see Capaldi 1990).

In the present study, we report the molecular and biochemical characteristics of a set of exonic *cox1* mutants in *S. cerevisiae* which have lost their ability to grow on respiratory substrates. The accumulation of Cox1p to Cox4p in the mitochondrial membranes of the *cox1* mutants com-

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Table 1 List of strains

Name	Nuclear genotype	Mitochondrial genotype	Origin
777-3A	*MATα ade1 op1*	*rho*$^+$*mit*$^+$	Kotylak and Slonimski (1977)
777-3A/G291 to G2394 777-3A/V44-V206	*MATα ade1 op1*	*rho*$^+$*mit*$^-$	Kotylak and Slonimski (1977)
BGT1	*MAT**a** ade2 ura3-1 trp1-1 his3-11,15 leu2-3,112 can1-100 COXIV::TRP1*	*rho*$^+$ *mit*$^+$	Kindly provided by B. Guiard
KM612-2D	*MATα ade1 OP*	*rho*$^+$ *mit*$^-$	Dujardin (1983)
KM612-2D/60	*MATα ade1 OP*	*rho°*	Ethidium bromide mutagenesis of KM612-2D (this work)
KL14-4A/60	*MAT**a** his1 trp2 OP*	*rho°*	Groudinsky et al. (1981)

pared to *cox2*, *cox3* and *cox4* mutants provides some insight into the stability of these subunits in these various cytochrome *c* oxidase-defective mutants.

Materials and methods

Strains and media. All the strains are listed in Table 1. The mit$^-$ mutants were isolated from the haploid strain 777-3A (*op1*), mapped to the *COX1*, *COX2* or *COX3* genes (Kotylak and Slonimski 1977) and were localized more precisely, for the *cox1* mutants, by deletion mapping (Netter et al. 1982, 1995). The *cox2* mutant (V44) and the *cox3* mutant (V206) have been previously described as defective in the synthesis of Cox2p and Cox3p respectively (Kruszewska et al. 1980; Baranowska et al. 1983). Growth analyses were performed on diploid strains constructed by crossing the various mutants with the strain KL14-4A/60. All the other experiments have been performed on haploid strains. The media N3, NE and NL were as described in Dujardin et al. (1980). YPGAL medium contained 2% galactose, 1% bactopeptone and 1% yeast extract. YPGALa was the same as YPGAL but with the addition of 2 μg/ml of adenine.

Sequence analysis of the cox1 mutations. For analysis of the mutations G1099, G1547 and G2508, single-stranded DNA was sequenced according to Sanger et al. (1977) after cloning into M13/mp18 or M13/mp19. To identify mutations G2567, G291, G450, W164, G1979, G2394 and G2276, double-stranded PCR products were cloned into pBluescript KS+ (Stratagene) and two clones per mutation were sequenced.

Pulse-labelling experiments and preparation of mitochondrial membranes. Pulse-labelling experiments were carried out according to Claisse et al. (1980) except that cells were re-suspended in 0.4 M mannitol, 50 mM Tris/H_2SO_4 pH 7.4, 2 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml antipaïn, 10 μg/ml chymostatin and 1 μg/ml pepstatin. Mitochondrial membranes were then prepared according to LaMarche et al. (1992). Protein concentrations were determined using Bio-Rad protein assay.

SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed using the Laemmli system (Laemmli 1970). The molecular weights of the polypeptides were estimated using a low-molecular-weight calibration kit from Bio-Rad.

Immunoblots experiments. Electrophoretic transfers were carried out according to Towbin et al. (1979). Immunodetection was carried out using an enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham International). The monoclonal antibodies anti-yeast cytochrome *c* oxidase Cox1p, Cox2p, Cox3p and Cox4p were purchased from Molecular Probes and have been described in Taanman and Capaldi (1993). The polyclonal antibody anti-yeast cytochrome *c* oxidase Cox6p was kindly provided by R. O. Poyton.

Activity and cytochrome spectra measurements. Oxygen consumption was measured with a Clark electrode (Gilson oxygraph), at 28 °C, using 2% ethanol as the substrate. The effect of the uncoupler was tested by the addition of 25 μM of Carbonyl-cyanide-p-trichlorophenylhydrazone and cyanide-sensitive respiration was measured in the presence of 0.5 mM KCN. Respiratory rates were expressed in nmoles O_2 consumed/min per 5×10^8 cells, taking into account that the solubility of O_2 is 243.75 μmoles/l of H_2O at 28 °C. Cytochrome *c* oxidation measurements were performed spectrophotometrically as described in Pajot et al. (1976). Cytochrome absorption spectra were recorded on whole-cell pastes at liquid nitrogen temperature, after dithionite reduction, according to Claisse et al. (1970).

Results

Sequences of the *cox1* mutations

The mutations had been previously localized by deletion mapping in the mitochondrial *COX1* gene (Netter et al. 1982, 1995). Sequencing revealed that the mutations are in positions consistent with their location determined by deletion mapping. All the results are listed in Table 2. Amongst the 13 mutants studied, two display a nonsense mutation: G2508 (S219ochre) and G291 (Y245ochre), and three a frameshift mutation: G1099 (N99ochre), G2567 (L109amber) and G1547 (L212amber), leading to a nonsense mutation downstream (see Table 3). Eight exhibit a single missense mutation, except for G2394 which is a double mutant in which the second mutation is silent (C12583T → Y394Y). The six missense mutations are located mainly in exons A4 and A5, while G1979 (G330D) and G2394 (G384D) are localized in exons A6 and A8 respectively. The implications of the amino-acid changes will be considered in the Discussion section.

Respiratory growth and activities of the *cox1* mutants

All the mutants have been previously selected for their inability to grow on glycerol medium (N3) (Kotylak and Slonimski 1977). We completed this analysis by testing the

Table 2 Nature and localization of the *COX1* mutations and analysis of *COX1* mRNA synthesis. The nucleotides are numbered from 1 to 13 003, starting at position –126 of the short strain (Bonitz et al. 1980) and interrupted for exons A5 to A7 by 3060 nucleotides of the long strain (Hensgens et al. 1983)

Mutant	Mutation	Nucleotide change	Exon localization	Amino-acid change	Amino-acid conservation[b]	*COX1* mRNA synthesis
777-3A/G1099	Frameshift	T6896TT	A4	See Table 3	na	+
777-3A/G2567	Frameshift	T6910Δ	A4	See Table 3	na	+
777-3A/G1547	Frameshift	G7219Δ	A4	See Table 3	na	+
777-3A/G2508	Nonsense	C7259A	A4	S219ochre	na	+
777-3A/G291	Nonsense	T8348A	A5	Y245ochre	na	nd
777-3A/G2276	Missense	C7073T	A4	S157L	Conserved (29/31)	+
777-3A/G450	Missense	G8340A	A5	E243K	Invariant (31/31)	+
777-3A/G3015[a]	Missense	G8343A	A5	V244M	Invariant (31/31)	+
777-3A/W113[a]	Missense	G8364A	A5	G251R	Conserved (22/31)	+
777-3A/W201[a]	Missense	A8407T	A5	K265M	Conserved (27/31)	nd
777-3A/W164	Missense	C8481T	A5	H290Y	Invariant (31/31)	+
777-3A/G1979	Missense	G9967A	A6	G330D	Conserved (24/31)	+
777-3A/G2394	Missense	G12552A + C12583T	A8	G384D + Y394Y	Conserved (29/31)	+

na = not applicable; nd = not done; Δ: deletion
[a] Sequenced by Lemarre et al. (1994)
[b] The amino-acid conservation has been considered in terms of the alignment of 31 sequences of Cox1p including bacteria, fungi, plants and animals

Table 3 Localization of the frameshift mutations and positions of the nonsense codons generated

Strain	Sequence								
777-3A	WT nucleotide sequence	ATT	AAT						
	Nucleotide position	6894							
	WT amino-acid sequence	I	N						
	Amino-acid position	98	99						
777-3A/G1979	Mutant nucleotide sequence	ATT	TAA						
	Mutant amino-acid sequence	I	ochre						
777-3A	WT nucleotide sequence	TTT	TGA	GTA	TTA	CCT	ATG	GGG	TTA
	Nucleotide position	6910							
	WT amino-acid sequence	F	W	V	L	P	M	G	L
	Amino-acid position	102							109
777-3A/G2567	Mutant nucleotide sequence	TTT	GAG	TAT	TAC	CTA	TGG	GGT	TAG
	Mutant amino-acid sequence	F	E	Y	Y	T	W	G	amber
777-3A	WT nucleotide sequence	GGT	ATT	ACA	ATG	TTA	TTA	TTA	
	Nucleotide position	7219							
	WT amino-acid sequence	G	I	T	M	L	L	L	
	Amino acid position	206						212	
777-3A/G1547	Mutant nucleotide sequence	GTA	TTA	CAA	TGT	TAT	TAT	TAG	
	Mutant amino-acid sequence	V	L	Q	C	Y	Y	amber	

thermosensitivity or cryosensitivity of these strains and their growth on other non-fermentable media. Strains carrying the various mitochondrial mutations were incubated on N3 medium at different temperatures, 20 °C, 28 °C and 36 °C, and were tested on NL (lactate) and NE (ethanol) media at 28 °C. After 3 days of incubation, no growth was observed under any condition. Extending the time of incubation leads to the appearance of rare revertants.

As has been previously shown for the cytochrome *bc1* complex (Oudshoorn et al. 1987; Tron and Lemesle-Meunier 1990), there is no direct relationship between growth on non-fermentable media of a strain altered in a given complex and the activity of this complex. Thus, measurements of the respiratory activity of the different *cox1* mutants were made in vivo by following the rate of O_2 consumption in intact cells. In all but one of the *cox1* mutants tested, the rate of oxygen uptake is not significantly different from a *rho*° strain lacking mitochondrial DNA (KM612-2D/60) or a *mit*$^-$ strain with a mutation located in the intron aI2, which leads to a lack of *COX1* mRNA (W165) (Carignani et al. 1986). Surprisingly, even though it does not grow on non-fermentable media, the mutant S157L displays a residual respiratory activity of 38 nmoles O_2 consumed/min/5×10^8 cells (about 15% compared to the wild-type (wt) strain). This activity is cyanide-sensitive.

Activity measurements were also performed in vitro, estimating the efficiency of the electron transfer chain by the ability of purified mitochondrial membranes to oxidize

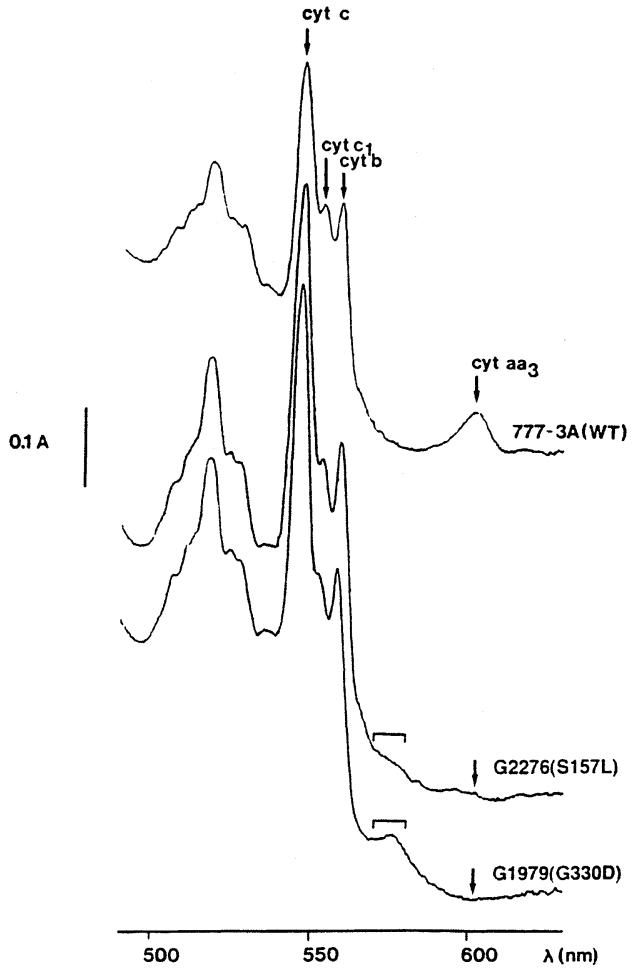

Fig. 1 Cytochrome spectra of whole cells from the wt strain 777-3A, mutants G1979 (G330D) and G2276 (S157L). Cells were grown for 2 days at 28 °C on solid YPGALa medium. Spectra were performed at –180 °C on dithionite-reduced whole-cell pastes (1-mm thick). In G1979 (G330D) and G2276 (S157L), the *square bracket* indicates the presence of a shoulder between 570 and 580 nm

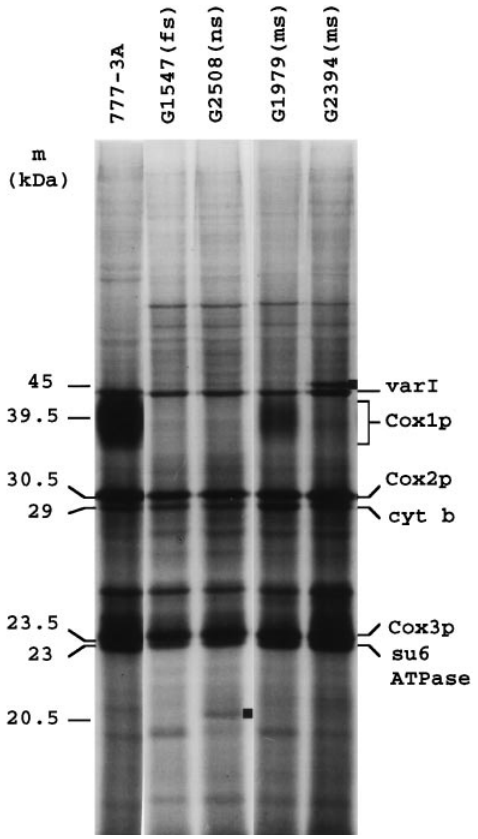

Fig. 2 Autoradiograms of mitochondrial translation products. Cells were pulse-labelled for 60 min with $^{35}SO_4$ in the presence of cycloheximide and mitochondrial membranes were purified and analyzed by an SDS polyacrylamide gel as described in the Materials and methods. *FS* frameshift; *NS* nonsense; *MS* missense. G1547 is representative of all the frameshift and nonsense mutants except G2508, while G1979 (G330D) is representative of all the missense mutants except G2394 (G384D). Note the presence of novel polypeptides (indicated by ■) in the strains G2508 and G2394 (G384D)

an exogenous electron donor, cytochrome *c*. The initial rate of the cyanide-sensitive cytochrome *c* oxidation of the wt strain is 650 nmoles of oxidized cytochrome *c*/ min per mg of protein. For the *cox1* mutants, the activities are not significatively different from the negative control (W165) except for the strain S157L which displays an activity of about 20% of that observed in wt and which is also cyanide-sensitive.

Cytochrome spectra of *cox1* mutants

Cytochrome spectra of the wt strain and mutants G330D (representative of the mutants lacking of any respiratory activity) and S157L are shown in Fig. 1. As expected, the wt strain presents a significant peak at 602 nm corresponding to the α band of cytochromes *a* and *a3*. S157L shows a slight shoulder at this position and its cytochrome *aa3*/ cytochrome *c* ratio is estimated to be about 7% of wt. In G330D, as in the 11 other mutants, no cytochromes *aa3* can be detected. In all mutants, the cytochrome *b*/cytochrome *c* ratio and the cytochrome *c1*/cytochrome *c* ratio are decreased (by about 20–40%) indicating a deficiency in the accumulation of cytochromes *bc1*. Such a decrease in all the cytochromes has already been reported (Lemesle-Meunier et al. 1993).

Analysis of the translation products in the *cox1* mutants

Since all the tested exonic *cox1* mutants displayed a significant level of *cox1* mRNA (data not shown), we examined whether the respiratory deficiency of the mutants was due to an alteration either at the translational or at the post-translational level. The mitochondrial translation products are shown in Fig. 2. The band corresponding to Cox1p (39.5 kDa) was lacking in the frameshift and nonsense mutants (represented by G1547), as well as in the missense mutant G384D, and was present, but reduced, in the other missense mutants (represented by G330D). In two mutants

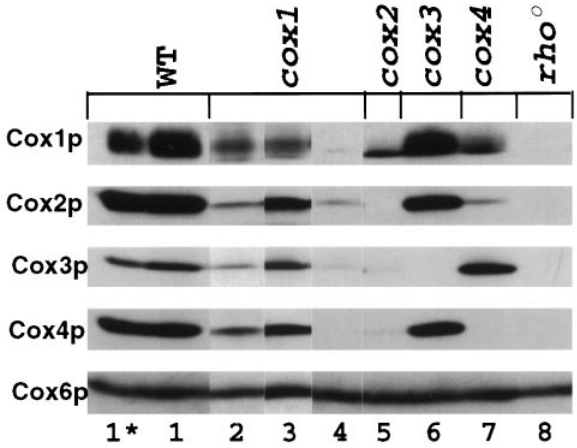

Fig. 3 Immunoblots of mitochondrial membranes from the wt strain, *cox1*, *cox2*, *cox3* and *cox4* mutants and the rho° strain. The loading corresponds to 40 µg of mitochondrial membranes except when specified by * which corresponds to 20 µg of mitochondrial membranes. *Lanes 1 and 1** WT strain, 777-3A. *Lane 2* G1979 (G330D), *cox1* missense mutant defective in cytochrome *c* oxidase activity. *Lane 3* G2276 (S157L), *cox1* missense mutant displaying 20% of cytochrome *c* oxidase activity. *Lane 4* G1547, frameshift *cox1* mutant. *Lane 5* V44, *cox2* mutant. *Lane 6* V206, *cox3* mutant. *Lane 7* BGT1, *cox4* mutant. *Lane 8* rho° strain

devoid of Cox1p, novel polypeptides were observed: in G384D we noted the presence of a 45-kDa polypeptide, which is not recognized by the monoclonal Cox1p antibody we used (data not shown), and in G2508 the additional polypeptide displays an apparent molecular weight of 20.5 kDa which is compatible with the predicted truncated polypeptide. In all the mutants, Cox2p and Cox3p, corresponding respectively to the 30.5-kDa and 23.5-kDa polypeptides, were synthesized.

Immunoblotting experiments with *cox* mutants

Using immunoblotting, we examined the stability of Cox1p, Cox2p and Cox3p and of the nuclear-encoded Cox4p, all of which are closely associated (Tsukihara et al. 1996), in the presence of mutations in each of the corresponding genes (*cox1*, *cox2*, *cox3* or *cox4* mutants). The same analysis was also performed on a *rho°* strain which lacks all three mitochondrial subunits. As a control, the stability of the nuclear-encoded Cox6p, which has no direct contact with Cox1p through Cox4p (Tsukihara et al. 1996), was also determined.

In the missense *cox1* mutants, the levels of the three mitochondrially encoded subunits and of Cox4p are reduced. This is illustrated in Fig. 3 with the mutant G330D (lane 2), which is devoid of any cytochrome *c* oxidase activity, and the mutant S157L (lane 3), which displays a residual cytochrome *c* oxidase activity of about 20%. In the frameshift and nonsense *cox1* mutants (represented by G1547, lane 4), Cox1p is absent and the levels of Cox2p, Cox3p and Cox4p are considerably decreased or else undetectable. In the *cox2* mutant (V44, lane 5) Cox1p is present in small amounts and Cox3p and Cox4p are barely detectable, whereas in the *cox3* mutant (V206, lane 6) significant amounts of Cox1p and Cox2p are observed and Cox4p accumulates to the wt level. In the absence of Cox4p (BGT1, lane 7) the levels of Cox1p and Cox2p are low, whereas Cox3p is even higher than in the wt strain. In the absence of the three mitochondrially encoded subunits (*rho°* strain, lane 8) Cox4p is absent. In all the mutants studied Cox6p accumulates as well as in the wt.

Discussion

Analysis of the amino-acid substitutions in the missense mutants

The analysis of the missense mutants reveals that, with the exception of S157L, the substitution of one amino acid has a drastic effect abolishing any accumulation of the cytochromes *a* and *a3* and any cytochrome *c* oxidase activity. These observations are correlated with a decrease in Cox1p translation and in the level of Cox1p, Cox2p, Cox3p and Cox4p accumulated in the membranes. All these observations led us to conclude that these mutants are altered in the assembly of the cytochrome *c* oxidase complex as a consequence of the instability of Cox1p. According to the crystal structure of bovine heart cytochrome *c* oxidase (Tsukihara et al. 1996) (see Fig. 4), five of the missense mutations are located in the trans-membrane helices: helix 4 (S157L), helix 6 (V244 M, E243 K and G251R) and helix 10 (G384D). Three mutations are in extra-membrane regions: K265 M and G330D in regions H6–7 and H8–9 respectively, orientated towards to the matrix side of the inner mitochondrial membrane, and H290Y in the H7–8 region on the periplasmic side.

Four mutations affect positions which are essential in the published structures (Iwata et al. 1995; Tsukihara et al. 1995, 1996). Our results are in good agreement with these data. The mutation H290Y alters an invariant histidine residue which is proposed to be one of the CuB ligands (Iwata et al. 1995; Tsukihara et al. 1995). In addition, Iwata et al. (1995) suggest that this residue could be involved in a histidine cycle/shuttle mechanism for the coupling of proton pumping to oxygen reduction. Three mutants (E243K, S157L and K265M) are altered in the positions of invariant or conserved amino acids (see Table 2) which have been proposed to be involved in proton channels either in the pathway for consumed protons (E243 and K265) (Iwata et al. 1995) or in the pathway for pumped protons (S157) (Tsukihara et al. 1996). S157L carries the only missense mutation, which led to a partial accumulation of an active complex in the mitochondrial membranes.

The three other mutants, G330D, G384D and G251R, do not affect invariant residues. However, we show here that they have important roles since they alter the level of Cox1p, Cox2p, Cox3p and Cox4p accumulated in the membranes and lead to an absence of detectable cytochrome *c* oxidase activity. All of them change a glycine residue. A

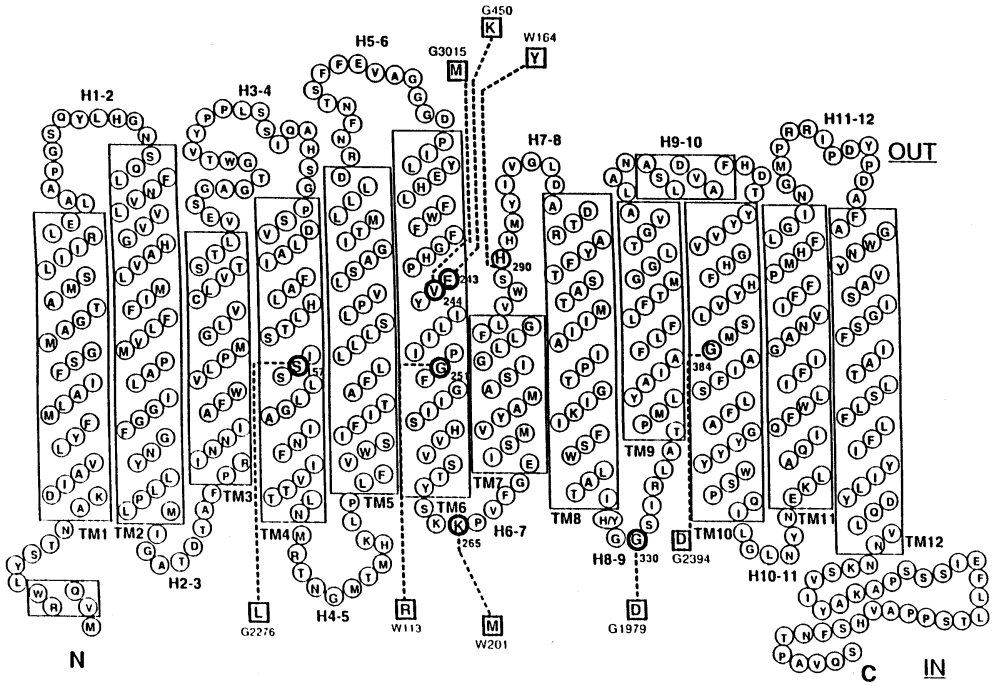

Fig. 4 Two-dimensional organization of the cytochrome *c* oxidase subunit I of *S. cerevisiae*. The distribution of the trans-membrane helices and of the two helices parallel to the membrane has been deduced from the structure of subunit I of cytochrome oxidase from beef heart (Tsukihara et al. 1996) and after alignment of its sequence with that of *S. cerevisiae*. *Rectangles* in the N-terminal and H9–10 regions are α helices found in the extra-membrane region. The localization of the eight missense mutations and the amino-acid changes observed in these mutants are indicated. The extra-membrane regions are *numbered* from H1–2 to H11–12, and the trans-membrane helices from TM1 to TM12 according to the nomenclature of Iwata et al. (1995)

similar substitution (G352 V) has been studied by Ortwein et al. (1997) but this resulted in a well-assembled but non-functional cytochrome *c* oxidase complex. In our case, the three amino-acid substitutions result in the appearance of a charge either in a trans-membrane region (TM6 for G251R and TM10 for G384D) or in a loop orientated to the matrix (H8–9 for G330D) (see Fig. 4). Consequently, we hypothesize that it is the presence of the charge which induces a change in the conformation of Cox1p, thereby altering its stability in the membranes.

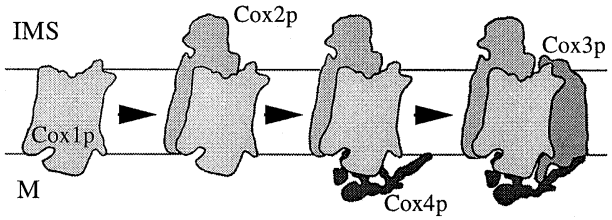

Fig. 5 Hypothetical model for the sequential assembly of the three mitochondrially encoded subunits (Cox1p, Cox2p and Cox3p) and the nuclear-encoded subunit (Cox4p) of cytochrome *c* oxidase into the mitochondrial membranes. *IMS* inter-membrane space; *M* matrix

Analysis of the stability of Cox1p, Cox2p, Cox3p, Cox4p and Cox6p in the mitochondrial membranes of mutants lacking one subunit

The comparison of the subunit content in the various mutants lacking either Cox1p, Cox2p, Cox3p or Cox4p shows that Cox1p, Cox2p, Cox3p and Cox4p accumulate in an inter-dependant way, whereas the accumulation of Cox6p appears to be independant from the four others. Indeed, Cox4p is not detectable in the rho° strain while Cox6p accumulates as well as in the wt cells. Cox4p is also absent in *cox1* and *cox2* mutants, but not in *cox3* mutants. Thus, the accumulation of Cox4p in the membranes appears to be dependent on the presence of Cox1p and Cox2p. By contrast, in all the mutants studied, Cox6p accumulates as well as in the wt strain. Similar observations have been made by Taanman et al. (1996) in the human cell line 143B206 (which is devoid of mitochondrial DNA), where the human homolog for Cox4p is reduced while the homolog for Cox6p is present in amounts close to the mtDNA-containing parental cell line. Recently, Glerum and Tzagoloff (1997) have reported that Cox4p is accumulated in comparable amounts in a nuclear mutant altered in the maturation/stability of *COX1* mRNA and in wt mitochondria, but in the mutant the protein is recovered almost entirely in the soluble fraction.

Taken together, these results confirm the interdependance of the assembly of Cox4p and Cox1p in the membranes. The difference in the accumulation of the two nuclearly encoded subunits Cox4p and Cox6p could be due to a distinct assembly pathway. This is supported by the crystallographic data (Tsukihara et al. 1997) showing that Cox4p and Cox6p are both extra-membrane subunits, but whereas Cox4p tightly interacts with Cox1p and Cox3p, Cox6p is without any direct contact with Cox1p.

Mitochondrial proteins of the inner membrane in yeast are rapidly degraded when not assembled. In particular, this has been shown by Nakai et al. (1994) for Cox2p and Cox3p which were degraded rapidly in a mutant lacking Cox4p. Several mitochondrial proteases have been identified like the hetero-oligomeric Afg3p/Rca1p complex which degrade Cox1p and Cox3p, or Yme1p which is involved in the degradation of Cox2p (for review see Langer

and Neupert 1996; Suzuki et al. 1997). However, Glerum and Tzagoloff (1997) have shown that in a *cox6* background, the steady state amount of Cox1p is not regulated by Afg3p/Rca1p and these authors have suggested that other proteases could exist. It would be interesting to determine which protease(s) is (are) involved in the subunit degradation in our cytochrome *c* oxidase mutants.

In conclusion, the deleterious effect produced by the absence of Cox1p on Cox2p, Cox3p and Cox4p suggests that Cox1p is necessary for the assembly of these subunits. In the *cox2* mutant, Cox3p and Cox4p were also absent indicating that their accumulation requires the presence of Cox2p. In the *cox3* mutant, Cox1p, Cox2p and Cox4p accumulate in a significant way and a slight peak of cytochrome *aa3* is observable in this strain, whereas no cytochrome *c* oxidase activity is detectable (data not shown). All these observations suggest that the assembly of Cox1p through Cox4p follows a sequential mode as schematized in Fig. 5. The first step could be the association of Cox2p with Cox1p, then Cox4p would associate with Cox1p, and finally Cox3p would stabilize the three other subunits. We will test this model by immunoprecipitation experiments. Also further experiments on other mutants altered in the seven remaining subunits will be necessary to better understand the sequence of assembly of the cytochrome *c* oxidase complex into the mitochondrial membranes.

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