Analysis of COX2 mutants reveals cytochrome oxidase subassemblies in yeast

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Cytochrome oxidase catalyses the reduction of oxygen to water. The mitochondrial enzyme contains up to 13 subunits, 11 in yeast, of which three, Cox1p, Cox2p and Cox3p, are mitochondrially encoded. The assembly pathway of this complex is still poorly understood. Its study in yeast has been so far impeded by the rapid turnover of unassembled subunits of the enzyme. In the present study, immunoblot analysis of blue native gels of yeast wild-type and Cox2p mutants revealed five cytochrome oxidase complexes or subcomplexes: *a*, *b*, *c*, *d* and *f*; *a* is likely to be the fully assem-

bled enzyme; *b* lacks Cox6ap; *d* contains Cox7p and/or Cox7ap; *f* represents unassembled Cox1p; and *c*, observed only in the Cox2p mutants, contains Cox1p, Cox3p, Cox5p and Cox6p and lacks the other subunits. The identification of these novel cytochrome oxidase subcomplexes should encourage the reexamination of other yeast mutants.

Key words: assembly, blue native protein electrophoresis, COX2 mutant, cytochrome *c* oxidase, haem *aa*₃, yeast.

INTRODUCTION

Deficiencies in the activity of the respiratory chain enzyme cytochrome oxidase are associated with a range of diseases in humans. Mutations in the core subunits of the enzyme and in the proteins required for its assembly have been identified in patients [1,2]. Cytochrome oxidase is a haem–copper metalloprotein that forms the terminal enzyme complex of the mitochondrial respiratory chain. It is embedded in the inner mitochondrial membrane and protonmotively catalyses the reduction of oxygen to water. The enzyme is composed of up to 13 subunits in eukaryotes. The yeast enzyme has 11 subunits, eight of which are nuclearly encoded. Their role is unclear, but some may be involved in enzyme assembly or stability. Three subunits (Cox1p, Cox2p and Cox3p) are encoded by the mitochondrial genome and form the catalytic core of the enzyme. Redox-active prosthetic groups are located within Cox1p (haems *a* + *a*₃ and Cu_B) and Cox2p (Cu_A).

The assembly of cytochrome oxidase is a complex process, requiring a large number of ancillary nuclear factors that have been identified by extensive study in yeast, *Saccharomyces cerevisiae* (reviewed in [2]). The assembly pathway is still poorly understood. Assembly of the mammalian enzyme is thought to occur in an ordered sequence [3], and assembly intermediates have been detected in metabolic labelling experiments [4]. Study of the enzyme in human cells from patients carrying mutations in the assembly factors COX10, SCO1 and SURF1 has revealed the presence of subcomplexes that are likely to represent assembly intermediates accumulated in the cells with a defective cytochrome oxidase assembly process [5]. Yeast would be the choice model organism to investigate the assembly pathway of such a complex enzyme. It has been used to study the molecular basis of some of the disease mutations (see for example [6–8]). A large number of mutants can be generated and the organism is amenable to site-directed mutagenesis of both nuclear and mitochondrial genes. However, only three cytochrome oxidase subassemblies, containing some nuclearly encoded subunits, have been identified in yeast so far [9]. As failure to assemble the enzyme seems to result in

a rapid degradation of the subunits, especially the core subunits Cox1p, Cox2p and Cox3p, by the mitochondrial proteases [10], the absence of haem *aa*₃ signal has been considered as the hallmark of cytochrome oxidase assembly mutants [11–13]. In the present study, we selected Cox2p mutants presenting optically detectable cytochrome oxidase with altered properties. We then used blue native gel electrophoresis to investigate the enzyme in mitochondrial membranes from these mutants and from wild-type cells, which allowed us to identify several novel subassemblies of the yeast enzyme.

MATERIALS AND METHODS

Media and chemicals

The following media were used for the growth of yeast: YPD (1 % yeast extract, 2 % peptone and 3 % glucose), YPG (1 % yeast extract, 2 % peptone and 3 % glycerol).

Generation of COX2 mutants

The mutagenesis and screening of respiratory-deficient mutants was performed as described in [14]. The strain BM1/1–16, which harbours the nuclear recessive mutation *op1* affecting the adenine nucleotide carrier of the inner mitochondrial membrane, was treated overnight with 8 mM MnCl₂, an mtDNA (mitochondrial DNA) mutagen. After mutagenesis, the cells were grown for a few generations in YPD medium, then diluted and plated on the same to obtain isolated colonies. The colonies were crossed with an OP rho⁰ (complete deletion of the mtDNA) strain. The resulting diploids were replicated on respiratory medium (YPG) to identify the respiratory-deficient mutants. Note that the use of a strain carrying *op1* prevents the accumulation of rho⁰ cells, which occurs at high frequency, since the mutation *op1* is lethal in combination with rho⁰ mutation. The screening of the respiratory-deficient colonies using diploids eliminates the nuclear recessive mutations. The mutations were then mapped by crossing the respiratory

Abbreviation used: mtDNA, mitochondrial DNA.

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mutants with tester ρ^- strains that retain only a part of the mtDNA, as described in [14]. When a mutant strain carrying an mtDNA point mutation is crossed with a ρ^- strain that retains the DNA sequence corresponding to that mutation, recombination between the mtDNA results in the production of respiratory-competent diploid cells. The ρ^- tester strain used in the present study to identify mutations in the coding region of the COX2 gene was constructed by biolistic transformation as described below. The mutations in COX2 were identified by sequencing. The missense mutations chosen for further study were then transferred into other nuclear backgrounds to facilitate genetic analysis. This was done in a two-step manner. The BM1/1-16 mutants were used for the cytoduction of the mitochondrial genome into the recipient strain JC8/56 (ρ^0). This strain carries the mutation *kar1-1* required for cytoduction [15]. These cytoductants were then used to transfer the mitochondrial genome into W303-1B/ ρ^0 . The resulting strains were used for the selection of revertants, i.e. respiratory-competent cells.

Generation of the COX2 ρ^- tester strain

The plasmid pBM2 carrying the wild-type sequence of the COX2 gene was constructed by blunt-end cloning of the PCR product of the coding region of COX2 into the pCRscript vector (Stratagene, Cambridge, U.K.). The plasmid was used for biolistic transformation of a recipient ρ^0 strain. The mitochondrial transformation by microprojectile bombardment was adapted from [16], as described in [8].

Reversion analysis

The chosen respiratory-deficient mutants were subcloned. Several subclones were grown in YPD and then incubated on respiratory medium (YPG). Respiratory-competent clones appeared after 1 or 2 weeks of incubation and were analysed as in [17] to determine the mitochondrial or nuclear heredity of the reversion mutation. The mitochondrial reversions in COX2 were identified by sequencing.

Spectrophotometric analysis

Spectroscopic measurements of haems in yeast cells and membrane samples were performed as described in [8].

Measurement of cytochrome *c* oxidase activity

Preparation of the mitochondrial membranes was as described in [18]. Cytochrome oxidase activity was measured with an oxygen electrode. Membrane samples were diluted to 2.5 nM cytochrome oxidase in 50 mM potassium phosphate, 10 mM ascorbate and 50 μ M *N,N,N',N'*-tetramethyl-*p*-phenylenediamine at pH 7. The reaction was initiated by the addition of cytochrome *c*. Initial rates were measured as a function of cytochrome *c* concentration, and V_m and K_m values were derived from Lineweaver–Burk plots ($1/V$ versus $1/[S]$, where V is the enzymatic reaction velocity and $[S]$ is the substrate concentration).

Immunodetection analyses

Immunodetection analyses were performed on crude mitochondrial membranes as described in [5]. Briefly, for one-dimensional denaturing gels, samples were dissociated in 50 mM Tris/HCl (pH 6.8), 12 % glycerol, 4 % SDS, 2 % 2-mercaptoethanol and 0.01 % Bromophenol Blue, and then 10–30 μ g of total protein/lane was resolved by electrophoresis on 10, 12.5 or 15 % polyacrylamide gels containing 0.1 % SDS and 5.5 M

urea. For one-dimensional blue native gels, samples were solubilized with dodecylmaltoside and 60 μ l of total protein/lane was resolved on 8–16 % polyacrylamide blue native gels [19]. For two-dimensional native/denaturing gels, samples were separated on 8–16 % polyacrylamide blue native gels as described above and then single sample lanes were soaked in 1 % 2-mercaptoethanol and 1 % SDS for 15 min before being washed twice in 50 mM Tris/HCl (pH 6.8) and 1 % SDS for 10 min. Each single gel slice was placed horizontally in the gel pouring equipment, resolving gel (15 or 20 % polyacrylamide, 0.1 % SDS and 5.5 M urea) was poured below it and then the slice was encased in 3 % polyacrylamide stacking gel and electrophoresed. We used subunit-specific mouse monoclonal antibodies against the yeast cytochrome oxidase subunits Cox1p, Cox2p, Cox3p, Cox4p, Cox5p, Cox6ap and Cox8p, and porin. In addition, a rabbit antiserum was used that recognizes the co-migrating subunits Cox7p and Cox7ap. All these antibodies were generated in the laboratory of Professor R. A. Capaldi (Institute of Molecular Biology, Eugene, OR, U.S.A.) and are described in [20]; most of them are commercially available (Molecular Probes, Eugene, OR, U.S.A.). The polyclonal antiserum against cytochrome *c*₁ was generously supplied by Professor B. L. Trumpower (Dartmouth Medical School, Hanover, NH, U.S.A.). Anti-mouse secondary antibodies were obtained from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.), DakoCytomation (Ely, Cambridgeshire, U.K.) and Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Anti-rabbit secondary antibodies were obtained from Bio-Rad.

Gel staining for cytochrome oxidase activity

Blue native gels were prepared with wild-type protein as described above and incubated for 15 h at 37 °C in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mg/ml cytochrome *c*, 2 μ g/ml catalase and 0.5 mg/ml diaminobenzidine [21].

RESULTS AND DISCUSSION

We investigated subcomplexes of yeast cytochrome oxidase using an immunodetection approach. New mutants harbouring mutations in Cox2p were generated and screened for the presence of altered cytochrome oxidase. Using a random mutagenesis approach, 319 respiratory growth-deficient mutants were obtained (as described in the Materials and methods section). By genetic mapping, 46 mutants were found to have mutations in the COX2 gene. Sequencing analysis showed that ten mutants harboured missense mutations localized in the C-terminal extramembrane domain of Cox2p. The cytochrome content in these mutants was monitored by optical spectroscopy as in [8]. In eight mutants, the cytochrome oxidase haem *aa*₃ signal could not be detected or was dramatically reduced, for example the double mutant C221W + L245W in Figure 1. Two mutants, G156E (Gly¹⁵⁶ → Glu) and R159K, resulted in partially assembled cytochrome oxidase with altered properties. These mutants were characterized in detail as we reasoned that they might provide interesting information on cytochrome oxidase assembly.

R159K showed a significant amount of cytochrome oxidase as judged by the optical spectra (Figure 1), as did G156E. However, the cells were unable to grow on respiratory medium. In both mutants, the amplitude of the haem *aa*₃ spectrum was decreased to 50 % of the wild-type level and the peak of absorption was shifted to 599 nm (603 nm in the wild-type cells), which indicates a perturbation in the haem environment. Cytochrome oxidase activity was monitored. Both mutants showed a decreased V_m value and an increased K_m value for cytochrome *c* (Table 1). The

Figure 1 Cytochrome level in mutants and wild-type cells

Optical spectra of whole cell samples. Optical spectra of reduced cell suspensions were obtained as described in the Materials and methods section. Curve *c*, cytochrome *c* (haem *c*); *b*, cytochrome *bc*₁ (haems *b*); *a*, cytochrome oxidase (haems *aa*₃).

Table 1 Growth rate, cytochrome *c* oxidase content and activity in mutants and wild-type

Mutation	CcO content* (nmol/g cells)	Growth rate† (% wild-type)	CcO activity‡	
			V_m (s^{-1})	K_m (μM cyt <i>c</i>)
Wild-type	0.8	100	625	7.4
G156E	0.4	< 5	47	53
R159K	0.4	< 5	208	43
R159K + D187N	0.6	60	650	14
R159K + S222Y	0.5	40	580	16

* The cytochrome oxidase (CcO) content was determined from the amplitude of the haem *aa*₃ optical spectra of whole cell suspensions as described in [12].

† The cells were incubated in YPG. The absorbance of the culture was monitored at different times. The doubling time of the wild-type cells was approx. 4 h.

‡ The cytochrome *c* oxidase activity of membrane samples was measured using an oxygen electrode (see the Materials and methods section). Initial rates were measured as a function of the cytochrome *c* (cyt *c*) concentration, and V_m and K_m values were derived from Lineweaver–Burk plots ($1/V$ versus $1/[S]$).

increase in K_m for cytochrome *c* is suggestive of a weakened interaction between the reaction partners, probably arising from a distortion of the binding site for cytochrome *c* on the surface of Cox2p. From these mutants, reversions were obtained as described in the Materials and methods section, which partially compensated the respiratory defect caused by G156E and R159K. The secondary mutations, D187N and S222Y/F, were located in Cox2p extramembrane domain, 25–30 Å (1 Å = 0.1 nm) from the primary sites. The revertants R159K + D187N and R159K + S222Y were further studied. Their respiratory growth competence was partially restored. The cytochrome oxidase content remained lower but the cytochrome oxidase activity was closer to wild-type (Table 1).

These mutants, G156E, R159K, R159K + D187N and R159K + S222Y, were then used to investigate cytochrome oxidase complexes using native gel electrophoresis. We also monitored the enzyme in wild-type and in C221W + L245W, a mutant that failed to assemble cytochrome oxidase. Mitochondrial membrane samples were subjected to native gel electrophoresis and antibodies raised against several cytochrome oxidase subunits were used to detect any complex or subcomplex (Figure 2).

In the control wild-type, two bands were detected by most of the antibodies tested. The upper band, denoted as *a*, was assumed to correspond to the monomeric holoenzyme complex; the lower band, *b*, was likely to be a partially degraded complex that lacks

Cox6ap, since it was not visible on probing with antibodies raised against this subunit. The relative proportions of complexes *a* and *b* were not equal: complex *b* was generally, but not always, present at higher levels. This may be due to subtle variations in sample treatment before loading on to the gels, suggesting that Cox6ap is only loosely associated with the enzyme complex. Solubilization of yeast cytochrome oxidase with Triton X-100 removes Cox6ap and Cox6bp [22]. In humans, an assembly subcomplex has been identified in which COX6A and COX7A/B (yeast Cox6ap and Cox7p respectively) are absent [4]. Surprisingly, Cox4p, detected in band *b*, was not detected in band *a*. This may be because the Cox4p epitope is not accessible to the anti-Cox4p monoclonal antibody in the fully assembled enzyme due to shielding by Cox6ap. Gel staining for cytochrome oxidase activity (Figure 3) showed that both bands *a* and *b* represented active forms of cytochrome oxidase.

No complexes or subcomplexes could be detected in the mutant C221W + L245W. Western-blot analysis of SDS denaturing gels (results not shown) showed that C221W + L245W lacked most of the cytochrome oxidase subunits assessed, with the exception of Cox4p. Cox4p is a matrix-side extrinsic polypeptide that has been shown to be present in soluble form when assembly is disrupted, suggesting that it is relatively stable compared with other subunits [23]. Thus C221W + L245W showed the well-documented phenotype of yeast mutants with impaired assembly of cytochrome oxidase, and very low steady-state level of unassembled subunits.

The other COX2 mutants revealed a new subassembly not detectable in the control, denoted as band *c*. G156E and R159K contained less than 10% of the wild-type levels of complexes *a* and *b*. In these mutants, the faster migrating band *c* could be seen. This band was not detected by antibodies raised against Cox2p, Cox4p, Cox6ap, Cox7p and Cox7ap, or Cox8p, suggesting that these subunits were absent from band *c*. This band comprises Cox1p, Cox3p, Cox5p and Cox6p. In the two revertants R159K + D187N and R159K + S222Y, higher levels of bands *a* and *b* were present compared with the single-site mutants, in addition to subcomplex *c*. It seems that the secondary mutations increase the content of fully assembled enzyme, which seems to be in agreement with the increased cytochrome *c* oxidase activity observed in the revertants (Table 1).

In addition to complexes or subcomplexes *a*, *b* and *c*, four other bands were identified in this analysis. Three faster migrating bands were detected separately by antibodies raised against Cox7p and Cox7ap (*d*), Cox8p (*e*) and Cox1p (*f*). A slower migrating band (*z*) was also detected by the Cox8p antibody. These bands were present in mutants, revertants and the wild-type. To determine whether the detection of bands *d*, *e*, *f* and *z* was specific and whether assembly complexes containing Cox7p and/or Cox7ap, Cox8p, or Cox1p really do exist, two-dimensional gels were run, native in the first dimension, then denaturing in the second dimension (Figure 4). R159K + S222Y was used since this was the strain in which highest levels of all the additional bands were found. On probing two-dimensional blots with the antibody raised against Cox1p, four spots corresponding to bands *a*, *b*, *c* and *f* detected on native blots could be clearly identified (Figure 4A). These spots all migrate at the same rate on the second-dimension denaturing gel, and the remainder of the blot was clear. This suggests that band *f* did indeed contain Cox1p. Further analysis showed that band *f* co-migrated with isolated Cox1p (results not shown). Band *f* thus represents unassembled Cox1p. However, it is not clear why the band would be absent from the C221W + L245W mutant.

Lower molecular mass proteins are more difficult to resolve on the second-dimension denaturing gel. When the antiserum

Figure 2 Subcomplexes of cytochrome oxidase in wild-type and COX2 mutants

Immunoblots of blue native gels loaded with 60 μ g of protein from mutant, revertant and wild-type were probed with subunit-specific antibodies, Cox1p, Cox2p, Cox3p, Cox4p, Cox5p, Cox6p, Cox6ap, Cox7p and Cox7ap (Cox7/7ap) and Cox8p, to determine the existence and composition of subcomplexes. Equal loading was confirmed by denaturing Western blots loaded with 10–30 μ g of protein and probed with an antibody specific for porin. Bands a–z represent complexes or subcomplexes of cytochrome oxidase.

Figure 3 Cytochrome oxidase activity of the two major bands of wild-type sample

Wild-type protein (60 μ g) was resolved on a blue native gel and then stained for cytochrome oxidase activity as described in the Materials and methods section.

against Cox7p and Cox7ap was used, spots corresponding to bands *a* and *b* could be distinguished. The signal then extended in a streak, making it difficult to identify further spots. However, the absence of any other signal suggests that band *d* was indeed a complex containing Cox7p and/or Cox7ap with other as yet undetermined proteins (Figure 4A). Since no other cytochrome oxidase subunits were detected in band *d*, it may be that band *d* is implicated in the maturation of Cox7p and/or Cox7ap. It is interesting to note that, in a recent study [9], a subcomplex containing the assembly factor Pet100p, Cox7p, Cox7ap and Cox8 was reported. Our analysis did not reveal the association of Cox8p with band *d* but it would be interesting to check for the presence of Pet100p. On probing with the Cox8p antibody, spots matching bands *a* and *b* were identifiable. However, spots corresponding to bands *e* and *z* did not co-migrate with bands *a* and *b* on the second-dimension denaturing gel: spot *e* was located

between 15 and 25 kDa, while spot *z* ran between 25 and 50 kDa (Figure 4B). Hence bands *e* and *z* did not represent assembly complexes containing Cox8p. In addition to the spots *a*, *b*, *e* and *z*, some smears were detected on the blot. Incubation of a companion blot with secondary antibody demonstrated that these smears were caused by non-specific reaction of the secondary antibody used in the experiment.

In summary, we observed five complexes or subcomplexes: *a*, likely to be the fully assembled enzyme; *b*, a subassembly lacking the peripheral subunit Cox6ap; *d*, which contains Cox7p and/or Cox7ap but no other cytochrome oxidase subunits; *f*, likely to be unassembled Cox1p; and *c*, observed only in the mutants and revertants, lacking Cox2p, Cox4p, Cox6ap, Cox7p, Cox7ap and Cox8p but containing Cox1p, Cox3p, Cox5p and Cox6p. We have not probed with Cox6bp.

What is the origin of band *c*? Band *c* has never been reported previously to our knowledge. It seems to be a stable product in the mutants. Band *c* might represent a degradation product of the fully assembled enzyme. Mutations in the extramembrane domain of Cox2p may alter its structure such that the enzyme can be assembled but is more susceptible to proteolytic attack. The entire Cox2p or just the C-terminal extramembrane region, likely to contain the antibody epitope, might be removed, and attachment of some of the peripheral subunits, namely Cox4p, Cox6ap, Cox7p, Cox7ap and Cox8p, may be weakened. However, that product would be relatively stable and protected against further proteolytic degradation.

It may be suggested that band *c* is an assembly intermediate. Cox1p, Cox3p, Cox5p and Cox6p would associate in a stable subcomplex but the assembly would not be able to proceed further. The intermediate would accumulate due to the absence of normal Cox2p, which might be required in a subsequent assembly step. This would be a situation similar to that observed in human cells harbouring mutations in assembly factors [5]. It has also been demonstrated in *Rhodobacter sphaeroides* that Cox1p is able to associate first with either Cox2p or Cox3p [24]. However, band *c* is not detected in the mutant C221W + L245W, suggesting that

Figure 4 Subunit composition of cytochrome oxidase subcomplexes in revertant verified by second-dimension electrophoresis

Blue native gel strips loaded with 60 μ g of protein from the revertant R159K + S222Y were electrophoresed and then mounted horizontally and resolved by denaturing gel electrophoresis in the second dimension, before immunoblotting. One-dimensional native blots are shown above two-dimensional blots to aid identification of spots. (A) The upper two-dimensional blot was probed with a cocktail of antibodies specific for Cox1p and Cox3p and the lower blot with an antiserum recognizing Cox7p and Cox7ap; 15% denaturing gel was used. (B) The blots were probed with an antibody against Cox8p. Non-specific signals are due to secondary antibody; 20% denaturing gel was used.

Cox2p is required for its formation. In the mutants G156E and R159K, initial folding of Cox2p may be comparatively normal, allowing the formation of an assembly intermediate containing Cox1p, Cox2p, Cox3p, Cox5p and Cox6p – which could not be formed in C221W + L245W and in other assembly mutants. Reduced stability of the mutant Cox2p could result in its removal from this subassembly while the other subunits remain attached to each other. A small proportion of the Cox2p-containing assembly intermediate would persist to form fully assembled cytochrome oxidase. In the revertants R159K + D187N and R159K + S222Y, the secondary mutations increase the stability of Cox2p such that the level of fully assembled enzyme present increases. The existence of an assembly intermediate containing Cox1p, Cox2p, Cox3p, Cox5p and Cox6p is in agreement with the assembly model of the mammalian enzyme [4,5]. The mammalian equivalents of Cox1p, Cox5p and Cox6p are thought to interact first to form a subcomplex and, since Cox2p and Cox3p are core

Scheme 1 Mammalian cytochrome oxidase assembly pathway

Assembly subcomplexes have been identified in human cells as reported in [4,5]. Yeast equivalents of mammalian subunits are given in parentheses.

subunits, they would be expected to associate soon afterwards (Scheme 1).

Further work is needed to investigate the origin of the sub-assemblies. These mutants may provide a useful tool to study the assembly pathway of cytochrome oxidase. The results reported here should encourage the reexamination of yeast cytochrome oxidase mutants.

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