

# Site-directed mutations in the mitochondrially encoded subunits I and III of yeast cytochrome oxidase

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Since yeast is amenable to mitochondrial transformation, designed mutations can be introduced in the mitochondrially encoded subunits of the respiratory complexes. In the present work, six mutations have been introduced by the biolistic method into yeast (*Saccharomyces cerevisiae*) cytochrome oxidase subunits I and III. The effects of these mutations on respiratory growth competence, cytochrome oxidase activity and optical properties were then characterized. Firstly, the conserved glutamate Glu-243 in the D-channel of subunit I was replaced by an asparagine or an aspartate residue. The effects of the mutations showed that Glu-243, which is essential for proton movement in bacterial oxidases, is also required for the activity of the eukaryotic enzyme. Secondly, four mutations associated with human disease were introduced in yeast, allowing detailed

analysis of their deleterious effects on cytochrome oxidase function: Met-273 → Thr, Ile-280 → Thr and Gly-317 → Ser, affecting residues located in or near the K-channel in subunit I, and a short in-frame deletion comprising residues Phe-102 to Phe-106 in subunit III (ΔF102–F106). The subunit III mutation was highly deleterious and abolished enzyme assembly. The change Gly-317 → Ser had no effect on respiratory function. However, mutations Met-273 → Thr and Ile-280 → Thr were mildly deleterious, decreased cytochrome oxidase activity and slightly perturbed the properties of the binuclear centre.

**Key words:** cytochrome *c* oxidase, disease-associated mutations, *Saccharomyces cerevisiae*.

## INTRODUCTION

Mitochondrial cytochrome oxidase is a complex multimeric enzyme, containing up to 13 subunits of dual genetic origin. Three subunits (subunits I, II and III) (Figure 1) are mitochondrially encoded, are homologous to subunits of the bacterial terminal oxidases and form the catalytic core of the enzyme. Subunit II forms part of the docking site for cytochrome *c* and binds Cu<sub>A</sub>, the first electron acceptor. Subunit I binds haem *a* and the binuclear centre (haem *a*<sub>3</sub> Cu<sub>B</sub>), the site of oxygen reduction. Subunit III has no redox centre and is involved in the assembly or stability of the complex. Cytochrome oxidase reduces oxygen to water, and couples that reaction to pumping of protons across the membrane. Movements of protons (for oxygen reduction and ‘pumped’ protons) requires proton-conducting pathway(s) within the protein. Based on analysis of the atomic structure and on site-directed mutagenesis studies of bacterial enzymes, two hydrophilic channels in subunit I, called the D- and K-channels, have been shown to fulfil this role (see [1] and references therein).

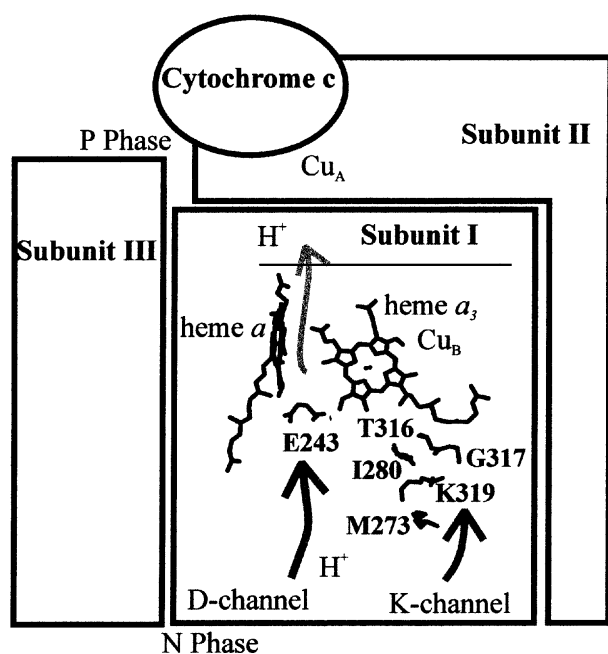
A large number of bacterial mutants have been generated, and have greatly helped our understanding of the catalytic mechanism of cytochrome oxidase. Nevertheless, it would be interesting to be able to study the effects of mutations in a eukaryotic enzyme, especially mutations associated with human diseases. Point mutations in the mitochondrial genes encoding subunits I, II and III of cytochrome oxidase (*COX1*, *COX2* and *COX3* respectively) have been found in patients with various disorders, such as sideroblastic anaemia [2], Lebers hereditary optical neuropathy (reviewed in [3]) and other neuromuscular disorders (e.g. see

[4,5]). Yeast and human cytochrome oxidases are highly similar. For instance, the sequences of their mitochondrially encoded subunits are 42–45% identical. In addition, yeast is amenable to mitochondrial transformation. Therefore yeast mutants could be produced and used as models to characterize the effects of these mutations on respiratory function.

In the present work, we studied the effect of mutations in yeast (*Saccharomyces cerevisiae*) cytochrome oxidase subunits I and III. Firstly, the protonable residue Glu-243, located in the D-channel of subunit I (Figure 1) and shown to be essential for the catalytic activity of bacterial enzymes [6], was replaced by an unprotonable residue (asparagine) or by another protonable residue (aspartate), in order to test whether this residue is required for the catalytic activity of the eukaryotic enzyme. Secondly, four human-disease-related mutations were introduced into yeast cytochrome oxidase in order to characterize their deleterious effects: a short in-frame deletion comprising residues Phe-102 to Phe-106 in subunit III (ΔF102–F106), which has been observed in a patient with recurrent myoglobinuria [4]; Gly-317 → Ser (G317S), found in fibroblasts from a patient presenting with a profound lactic acidemia and cytochrome oxidase deficiency (J.-W. Taanman, personal communication); and Met-273 → Thr (M273T) and Ile-280 → Thr (I280T), which have been observed in patients suffering from sideroblastic anaemia [2]. Met-273, Ile-280 and Gly-317 are highly conserved residues that are located in the vicinity of the K-channel in subunit I (Figure 1). The yeast mutants were generated by the biolistic method [7]. The effects of the mutations on respiratory growth ability and on cytochrome oxidase content, activity and optical properties were then monitored.

Abbreviations used: *COX1*, *COX2* and *COX3*, mitochondrial genes encoding cytochrome oxidase subunits I, II and III respectively; mit<sup>−</sup>, point mutation in the mitochondrial genome affecting single function.

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**Figure 1** Location of the mutated residues in cytochrome oxidase subunit I

The structure has been drawn using QUANTA, from the co-ordinates of the bovine enzyme [20]. The numbering is according to the yeast sequence. The redox centres, haem *a*, haem *a*<sub>3</sub> and Cu<sub>B</sub>, are bound to the mitochondrially encoded subunit I. The D-channel leads from the N phase (matrix site of the mitochondrial inner membrane) to Glu-243. The K-channel leads from the N phase to the binuclear centre (haem *a*<sub>3</sub> + Cu<sub>B</sub>), site of oxygen reduction.

## MATERIALS AND METHODS

### Media and yeast strains

The following media were used for the growth of yeast: YPD [1% (w/v) yeast extract, 2% (w/v) peptone and 3% (w/v) glucose], YG [1% (w/v) yeast extract and 3% (v/v) glycerol], Ygal [1% (w/v) yeast extract and 3% (w/v) galactose], and transformation medium [0.7% (w/v) yeast nitrogen base, 3% (w/v) glucose, 2% (w/v) agar, 1 M sorbitol and 0.8 g/l of a complete supplement mixture minus uracil, supplied by Anachem]. The following yeast strains were used for transformation and screening: the recipient strain for biolistic transformation was W303-1B/*rho*<sup>0</sup> (Mat  $\alpha$  *ura3* *his3* *leu2* *ade2* *trp1*); the wild-type strain CKBM and the *mit*<sup>-</sup> tester strains CKL174 (*COX1* I67N), CKL13 (*COX1* G253D) and CKL163 (*COX3* S203L) (where *mit*<sup>-</sup> represents a point mutation in the mitochondrial genome affecting single function) were isonuclear Mat  $\alpha$  *leu1* *kar1-1* with a *rho*<sup>+</sup> intronless mitochondrial DNA [8].

### Site-directed mutagenesis

The plasmid pYGT21, which carries the *HpaII*–*EcoRI* yeast mitochondrial DNA fragment containing the wild-type intronless sequence of the *COX1* gene, was cloned into the *AccI*–*EcoRI* site of pUC13 and kindly provided by Dr J. Lazowska (CNRS, Gif sur Yvette, France). The plasmid pMC241, carrying the *COX3* gene cloned in pBluescript [9], was kindly given by Professor T. Fox (Cornell University, Ithaca, NY, U.S.A.). Mutagenesis was performed using a Quickchange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations.

The mutations introduced were as follows. (1) Point mutations in the subunit I gene: E243N, GAA → AAC; E243D, GAA → GAC; M273T, ATG → CTG; I280T, ATT → ACA; G317S, GGA → TCA. (2) Five-codon deletion in the subunit III gene: I101  $\Delta$ F102–F106 W107, ATC *ttt* *gct* *ggt* *tta* *ttc* TAG → ATC TAG. After verification of the sequence, the plasmids carrying the mutated genes were used for biolistic transformation.

### Biolistic transformation

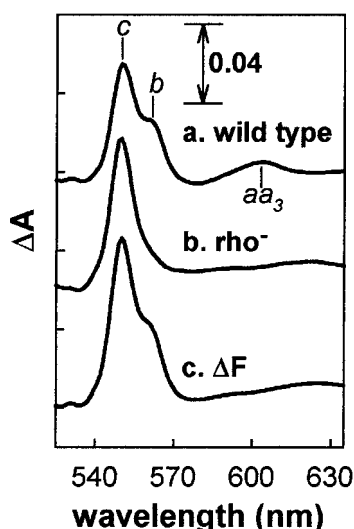
Portions of approx. 3  $\mu$ g of the plasmids carrying the mutated *COX* genes and 0.5  $\mu$ g of YEP352 plasmid (which contains the *URA3* gene, allowing the selection of *Ura*<sup>+</sup> nuclear transformants) were mixed with 50  $\mu$ l of 0.7  $\mu$ m tungsten particles (at a concentration of 60 mg/ml). The particles (supplied by Bio-Rad) were prepared and coated with DNA according to the manufacturer's protocol. Aliquots of the coated particles were used for the transformation of the recipient strain W303-1B/*rho*<sup>0</sup>. Approx. 10<sup>7</sup> cells of the recipient strain were spread evenly on transformation medium (selective for *Ura*<sup>+</sup> transformants). The particle bombardments were performed with a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) according to the manufacturer's recommendations.

### Screening of transformants

Colonies of *Ura*<sup>+</sup> transformants (which have received YEP352 plasmid) appeared after 4–5 days of incubation. In order to identify the mitochondrial transformants (or synthetic *rho*<sup>-</sup> clones [7]), the *Ura*<sup>+</sup> colonies were crossed with the respiratory-deficient (*mit*<sup>-</sup>) tester strains CKL174 (*COX1* I67N) or CKL163 (*COX3* S203L). CKL174 was used to screen for the presence of plasmid-borne *COX1*, while CKL163 was used to screen for the presence of plasmid-borne *COX3*. The diploids were replica-plated on respiratory medium (YG). Since the 'tester' deficiency mutation could be corrected by recombination with the plasmid-derived mitochondrial sequence, mitochondrial transformants were identified by their ability to form respiratory-competent diploids when crossed with their tester strain. The mitochondrial transformants were then subcloned and tested again. The frequency of mitochondrial transformants was approx. 1 in 1000 nuclear transformants, which was in agreement with values reported previously [7,10].

### Replacement of the wild-type *COX1* gene by the mutated form in a *rho*<sup>+</sup> mitochondrial genome

The mutated gene was introduced into a *rho*<sup>+</sup> genome by double recombination, by crossing the synthetic *rho*<sup>-</sup> with a *rho*<sup>+</sup> strain as described in [10]. The *rho*<sup>-</sup> strains E243N and  $\Delta$ F102–F106 were mated with the wild-type *rho*<sup>+</sup> strain CKBM. Then 200–300 diploid colonies resulting from the crossing were picked and their respiratory growth competence was tested. The respiratory-deficient diploids (parental *rho*<sup>-</sup> and recombinant *mit*<sup>-</sup>) were picked and their optical spectra were monitored in order to identify recombinant *mit*<sup>-</sup>. *mit*<sup>-</sup> strains can easily be distinguished from *rho*<sup>-</sup> strains on the basis of their optical signal, since *rho*<sup>-</sup> strains lack both cytochrome *b* and cytochrome oxidase, as illustrated in Figure 2. The introduction of the mutations was confirmed by sequencing. A slightly different procedure was followed for mutants E243D, M273T, I280T and G317S. The synthetic *rho*<sup>-</sup> strains were mated with the respiratory-deficient strain CKL13 (*COX1* G253D). Respiratory-competent diploids issued from the crossing were selected and subcloned. Sequencing showed that the new mutations had been incorporated by double



**Figure 2** Optical spectra of wild-type,  $\rho^-$  and  $\text{mit}^-$  cells

The cells were resuspended in 10% (v/v) Ficoll and 50 mM Tris/HCl, pH 7, to approx. 100 mg/ml. The samples were reduced by the addition of solid sodium dithionite. Solutions of milk matching the light scattering of the cell suspensions in the 520–650 nm region were used as baseline. In order to remove distortions caused by imperfect matching of the baselines to the samples, a quadratic baseline compensation was carried out on the data, as described in [11], using the three wavelengths 542, 575 and 630 nm. The optical spectrum of wild-type cells (trace a) shows the cytochrome *c*, *b* and  $aa_3$  signals, while the spectrum of  $\rho^-$  cells (trace b) lacks both the cytochrome *b* and cytochrome oxidase signals. The spectrum of the recombinant  $\text{mit}^-$   $\Delta F102\text{--}F106$  (trace c) shows the cytochrome *b* signal, but lacks the cytochrome oxidase signal.

crossing-over in the  $\rho^+$  genome, while the G253D mutation had been replaced by the wild-type codon.

### Spectrophotometric measurements

Difference spectra were generated by sequential scanning of the same sample with a single-beam instrument built in-house. The difference spectra of cell suspensions were obtained as follows. The cells, grown on Ygal plates, were resuspended in 10% (v/v) Ficoll and 50 mM Tris/HCl, pH 7, at a concentration of approx. 120 mg of cells/ml. The samples were oxidized with 0.025%  $\text{H}_2\text{O}_2$  in the presence of 10  $\mu\text{M}$  myxothiazol and a first spectrum was recorded. The samples were then reduced by dithionite, and a second spectrum was taken. A third spectrum was recorded after addition of 10 mM cyanide. Difference spectra (reduced minus oxidized, and reduced plus cyanide minus reduced) were then constructed. The reaction of cytochrome oxidase with CO was monitored as described in [11–13]. Room-temperature photolysis of CO was achieved with short actinic light pulses (10 ns half peak width; 532 nm; > 100 mJ/pulse) provided by a frequency-doubled Nd-YAG laser. Photolysis signals were recorded at wavelengths of 430 and 445 nm, and 25 transients were signal-averaged at each wavelength. The data are plotted as the signal at 430 nm minus the signal at 445 nm. First-order decays were fitted to the traces to give the observed recombination rates ( $k_{\text{obs}}$ ).

### Quantification of cytochrome oxidase

Quantification of cytochrome oxidase was based on the haem *a* signal of the reduced-minus-oxidized spectra in the visible region. Cytochrome oxidase content was estimated by using a molar absorption coefficient ( $\epsilon$ ) of 14  $\text{mM}^{-1}\cdot\text{cm}^{-1}$  at [604–(594 +

614)/2] nm [12]. The amount of cyanide-binding cytochrome oxidase was estimated from the cyanide binding spectra by using  $\epsilon = 18.7 \text{ mM}^{-1}\cdot\text{cm}^{-1}$  at (592–612) nm [12]. The amount of CO-binding enzyme was estimated from the amplitude of the CO photolysis spectra by using  $\epsilon = 113 \text{ mM}^{-1}\cdot\text{cm}^{-1}$  at (430–445) nm [11,12].

Cytochrome *c* content was estimated from the reduced-minus-oxidized spectra by using  $\epsilon = 18 \text{ mM}^{-1}\cdot\text{cm}^{-1}$  at (550–542) nm.

### Preparation of mitochondria

Mitochondria were prepared from cells grown in Ygal medium by an enzymic method, as described in [14].

### Respiratory activity of cell suspensions and mitochondria

Oxygen consumption activity was measured in an oxygen electrode. The data are presented as electrons consumed per cytochrome oxidase per s. In order to measure the oxygen consumption of intact cells, cell suspensions were diluted to approx. 100 mg in 2 ml of a solution containing 50 mM potassium phosphate, 150 mM glucose and 2.5  $\mu\text{M}$  FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) at pH 7, and the rate of oxygen consumption was recorded. Cytochrome *c* oxidase activity was measured using isolated mitochondria. Mitochondria were resuspended in 2 ml of a solution containing 50 mM potassium phosphate, 0.1% lauryl maltoside, 10 mM ascorbate and 50  $\mu\text{M}$  TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) at pH 7. The reaction was initiated by the addition of 80  $\mu\text{M}$  cytochrome *c*.

### RESULTS

Six yeast mutant strains with site-directed mutations in the mitochondrially encoded subunits I and III of cytochrome oxidase were generated by the biolistic method, as described in the Materials and methods section. Mutants E243N and E243D affect the conserved glutamate in the D-channel; M273T, I280T and G317S change conserved residues in the vicinity of the K-channel; and  $\Delta F102\text{--}F106$  is an in-frame deletion of five residues in subunit III. The effects of the site-directed mutations on respiratory growth competence and on cytochrome oxidase content, optical properties and activity in cells and in isolated mitochondria were then characterized.

The respiratory growth rates of the mutant strains were compared with that of their wild-type strain (Table 1). The mutation G317S had no effect on the respiratory growth competence of the cells. The growth rates of strains E243D, M273T and I280T were decreased to 30–40%, whereas E243N and  $\Delta F102\text{--}F106$  cells were respiratory growth deficient.

Cytochrome oxidase content was estimated from the haem *a* signal of the reduced-minus-oxidized spectra of cell suspensions, as described in the Materials and methods section. As shown in Table 1, the cytochrome oxidase content was affected little or not at all by the mutations in *COX1*, but was dramatically decreased in the *COX3* mutant  $\Delta F102\text{--}F106$ , in which no cytochrome oxidase signal was detectable. In mutant E243N, the cytochrome oxidase level was lower than that in the wild type, while the cytochrome *c* level was increased.

In order to assess possible alterations in the binuclear centre environment, the reactions of reduced haem  $a_3$  with cyanide and CO were monitored in whole cells. Cyanide reacts with reduced haem  $a_3$  and induces an optical change which is easily monitored in the visible region. The amount of cyanide-ligated haem  $a_3$  was estimated from the cyanide binding spectra and compared with the total amount of cytochrome oxidase (estimated on the basis

**Table 1** Characterization of cytochrome oxidase in whole cells

For assessment of growth rate, the cells were incubated in YG medium, and the cell density of the cultures was monitored at various times. The data are presented as a percentage of the growth rate of wild-type cells. Cytochrome oxidase (CcO) and cytochrome *c* (Cyt. *c*) contents were estimated as described in the Materials and methods section, and are given in nmol per g of cells. Percentage ligand binding (cyanide or CO) was calculated from the difference spectra compared with the signal intensity expected for 100% reactivity. The measurements were made on three to four samples. The kinetics of CO recombination ( $k_{\text{obs}}$ ) were determined as described in the Materials and methods section. Oxygen consumption activity was measured as described in the Materials and methods section, and the data are presented as electrons consumed per cytochrome oxidase per s. ND, no growth, optical signal or activity could be detected.

Strain	Growth rate (%)	Content (nmol/g)		Ligand binding (%)		$k_{\text{obs}}$ (s <sup>-1</sup> )	Activity (s <sup>-1</sup> )
		Cyt. <i>c</i>	CcO	Cyanide	CO		
Wild type	100	13	1.6	> 95	95 ± 5	60–65	240
G317S	100	12	1.5	> 95	> 95	60–65	260
I280T	25–30	16	1.8	85 ± 10	80 ± 5	60	100
M273T	30–40	13	1.7	> 95	95 ± 5	100	135
E243D	30	14	1.6	95 ± 5	95 ± 5	60–65	140
E243N	ND	18	1.2	80 ± 5	70 ± 5	60–65	15
ΔF102–F106	ND	20	ND				ND

**Table 2** Cytochrome oxidase activity and reaction with CO in isolated mitochondria

Preparation of mitochondria, quantification of cytochrome oxidase (estimated from dithionite-reduced minus ferricyanide-oxidized spectra), measurement of cytochrome oxidase activity and kinetics of the reaction with CO ( $k_{\text{obs}}$ ) were carried out as described in the Materials and methods section.

Strain	Activity (s <sup>-1</sup> )	$k_{\text{obs}}$ (s <sup>-1</sup> )
Wild type	430	65
E243D	180 (42%)	65
G317S	490	65
M273T	170 (40%)	90–100
I280T	200 (46%)	65

of the haem *a* signal). The extent of reactivity, i.e. the percentage of enzyme that binds cyanide, was greater than 90–95% in the wild-type, G317S, M273T and E243D strains. It was slightly decreased in strain E243N (75–85%). In strain I280T, the amount of cyanide-binding enzyme varied from 75% to 90%.

Reactivity with CO was monitored by laser-flash photolysis [12,13]. Like cyanide, CO binds to reduced haem *a*<sub>3</sub> and induces an optical change. The CO–haem *a*<sub>3</sub> compound is labile and can be photolysed by a laser flash. The optical signal induced by the laser-flash photolysis of CO and by the subsequent re-binding of CO to haem *a*<sub>3</sub> (in a ms timescale) can be monitored easily in the Soret region (430–445 nm). The amount of CO-ligated haem *a*<sub>3</sub> was estimated from the magnitude of the photolysis signal and compared with the total amount of cytochrome oxidase. The amount of CO-reacting enzyme was greater than 90% in the wild type and in strains G317S, M273T and E243D, but was decreased in strains I280T (75–85%) and E243N (65–75%). The decrease in the extent of ligand reaction observed in strain E243N, and to a lesser degree in strain I280T, suggested that a minor portion of the enzyme populations had an altered (not reacting) binuclear centre. The re-binding of CO to haem *a*<sub>3</sub> after photolysis was also monitored. The kinetics of CO recombination were monophasic (for at least 80% of the signal), which suggested that the mutations did not cause any significant structural distortions around the binuclear centre. An apparent rate constant ( $k_{\text{obs}}$ ) of 60–65 s<sup>-1</sup> was observed for the wild type and for strains G317S, I280T, E243D and E243N, which was in agreement with values reported previously for wild-type enzymes (see [12] and references therein). With strain M273T, the rate of CO recombination was

slightly higher (100 s<sup>-1</sup>). A similar rate was observed previously for human cells carrying the M273T mutation [13]. The reaction of cytochrome oxidase with CO in isolated mitochondria was also monitored. As observed with intact cells, the rate of CO recombination was higher in strain M273T (Table 2).

Oxygen consumption activity was measured in intact cells. As expected, strain G317S showed a wild-type activity, while the activity was decreased to approx. 50% in strains M273T, I280T and E243D and to 6% in E243N. No respiratory activity was detected in strain ΔF102–F106. The decreased respiratory activity in M273T, I280T and E243D was confirmed by monitoring cytochrome *c* oxidase activity in isolated mitochondria (Table 2). Turnover was approx. 430 s<sup>-1</sup> for the wild-type enzyme and approx. 180 s<sup>-1</sup> for the mutants.

## DISCUSSION

### Mutation of residue Glu-243

In bacterial terminal oxidases, it has been shown that Glu-243, located in the D-channel (Figure 1), plays a pivotal role in the catalytic mechanism of the enzyme. The replacement of this glutamate by unprotonable residues caused a dramatic loss of enzymic activity [6]. Several steps in the catalytic cycle involving proton movements were affected in the mutants [15–18], indicating a crucial function for Glu-243 in proton translocation. The crystal structure of the bovine enzyme, by contrast, seemed to show that residue Glu-243 was at a 'dead end' of a possible hydrogen-bond network. It was suggested that the D-channel and Glu-243 might not have an essential proton-conveying function, and an alternative proton pathway was proposed [19]. In the present study, Glu-243 in yeast cytochrome oxidase was replaced by aspartate, another protonable residue with a shorter side chain, or by asparagine, an unprotonable residue. The mutation Glu-243 → Asn had a dramatic effect on enzyme activity, which was decreased to a few percent of that of the wild-type enzyme. Analysis of the optical properties of the enzyme in cells showed that reduced haem *a*<sub>3</sub> reacted normally with ligands, which was in agreement with observations on the bacterial enzyme [15]. A minor population (25–35%) did not react with CO and cyanide. This has been observed previously with E243Q *Rhodobacter sphaeroides* enzyme. It was suggested that the non-reactive population arose from some misfolding of the mutant protein [15]. The replacement of glutamate by the protonable residue aspartate (which shortens the side chain by one methylene group) decreased enzyme activity to around 40% of the wild

type. This behaviour was again very similar to that observed with the analogous E243D *Rh. sphaeroides* enzyme [15].

It therefore seems that, as in bacterial enzyme, Glu-243 (or at least a protonable residue) is required for the catalytic activity of yeast cytochrome oxidase.

#### Disease-associated mutations: M273T, I280T and G317S in subunit I, and $\Delta$ F102–F106 in subunit III

The three highly conserved residues Met-273, Ile-280 and Glu-317 are located in the vicinity of the K-channel (Figure 1). The precise role of the K-channel is still debated (e.g. see [1] and references therein). However, the channel is essential for the catalytic activity of the enzyme, and is clearly present in the crystal structure of the bovine enzyme [20].

The mutation G317S has been found in fibroblasts from a patient presenting with lactic acidemia and cytochrome oxidase deficiency. As shown in the present study, this mutation has no effect on yeast cytochrome oxidase. The respiratory growth competence was not affected, and no decrease in cytochrome oxidase content or activity was observed. Gly-317 is a highly conserved residue located next to Thr-316, which is part of the K-channel [20,21]. The replacement of Gly-317 by serine may perturb Thr-316, but is without effect on the catalytic activity of the enzyme. Mutations of conserved residues even located in structures such as the K-channel may have no or little effect if the changes do not disrupt the network of water molecules and hydrophilic residues that works as a functional unit. It has been shown recently that, in human cells, transfer of the mutated mitochondria to control rho<sup>0</sup> cells restored enzyme activity, indicating that the deficiency was caused by a nuclear mutation and not by the mitochondrial mutation (J.-W. Taanman, personal communication), which is in agreement with the data reported here.

Mutations I280T and M273T have been observed in haematopoietic cells from patients suffering from acquired idiopathic sideroblastic anaemia [2]. Analysis of the homoplasmic trans-mitochondrial mutant clones showed that these mutations had only a mild effect on respiration, and did not seem to hamper cell growth. In the I280T cell line, the cytochrome oxidase activity of permeabilized cells was decreased by around 35%. In M273T cell lines, a slight decrease in the oxygen consumption of intact cells was observed, with slightly higher lactate production. However, this decrease in respiratory activity was not apparent in digitonin-permeabilized cells. Despite these inconclusive results with regard to activity, the mutation did seem to affect the enzyme, since a higher rate of reaction with CO was observed [13].

In the present study, the same mutations (M273T and I280T) were introduced into the yeast enzyme in order to assess their effects. The respiratory growth competence of the yeast mutants was altered, and cytochrome oxidase activity was decreased by a factor of two. The cytochrome oxidase content (based on the haem *a* signal) was not affected. In strain I280T, the enzyme population seemed to be heterogeneous. The major population (75% or more; see Table 1) reacted normally with CO and cyanide, whereas a minor population could not bind these ligands. This decrease in ligand reactivity is in agreement with observations with human cells. In an I280T human cell line, estimates of the cytochrome oxidase content, based on the extent of reaction with CO, showed a lower level of enzyme (approx. 60% of the control). We concluded that the mutation I280T might affect the assembly or stability of the enzyme [13]. We show here that this mutation did not affect the overall cytochrome oxidase content, but caused a slight increase in non-reactive

binuclear centres. The non-reactive form may arise from some misfolding of the mutant enzyme, as has been suggested previously [15]. In strain M273T, the kinetics of the reaction of CO with cytochrome oxidase were faster ( $k_{\text{obs}}$  100 s<sup>-1</sup>) than in the wild type ( $k_{\text{obs}}$  60 s<sup>-1</sup>), which is also in agreement with the observations from M273T human cell lines. This increase in the rate of CO reaction suggested a slight alteration of the binuclear centre environment. This has already been reported for two other yeast mutations, *COX1* G253D and *COX1* T316K, both of which are located in the K-channel [11].

Even though mutations M273T and I280T had little effect in human cell lines under laboratory conditions [13], the present work on similar yeast mutants showed that the mutations could have a mildly deleterious effect on respiration. Residues Met-273 and Ile-280 are close to Lys-319, Thr-316 and Thr-309, which are part of the K-channel. It has been shown, for instance, that mutation of Lys-319 in bacteria inhibited the catalytic activity of the enzyme by hindering electron transfer from haem *a* to the binuclear centre [22]. It is likely that mutations M273T and I280T affect the function of this channel and slow down electron transfer into the binuclear centre, which causes the decrease in oxygen consumption. Their pathogenic significance is not clear. However, it is possible that the high energy demands of erythropoietic cells could not be fully met by cells with a mildly decreased respiratory function.

The short deletion of five residues ( $\Delta$ F94–F98 in human;  $\Delta$ F102–F106 in yeast) in a conserved region of subunit III has been observed in a patient with myoglobinuria and cytochrome oxidase deficiency [4]. The mutation is located in a trans-membrane helix, in contact with subunit I. The effect of this mutation has been studied in detail in homoplasmic trans-mitochondrial cell lines [23]. These cells had no detectable cytochrome oxidase activity. Immunoprecipitation experiments were performed to monitor the assembly of subunits I, II and III, and showed that no assembled complex was detected in the mutant cells. In the present study, a similar five-residue deletion was introduced in yeast and the effect of the mutation was characterized. The behaviour of the yeast deletion mutant was very similar to that of the human mutant. The yeast cells were respiratory growth deficient, and no cytochrome oxidase was optically detectable; this suggests that, in yeast, like in human cells, the deletion dramatically altered enzyme assembly. It is not surprising that a deletion of five amino acids in a membrane helix should distort the subunit and affect contact with subunit I and the assembly of the complex. Previous analysis of deficient mutations and second-site reversions in cytochrome oxidase subunits I and II have suggested that interface regions between subunits play an essential role in the assembly or stability of the enzyme [24].

From this first study of 'human' mutations in yeast cytochrome oxidase, it appears that the yeast mutants behave like their human counterparts, and can be used as models to characterize in detail the effects of disease-related mutations on respiratory function. The possibility of studying in yeast the effects of human mutations would be of particular value when lines of mutant human cells are not available. In addition, when the mutations cause respiratory deficiency in yeast models, revertants could be selected and compensatory mutations identified.

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#### REFERENCES

- Wikström, M., Jasaitis, A., Backgren, C., Puustinen, A. and Verkhovsky, M. I. (2000) The role of the D- and K-pathways of proton transfer in the function of the haem-copper oxidases. *Biochim. Biophys. Acta* **1459**, 514–520

- 2 Gattermann, N., Retzlaff, S., Wang, Y. L., Hofhaus, G., Heinisch, J., Aul, C. and Schneider, W. (1997) Heteroplasmic point mutations of mitochondrial DNA affecting subunit I of cytochrome c oxidase in two patients with acquired idiopathic sideroblastic anemia. *Blood* **90**, 4961–4972
- 3 Wallace, D. C. (1994) Mitochondrial DNA sequence variation in human evolution and disease. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8739–8746
- 4 Keightley, J. A., Hoffbuhr, K. C., Burton, M. D., Salas, V. M., Johnston, W. S. W., Penn, A. M. W., Buist, N. R. M. and Kennaway, N. G. (1996) A microdeletion in cytochrome c oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria. *Nat. Genet.* **12**, 410–416
- 5 Rahman, S., Taanman, J.-W., Cooper, J. M., Nelson, I., Hargreaves, I., Meunier, B., Hanna, M. C., Garcia, J., Capaldi, R. A. and Lake, B. D. et al. (1999) A mis-sense mutation of cytochrome oxidase subunit II causes defective assembly and myopathy. *Am. J. Hum. Genet.* **65**, 1030–1039
- 6 Mitchell, D. M., Aasa, R., Ådelroth, P., Brzezinski, P., Gennis, R. B. and Malmström, B. G. (1995) EPR studies of wild-type and several mutants of cytochrome c oxidase from *Rhodobacter sphaeroides*: Glu<sup>286</sup> is not a bridging ligand in the cytochrome a<sub>3</sub> Cu<sub>2</sub> center. *FEBS Lett.* **374**, 371–374
- 7 Fox, T. D., Sanford, J. C. and McMullin, T. W. (1988) Plasmids can stably transform yeast mitochondria lacking endogenous mtDNA. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7288–7292
- 8 Meunier, B., Lemarre, P. and Colson, A. M. (1993) Genetic screening in *Saccharomyces cerevisiae* for large numbers of mitochondrial point mutations which affect structure and function of catalytic subunits of cytochrome-c oxidase. *Eur. J. Biochem.* **213**, 129–135
- 9 Costanzo, M. C. and Fox, T. D. (1993) Suppression of a defect in the 5' untranslated leader of mitochondrial COX3 mRNA by a mutation affecting an mRNA-specific translation activator protein. *Mol. Cell. Biol.* **13**, 4806–4813
- 10 Fox, T. D., Folley, L. S., Mulero, J. J., McMullin, T. W., Thorsness, P. E., Hedin, L. O. and Costanzo, M. C. (1991) Analysis and manipulation of yeast mitochondrial genes. *Methods Enzymol.* **194**, 149–165
- 11 Brown, S., Colson, A.-M., Meunier, B. and Rich, P. R. (1993) Rapid screening of cytochromes of respiratory mutants of *Saccharomyces cerevisiae* – application to the selection of strains containing novel forms of cytochrome c oxidase. *Eur. J. Biochem.* **213**, 137–145
- 12 Meunier, B. and Rich, P. R. (1998) Quantitation and characterisation of cytochrome c oxidase in complex systems. *Anal. Biochem.* **260**, 237–243
- 13 Bröker, S., Meunier, B., Rich, P. R., Gattermann, N. and Hofhaus, G. (1998) MtDNA mutations associated with sideroblastic anaemia cause a defect of mitochondrial cytochrome c oxidase. *Eur. J. Biochem.* **258**, 132–138
- 14 Daum, G., Bohni, P. C. and Schatz, G. (1982) Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* **257**, 13028–13033
- 15 Jünemann, S., Meunier, B., Fisher, N. and Rich, P. R. (1999) Effects of mutation of the conserved glutamic acid-286 in subunit I of cytochrome c oxidase from *Rhodobacter sphaeroides*. *Biochemistry* **38**, 5248–5255
- 16 Adelroth, P., Svensson, M., Mitchell, D. M., Gennis, R. B. and Brzezinski, P. (1997) Glutamate 286 in cytochrome aa3 rhodobacter sphaeroides is involved in proton uptake during the reaction of the fully-reduced enzyme with dioxygen. *Biochemistry* **36**, 13824–13829
- 17 Konstantinov, A. A., Siletsky, S., Mitchell, D., Kaulen, A. and Gennis, R. B. (1997) The roles of the two proton input channels in cytochrome c oxidase from *Rhodobacter sphaeroides* probed by the effects of site-directed mutations on time-resolved electrogenic intraprotein proton transfer. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9085–9090
- 18 Svensson-Ek, M., Thomas, J. W., Gennis, R. B., Nilsson, T. and Brzezinski, P. (1996) Kinetics of electron and proton transfer during the reaction of wild type and helix VI mutants of cytochrome b<sub>o</sub>3 with oxygen. *Biochemistry* **35**, 13673–13680
- 19 Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P. and Mizushima, T. et al. (1998) Redox-coupled crystal structural changes in bovine heart cytochrome c oxidase. *Science* **280**, 1723–1729
- 20 Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å. *Science* **272**, 1136–1144
- 21 Ostermeier, C., Harrenga, A., Ermler, U. and Michel, H. (1997) Structure at 2.7 Å resolution of the *Paracoccus denitrificans* two-subunit cytochrome c oxidase complexed with an antibody F<sub>v</sub> fragment. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10547–10553
- 22 Jünemann, S., Meunier, B., Gennis, R. B. and Rich, P. R. (1997) Effects of mutation of the conserved lysine-362 in cytochrome c oxidase from *Rhodobacter sphaeroides*. *Biochemistry* **36**, 14456–14464
- 23 Hoffbuhr, K. C., Davidson, E., Fillano, B. A., Davidson, M., Kennaway, N. G. and King, P. M. (2000) A pathogenic 15-base pair deletion in mitochondrial DNA-encoded cytochrome c oxidase subunit III results in the absence of functional cytochrome c oxidase. *J. Biol. Chem.* **275**, 13994–14003
- 24 Meunier, B. and Rich, P. R. (1998) Second-site reversion analysis is not a reliable method to determine distance in membrane proteins: an assessment using mutations in yeast cytochrome c oxidase subunits I and II. *J. Mol. Biol.* **283**, 727–730

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