Cytochrome Oxidase Assembly does not Require Catalytically Active Cytochrome c

Antoni Barrientos, Danielle Pierre, Johnson Lee, and Alexander Tzagoloff &

Department of Biological Sciences
Columbia University, New York, NY, 10027

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&Corresponding author: Alexander Tzagoloff
Department of Biological Sciences
Columbia University
New York, New York 10027, USA
Tel.: 212-854-2920
Fax: 212-865-8246
Email: spud@cubpet2.bio.columbia.edu
SUMMARY

Cytochrome c oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen. COX assembly requires the coming together of nuclear and mitochondrially encoded subunits, and the assistance of a large number of nuclear gene products acting at different stages of maturation of the enzyme. In *Saccharomyces cerevisiae*, expression of cytochrome c, encoded by *CYC1* and *CYC7*, is required not only for electron transfer but also for COX assembly through a still unknown mechanism. We have attempted to distinguish between a functional and structural requirement of cytochrome c in COX assembly. A *cyc1/cyc7* double null mutant strain was transformed with the *cyc1-166* mutant gene (Schweingruber, M. E., et al (1979) J. Biol. Chem. 254, 4132-4143) that expresses stable but catalytically inactive iso-1-cytochrome c. The COX content of the *cyc1/cyc7* double mutant strain harboring non-functional iso-1-cytochrome c has been characterized spectrally, functionally and immunochemically. The results of these studies demonstrate that cytochrome c plays a structural rather than functional role in assembly of cytochrome c oxidase. In addition to its requirement for COX assembly, cytochrome c also affects turnover of the enzyme. Mutants containing wild type apocytochrome c in mitochondria lack COX suggesting that only the folded and mature protein is able to promote COX assembly.
Saccharomyces cerevisiae contains two genes for cytochrome c. Iso-1-cytochrome c, encoded by CYC1 (1), accounts for approximately 95% of the total cytochrome c in mitochondria (2). The homologous and less abundant iso-2-cytochrome c is encoded by CYC7 (3). Even though iso-2-cytochrome c represents only 5% of the total cytochrome c, it is sufficient to support respiration and growth, albeit at a somewhat reduced rate, on non-fermentable carbon sources such as glycerol and ethanol (4). Mutations in both isoforms lead to a respiratory defect (3). In addition to the absence of cytochrome c, the double mutant is also deficient in cytochrome oxidase (COX) (5). The same phenotype is observed in cyc3 mutants that are blocked in the covalent attachment of heme to apocytochrome c (6). The biochemical defect of cytochrome c mutants has all the hallmarks of COX assembly mutants. Mitochondria lack the absorption bands corresponding to cytochrome aa3 and the steady-state concentrations of the mitochondrialy-encoded subunits 1, 2, and 3 of COX are reduced, presumably as a result of increased turnover of unassembled subunits (5).

The function of cytochrome c in COX assembly is not understood. Oxidized cytochrome c can accept electrons from cytochrome c1 of the bc1 complex and cytochrome b2 of lactate dehydrogenase. The principle function of reduced cytochrome c is to donate electrons to cytochrome oxidase. Conceivably, cytochrome c could also promote an oxidation or reduction event essential for COX assembly. The obvious possibility that cytochrome c may be involved in heme A biosynthesis has been excluded (7). Alternatively, cytochrome c could be required in a structural capacity. For example, its interaction with a COX intermediate may be necessary for some step in the assembly pathway.

In the present study we have tried to distinguish between a functional and structural requirement of cytochrome c in COX assembly. A cyc1/cyc7 double null mutant was transformed with a cyc1 mutant gene that was previously reported to express stable but catalytically inactive cytochrome c (8). The COX content of the cyc1/cyc7 mutant containing the non-functional form of iso-1-cytochrome c has been characterized spectrally, functionally and immunochemically. The results of these analyses are consistent and point to a structural rather than functional role of cytochrome c in COX assembly.
MATERIALS AND METHODS

Yeast Strains and Media - The genotypes and sources of the strains of *S. cerevisiae* used in this study are listed in Table I. Yeast strains were routinely grown in 2% galactose, 1% yeast extract, and 2% peptone (YPGal). The compositions of solid media have been described elsewhere (12).

Preparation of Yeast Mitochondria - Wild-type and mutant yeast were grown to stationary phase in YPGal. Unless otherwise indicated, mitochondria were prepared by the procedure of Faye et al. (13), except that Zymolyase 20,000 (ICN Biomedicals, Inc.) instead of Glusulase was used at 24°C to convert cells to spheroplasts.

Construction of a *cyc1* and *cyc7* null alleles - *CYC1* was cloned as a Xmal-HindIII fragment into pUC18. The resultant plasmid pCYC1/ST1 was used to delete the gene with the bi-directional primers: 5’-GGCGGTACCTATTAATTTAGTGTGTATTG and 5’-GGCGGTACCAACAGGCCCCTTTTCCTTTGTC. The PCR-amplified product containing 5’ and 3’ flanking sequences but lacking the entire *CYC1* coding sequence was digested with *Kpn*I and ligated to a 1 kb *Kpn*I fragment containing the yeast *URA3* gene. This plasmid (pCYC1/ST3) was used as a source of a linear 1.5 kb *Eco*RI-HindIII fragment with the Δ*cyc1::URA3* allele.

To delete *CYC7*, the gene was first PCR-amplified from yeast nuclear DNA with primers: 5’-GGCGGATCC-GAAGGGTCTGCAGTCCCCCGCC and 5’-GGCGGATCCC-TGTAAGCGGAAGCGC-CTCCAG. The 800 bp fragment containing *CYC7* and flanking sequences was digested with *Bam*H1 and cloned in YEp352B (this plasmid is identical to YEP352 (14) except that the multiple cloning sequence is replaced by a single *Bam*H1 site) yielding pCYC7/ST2. *CYC7* was deleted from CYC7/ST2 with the bi-directional primers: 5’-GGCG-AATTCGTTTTGTTTATG-ATGTAATGTAGTT and 5’-GGCGAATTCGG-CTATGTCGTCGGAGGAG. The linear product containing 5’ and 3’ flanking sequences but lacking *CYC7* was digested with *Eco*RI and ligated to a 1.7 kb *Eco*RI fragment containing the yeast *TRP1* gene. The resultant plasmid pCYC7/ST4 was digested with *Bam*H1 to obtain a linear 2 kb fragment with the Δ*cyc7::TRP1* allele.
The respiratory competent haploid yeast W303-1B was transformed with the linear 1.5 kb EcoR1-HindIII fragment containing the cyc1 null allele. A uracil prototrophic transformant was verified by PCR to be deleted for CYC1. This mutant, designated W303ΔCYC1, was transformed with the linear fragment containing the cyc7 null allele. Several uracil- and tryptophan-independent clones obtained from the transformation were confirmed by PCR to have the CYC7 deletion. One of the double mutants W303ΔCYC1,7 was used for further studies.

**Construction of the cyc1 mutant gene** - The cyc1-166 mutation (8) was introduced into CYC1 by PCR amplification of the region between the Xma1 and internal KpnI site of pCYC1/ST1 with primers 5'-GGACCCGGGAGCAAGATCAAGATG and 5'-CTTGGGTACCAGGAATATATTTCTTTGTTAGTCAAGTACTCTGACATGTTATTTCGTCCGACAAACG. The fragment with the mutation was digested with a combination of Xma1 and KpnI and was substituted for the corresponding fragment in pCYC1/ST1 yielding pCYC1/W65S. The mutant gene was recovered as an XmaI-HindIII fragment and was transferred to YIp351 and YEp351 (14) yielding pCYC1/ST11 and pCYC1/ST12, respectively. The mutation was confirmed by sequencing of the insert in pCYC1/ST11. The mutant gene was integrated at the leu2 locus of W303-1B after linearization of pCYC1/ST11 at the ClaI site of the LEU2 marker in the plasmid.

**Cytochrome oxidase assays** - COX was assayed either spectrophotometrically by following the oxidation of ferro-cytochrome c at 550 nm (15) or polarographically by measuring oxygen utilization with ascorbate plus N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as the substrate. In the latter assay the reaction contained 10 mM potassium phosphate, pH 7.5, 5 mM ascorbic acid, 0.1 mM TMPD and 50-100 µg mitochondrial protein. Both assays were carried out at 24°C.

**Miscellaneous procedures** - Standard procedures were used for the preparation and ligation of DNA fragments, and for transformation and recovery of plasmid DNA from E. coli (16). Yeast strains were transformed by the method of Schiestl and Gietz (17). Mitochondrial protein synthesis was assayed *in vivo* in the presence of cycloheximide as described previously (11). Proteins were separated by PAGE in the buffer system of
Laemmli (18). Cytochrome c was detected on Western blots using a rabbit polyclonal antibody raised against SDS-denatured yeast cytochrome c purchased from Sigma Chemical Co. (St Louis, MO). Antibody-antigen complexes were visualized by a secondary reaction with the Super Signal detection kit (Pierce, Rockford, IL). Alternatively Western blots were first treated with antibody against cytochrome c, followed by incubation with $^{125}$I-protein and quantitation of the signals with a Storm PhosphorImager (Molecular Dynamics, Inc, Sunnyvale, CA). Protein concentrations were determined by the method of Lowry et al. (19).

RESULTS AND DISCUSSION

Phenotype of the cyc1, and the cyc1/7 null mutants - Deletion of CYC1 reduces the rate but does not abolish growth of yeast on rich glycerol/ethanol medium (ref. 4, Fig. 1A). In contrast, deletion of both CYC1 and CYC7 completely blocks growth on the non-fermentable carbon sources (Fig. 1A). Wild type yeast has been shown to contain 95% iso-1-cytochrome c encoded by CYC1 gene and 5% iso-2-cytochrome c encoded by CYC7 (2). Western blot analysis of mitochondrial iso-2-cytochrome c expressed in the cyc1 null mutant is 12% of the total cytochrome c detected in the parental wild type strain (Fig. 1B). This value is 2-3 times higher than the 5% iso-2-cytochrome c reported previously. This could be due to a difference in the strains or carbon sources used in two studies. It is also possible that the absence of CYC1 results in an increased expression of iso-2-cytochrome c.

The spectral properties of the single and double null mutants are consistent with earlier studies showing that cyc1 and cyc7 mutants are able to synthesize COX while cyc1/cyc7 double mutants are totally deficient in the cytochrome $aa_3$ components of this respiratory complex (ref. 4, Fig. 2). The spectral properties of the mutants correlate with the results of enzymatic assays. Spectrophotometric and polarographic assays indicate a reduction of COX activity in the single cyc1 mutant and a complete absence of activity in the double mutant (Table II).
Most COX mutants display a decreased incorporation of radioactive precursors into Cox1p but not Cox2p or Cox3p when mitochondrial translation is measured in vivo in the presence of cycloheximide to inhibit cytoplasmic protein synthesis (Barrientos and Tzagoloff, unpublished). This characteristic is shared by the cyc1/cyc7 double mutant (Fig. 3). The in vivo assays also show that the absence of cytochrome $c$ does not significantly affect the stability of Cox2p and Cox3p during a 90 min chase period. The double mutant also displays greatly reduced steady-state levels of the mitochondrially translated Cox1p and Cox2p as reported previously (ref. 4. and Fig.4). Although there is some reduction in Cox3p and some of the imported subunits (Cox4p and Cox5p), these constituents appear to be more stable (Fig. 4).

**Phenotype of a cyc1/cyc7 double mutant expressing iso-1-cytochrome c with a W65S mutation** - Several mutant alleles of cyc1 were previously shown to express catalytically inactive forms of iso-1-cytochrome $c$ (4). One such allele (cyc1-166), coding for a W65S amino acid substitution, produces a protein that is stable at 24°C but is unable to mediate electron transfer from the $bc_1$ complex to COX (8). These properties of iso-1-cytochrome $c$ with the W65S mutation made it possible to examine if COX assembly depends on a redox active protein.

A gene with the cyc1-166 mutation was made by PCR amplification of the appropriate coding region with the mutation in one of the primers. The mutant gene was integrated into the chromosomal DNA of the cyc1/cyc7 double mutant by targeted insertion at the leu2 locus (W303ΔCYC1,7/ST11). The mutant was also transformed with the gene on a high copy plasmid (W303ΔCYC1,7/ST12). Even though mitochondria from both transformants had immunochemically detectable cytochrome $c$ (Fig. 1B), neither the multicopy nor the integrated mutant gene was able to rescue the growth defect of the cyc1/cyc7 mutant strain on glycerol at either 24°C or 30°C (Fig. 1A). Western blot analysis of mitochondria from the transformant with the integrated mutant gene indicated that the level of iso-1-cytochrome $c$ is approximately 85% of the iso-1-cytochrome $c$ in wild type (Fig. 1). The mitochondrial concentration of iso-1-cytochrome $c$ was somewhat lower in the transformant with the gene on a high-copy plasmid (68% of wild type). The failure of the W65S mutant protein to support growth on non-
fermentable carbon sources is in agreement with the previously noted deleterious effect of the mutation on the catalytic activity of iso-1-cytochrome c (8).

The *cyc1-166* mutant was reported to contain spectrally detectable cytochrome *c* but at lower concentrations than wild type yeast (8). This was also true of the *cyc1/cyc7* null strain transformed with the *cyc1-166* gene in the integrative or episomal plasmid (Fig. 2A). The spectra of mitochondria from both strains indicated only a partial restoration of cytochrome *c* (compare the double mutant with the ST11, ST12 transformants in Fig. 2A). The concentrations of cytochrome *c* in mitochondria of the transformant with the integrated mutant gene and of the wild type strain were determined from spectra of extracts obtained by sonic disruption of mitochondria in the presence of salt. Under these conditions most of the cytochrome *c* is rendered soluble while other cytochromes associated with the respiratory chain complexes remain in the membrane fraction (Fig. 2B). The concentrations of cytochrome *c* in wild type and the mutant mitochondria were estimated to be 0.32 and 0.08 nmoles/mg protein, respectively. The mutant iso-1-cytochrome *c*, represents only 25% of total cytochrome *c* in wild type. Since the amount of iso-2-cytochrome *c* in the mutant detected immunologically is 75% of wild type (Fig. 1B), only 30% of the W65S protein contains heme.

The status of COX in the *cyc1/cyc7* null strain harboring the *cyc1-166* allele was examined in several ways. Spectra of mitochondrial cytochromes indicated the presence of cytochromes *aa*$_3$ when the mutant protein was expressed either from the chromosomally integrated or plasmid-borne gene (Fig. 2). The presence of the integrated or episomal copy of the *cyc1-166* allele restored cytochrome *aa*$_3$ to more than 50% of the level seen in wild type mitochondria (Fig. 2A). The ability of iso-1-cytochrome *c* with the W65S mutation to rescue the COX deficiency of the *cyc1/cyc7* strain was confirmed by enzyme assays and by Western analysis of COX subunits proteins, which indicated that the steady-state levels of the mitochondrially encoded Cox1p, Cox2p, and Cox3p were restored to nearly wild type levels (compare Figs. 4 and Fig. 6C).

Two different assays were used to measure COX activity. The first relied on the reduction of endogenous cytochrome *c* in mitochondria by ascorbate in the presence of TMPD. Using this assay the *cyc1* null mutant had 40% of wild type COX, while no activity was detected in the *cyc1/cyc7* double mutant (Table II). Predictably, the double
mutant transformed with the *cyc1-166* gene was also completely inactive in catalyzing ascorbic acid oxidation by oxygen (Table II). When the assays were repeated in the presence of exogenous cytochrome *c*, the specific activity of COX in the *cyc1* mutant was raised to 70% of wild type, while the *cyc1/cyc7* strain with the integrated *cyc-166* gene was comparable to that of wild type (Table II). Similar results were obtained when the COX activity was assayed spectrophotometrically by measuring oxidation of substrate amounts of reduced cytochrome *c* (Table II). Surprisingly, the COX activity measured by both assays was lower in the transformant expressing the W65S protein from a multicopy plasmid. This was not true of the NADH-cytochrome *c* reductase activities, which were nearly the same in all the strains (Table II). The spectrum of mitochondrial cytochromes also showed a lower concentration of cytochromes *aa*<sub>3</sub> in the high copy transformant than in the strain with the integrated gene (Fig. 2).

The lower COX activity in the multicopy transformant could be explained by a kinetic block due to limited accessibility or exchange of the wild type substrate cytochrome *c* with the W65S mutant protein. This was tested by depletion of the iso-1-cytochrome *c* from mitochondria prior to the assay. Following sonic irradiation of wild type mitochondria in the presence of 1 M KCl, COX activity was reduced to 15% of the starting values (Table III). The specific activity returned to normal levels when the depleted mitochondria were assayed polarographically or spectrophotometrically in the presence of added cytochrome *c*. Under these conditions, however, the specific activity of mitochondria from the high copy transformant measured in the presence of cytochrome *c* was even lower after depletion (Table III). At present, therefore, the reason for the observed difference COX between the single and multicopy transformants is not clear.

*Only a fraction of the W65S mutant iso-1-cytochrome c is in a protease protected compartment of mitochondria*- As indicated, only 30% of the W65S mutant iso-1-cytochrome *c* in mitochondria of cells with the integrated *cyc1-166* allele contains heme. The intra-mitochondrial location of the wild type and of the W65S mutant proteins was compared by testing their sensitivity to proteinase K in mitochondria and mitoplasts. Most of the iso-1-cytochrome *c* in the strain with the chromosomally integrated *cyc1-166*
allele was found to be susceptible to digestion by the protease in intact mitochondria (Fig. 5A). A small fraction corresponding to 10%, however, was in a proteinase K protected compartment. Even though all the protein sedimented with mitoplasts, it was completely sensitive to proteinase K. In contrast, cytochrome c in wild type mitochondria is digested by the protease only when they are converted to mitoplasts (Fig. 5B). Sco1p, an inner membrane protein previously shown to face the intermembrane space (22), cytochrome b$_2$, a soluble intermembrane marker, and α-ketoglutarate dehydrogenase, a soluble matrix protein showed the expected properties in the mitochondria and mitoplasts (Fig. 3B). These results indicate that some 90% of the mutant protein is associated with mitochondria in a manner that makes it accessible to proteinase K. Whether it is bound to the outer membrane or is only partially inserted into the intermembrane space has not been determined.

The small fraction of W65S protein resistant to proteinase K in mitochondria probably corresponds to mature protein located in the intermembrane space while the more abundant fraction digested by the protease, is probably mostly mutant apoprotein. Since transport of cytochrome c to the intermembrane space of mitochondria has been shown to be coupled to heme addition (23), the protease sensitivity of most of the W65S protein suggests notwithstanding the fact that the tryptophan at residue 65 is not covalently linked to heme, its replacement by a serine must reduce the efficiency of heme attachment to the apoprotein. Mutations in the cysteine ligands of heme have also been shown to impede apocytochrome c import into mitochondria in vitro (23). In these studies most of the mutant apocytochrome c was also found to cosediment with mitochondria even though it was not protected against the protease (23).

**Role of cytochrome c in stability of COX**- The absence of COX in cyc1/cyc7 mutants has been interpreted to indicate that cytochrome c plays a role in assembly of this respiratory complex (5). Alternatively, cytochrome c could protect COX against proteolysis. The partial instability of the W65S protein at 37°C (8) made it possible to examine the effect of cytochrome c on the half-life of COX. The wild type parental strain and the cyc1/cyc7 mutant with the chromosomally integrated cy1c-166 gene were grown to early stationary phase at 24°C in rich galactose medium. The cultures were
treated with cycloheximide to prevent synthesis of new proteins and were further incubated at 37°C for different times. Approximately 80% of the protein was degraded as early as 1 hour after the temperature switch. Longer times at 37°C led to progressively greater destruction with only 4% of the starting protein remaining after 14 hours incubation. Wild type cytochrome c was also reduced at the higher temperature, although the extent, even after overnight incubation, was much less (Fig. 6A).

Incubation of the mutant cells at 37°C caused greater than 90% loss of COX activity after 14 hours of incubation (Fig. 6A). The decrease in the specific activity was accompanied by a partial reduction in the cytochromes aa3 absorption bands (Fig. 6B). The loss of enzymatic activity, however, preceded the reduction in the cytochrome aa3 bands. For example, even though less than 25% of the starting activity remained after 4 hours at 37°C, the cytochromes aa3 peaks were only marginally reduced (Fig. 6A and 6B). Western analyses of COX subunits also indicated very partial losses of Cox1p, Cox2p, Cox3p and Cox5p during the first 4 hours at 37°C (Fig. 6C). The most significant change was a large decrease of Cox1p in the mutant after the overnight incubation. In the wild type strain COX was highly stable at 37°C, with more than 90% of enzyme and activity after 14 hr of incubation at the high temperature (Fig. 6A). Western analysis also failed to reveal any significant reductions in the COX subunits after different times at 37°C. The NADH-cytochrome c reductase activities decreased to approximately the same extent in the wild type and the mutant. At present it is not clear if this is caused by a destabilization of the dehydrogenase, the bc1 complex, or the coenzyme Q pool at 37°C.

The spectrum of mitochondria from mutant cells grown exponentially at 37°C, exhibited an almost complete absence of cytochromes aa3 similar to the cyc1,7 double mutant (Fig. 6D).

The marked decrease of COX activity in mutant cells exposed to 37°C for 4 hours even though cytochromes aa3 and steady-state concentrations of COX subunits are unaffected during this period, suggests some more subtle changes in the quaternary structure or some other aspect of the enzyme. This is also seen in the wild type strain but to a lesser degree. These results suggest that the main role of cytochrome c is in assembly but that it also contributes towards the stability of the enzyme. The latter role is especially evident after prolonged incubation at 37°C, which leads to turnover of Cox1p,
more extensive loss of cytochromes $aa_3$, and a virtually complete absence of enzyme activity (Fig. 6). The degradation of COX in mutant cells undergoing cytochrome $c$ degradation may be similar to the loss of COX induced by mitochondrial cytochrome $c$ release in wild type yeast committed to programmed cell death (24).

*Apocytochrome c does not promote COX assembly*— Apocytochrome $c$ is imported into the intermembrane space of mitochondria where it is matured by covalent attachment of protoheme (23, 25). This reaction, catalyzed by the heme lyase product of CYC3, fixes the mature heme protein in the intermembrane space. Apocytochrome $c$ exits from the intermembrane space when heme attachment is blocked as a result of mutations in the lyase. Hence cyc3 mutants like cyc1/cyc7 mutants are COX deficient (ref. 5, Fig 7A).

To test if apocytochrome $c$ is able to promote COX assembly, a cyc3 null mutant was transformed with CYC1 and CYC7 on high copy plasmids (ST13 and ST5 respectively. Both yeast transformants accumulate some apocytochrome $c$ (Fig. 7B). The concentration of apocytochrome $c$ in mitochondria of the cyc3 mutant transformed with the respective genes was lower than in wild type and was further reduced after treatment with proteinase K. The mitochondrial concentrations of iso-1 or iso-2-apocytochrome $c$ in the proteinase K protected compartment is comparable to the amount of iso-2-cytochrome $c$ present in the cyc1 mutant, which is able to express at least 70% of the normal amount of COX (Fig. 8). Neither of the two cyc3 transformants, however, contained COX either by spectral (Fig. 7A) or enzymatic criteria (not shown). Additionally, Western analysis of COX subunits indicated that despite the presence of apocytochrome $c$ in mitochondria of the two transformants there was no increase in the steady-state concentrations of Cox2p and Cox3p. These results indicate that apocytochrome $c$ is unable to promote COX assembly.

The present study shows that assembly of COX depends on the presence of cytochrome $c$ in mitochondria even when the latter is unable to function in electron transport. The requirement for cytochrome $c$, therefore, is not related to either reduction or oxidation of some group in a subunit or assembly intermediate of COX. We estimate that the molar concentration of cytochrome $c$ in mitochondria of wild type yeast is approximately the same as that of COX. Assembly of COX, therefore, does not depend
on stoichiometric concentration of cytochrome c. Iso-2-cytochrome c whose concentration in a cyc1 mutant is only 12% of the total amount of cytochrome c in wild type yeast is able to support the expression of 70% of normal amounts of COX. This lack of requirement for stoichiometry is also supported by the results obtained with the W65S mutant. Since the apoprotein cannot substitute for the mature cytochrome, the function of cytochrome c probably depends on a properly folded protein. This is consistent with a structural role in COX assembly. In addition to its requirement for assembly, the results of obtained with the W65S mutant exposed to 37°C indicate that cytochrome c also affects turnover of COX.
FOOTNOTES

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Abbreviations: PCR, polymerase chain reaction; TMPD; N,N,N',N'-tetramethyl-p-phenylenediamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DOC, potassium deoxycholate; PMSF, phenylmethylsulfonyl fluoride.
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Table I. Genotypes and sources of *S. cerevisiae* strains

| Strain            | Genotype                                                                 | Source               |
|-------------------|---------------------------------------------------------------------------|----------------------|
| W303-1A           | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1                                 | This study           |
| W303ΔCYC1         | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc1::URA3                     | This study           |
| W303ΔCYC1,7       | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc1Δ::URA3 cyc7Δ::TRP1          | This study           |
| W303ΔCYC1,7/ST7   | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc1Δ::URA3 cyc7Δ::TRP1 leu2::pCYC1/ST7 | This study           |
| W303ΔCYC1,7/ST11  | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc1Δ::URA3 cyc7Δ::TRP1 leu2::pCYC1/ST11 | This study           |
| W303ΔCYC1,7/ST7   | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc1Δ::URA3 cyc7Δ::TRP1          | This study           |
| W303ΔCYC1,7/ST12  | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc1Δ::URA3 cyc7Δ::TRP1 leu2::pCYC1/ST7 | This study           |
| aW303ΔCYC3        | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc3Δ::URA3                     | This study           |
| aW303ΔCYC3/ST13   | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc3Δ::URA3 + pCYC1/ST13        | This study           |
| W303ΔSCO1         | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 sco11::URA3                   | (9)                  |
| W303ΔCOX15        | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox15::HIS3                    | (10)                 |
| W303ΔMSS51        | ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 mss51::HIS3                   | (11)                 |

* Dr. Rodney Rothstein, Department of Development and Human Genetics, Columbia University.
Table II. Respiratory and enzymatic activities in cyc mutants and transformants

| Strain                  | Specific Activity                  |
|-------------------------|-----------------------------------|
|                         | Ascorbate-TMPD oxidation\(^1\)  |
|                         | - Cyt. c | + Cyt. c   |
|                         | Cyt. c - oxidase\(^2\)            |
|                         | NADH- cyt. c Reductase\(^3\)      |
| W303-1B                 | 0.50     | 1.06       | 5.08  | 2.74 |
| W303ΔCYC1               | 0.20     | 0.72       | 3.56  | 2.73 |
| W303ΔCYC1,7             | 0.00     | 0.00       | 0.00  | 2.49 |
| W303ΔCYC1,7/ST7         | 0.49     | 1.07       | 4.13  | 2.76 |
| W303ΔCYC1,7/ST11        | 0.00     | 1.01       | 4.41  | 2.47 |
| W303ΔCYC1,7/ST12        | 0.00     | 0.47       | 2.35  | 2.38 |

\(^1\) The specific activity is expressed as μmol O\(_2\) consumed per min per mg protein.

\(^2,3\) The specific activities are expressed as μmol of cytochrome c oxidized or reduced per min per mg protein. The assays were done with mitochondria solubilized in 0.2% potassium deoxycholate (DOC). All the values are the average of two independent assays.
Table III. Effect of cytochrome c depletion on the NADH-cytochrome c and COX activities of cyc mutants.

| Strain                  | Specific Activity |         |         |         |         |         |
|-------------------------|-------------------|---------|---------|---------|---------|---------|
|                         | Ascorbate-TMPD    | Cyt. c  | DOC     | +KCl, DOC | Cyt. c oxidase | NADH- cyt. c Reductase |
|                         | oxidation<sup>1</sup> |         |         |         |         |         |
| Intact mitochondria     |                   | - Cyt. c | + Cyt c | DOC     | +KCl, DOC |         |
| W303-1B                 | 0.41              | 0.85    | 6.48    | 6.47    | 2.53    |         |
| W303ΔCYC1               | 0.26              | 0.72    | 4.97    | 6.06    | 2.10    |         |
| W303ΔCYC1,7/ST11        | 0.00              | 0.78    | 4.03    | 1.80    | 2.32    |         |
| W303ΔCYC1, 7/ST12       | 0.00              | 0.38    | 2.94    | 1.22    | 2.04    |         |
| Cytochrome c depleted mitochondria |         |         |         |         |         |         |
| W303-1B                 | 0.06              | 0.84    | 6.30    | ND      | ND      |         |
| W303ΔCYC1               | 0.04              | 0.77    | 5.57    | ND      | ND      |         |
| W303ΔCYC1,7/ST11        | 0.00              | 0.71    | 3.73    | ND      | ND      |         |
| W303ΔCYC1,7/ST12        | 0.00              | 0.16    | 0.91    | ND      | ND      |         |

Enzyme activities were measured in intact isolated mitochondria and in mitochondria depleted of cytochrome c. The values reported are the average of two independent assays. ND, not determined.

<sup>1</sup> Specific activity is expressed as µmol O<sub>2</sub> consumed per min per mg protein.

<sup>2,3</sup> Specific activities are expressed as µmol of cytochrome c oxidized or reduced per min per mg protein. Cytochrome c oxidase and NADH-cytochrome c reductase were measured in mitochondria solubilized in 0.2% potassium deoxycholate (DOC) in the absence or presence of 0.5M KCl.
LEGENDS TO FIGURES

Fig. 1. Growth properties of mutant and transformants. A. Serial dilutions of the indicated strains were plated on rich glucose (YPD) medium and incubated at 30°C for 1 day. The same dilutions were also plated on rich glycerol, ethanol (YPEG) medium and incubated at 24°C and 30°C for 2 days. W303-1B (W303), the respiratory competent parental strain; W303ΔCYC1 (ΔCYC1), a cyc1 null mutant; W303ΔCYC1,7 (ΔCYC1,7), a cyc1/cyc7 double mutant: W303ΔCYC1,7 (ΔCYC1,7/ST7), the double mutant with the wild type CYC1 gene integrated in chromosomal DNA; W303ΔCYC1,7 (ΔCYC1,7/ST11), the double mutant with the cyc1-166 mutant gene integrated in chromosomal DNA; W303ΔCYC1,7 (ΔCYC1,7/ST12); the double mutant transformed with the cyc1-166 mutant gene on a high copy plasmid. B. Mitochondria (20 µg protein) were separated SDS-PAGE on a 12% polyacrylamide gel and were transferred to nitrocellulose. The Western blot was probed with an antibody against yeast cytochrome c and the antibody-antigen complexes were visualized with the SuperSignal chemiluminescent substrate kit (Pierce, Rockford, IL). The sources of mitochondria are the same as in panel A. The two additional strains are: W303ΔCYC1,7/ST7 and W303ΔCYC1,7/ST13, the cyc1/cyc7 double mutant with the wild type CYC1 gene integrated at the leu2 locus and on a multicopy plasmid, respectively. The AU values are the relative absorbance units obtained from a duplicate Western in which the bands were visualized by a secondary reaction with ¹²⁵I-protein A and exposed in a PhosphorImager (Molecular Dynamics).

Fig. 2. Spectra of mitochondrial cytochromes. A. Mitochondria were prepared from the following strains grown at 24°C on rich galactose medium (YPGal): W303ΔCYC1 (ΔCYC1), the cyc1 null mutant; W303ΔCYC1,7 (ΔCYC1,7), the cyc1, cyc7 double mutant; W303ΔCYC1,7 (ΔCYC1,7/ST11), the double mutant with the cyc1-166 gene integrated in chromosomal DNA; W303ΔCYC1,7 (ΔCYC1,7/ST12);
the double mutant transformed with the *cyc1-166* gene on a high copy plasmid. The respiratory competent parental W303-1B (W303) strain was grown at 30°C. Mitochondrial cytochromes were extracted with potassium deoxycholate at a final concentration of 5 mg protein/ml as described previously (20). Difference spectra of the reduced (sodium dithionite) versus oxidized (potassium ferricyanide) extracts were recorded at room temperature. The α absorption bands corresponding to cytochromes a and a₃ have maxima at 603 nm (a), of cytochrome b (b) at 560 nm and of cytochrome c and c₁ (c) at 550 nm. B. Mitochondria obtained from the wild type strains W303-1B (W303) and the mutant W303ΔCYC1,7/ST11 (ΔCYC1,7/ST11) were sonically irradiated in the presence of 1 M KCl at a protein concentration of 13.2 mg/ml. The suspensions were centrifuged at 230,000 x gav for 15 min. The supernatants were collected and difference spectra of the reduced versus oxidized extract were recorded.

Fig. 3. *In vivo* synthesis of COX subunits in mutants and transformants. Synthesis of mitochondrial translation products was assayed in the following strains: W303-1B (W303), the respiratory competent parental strain; W303ΔCYC1 (ΔCYC1), the *cyc1* null mutant; W303ΔCYC1,7 (ΔCYC1,7), the *cyc1*, *cyc7* double mutant; W303ΔCYC1,7 (ΔCYC1,7/ST7), the double mutant with the wild type *CYC1* gene integrated in chromosomal DNA; W303ΔCYC1,7 (ΔCYC1,7/ST11), the double mutant with the *cyc1-166* mutant gene integrated in chromosomal DNA. Incorporation of ²⁵⁵S-methionine into the mitochondrial translation products was allowed to proceed for 20 min at 30°C as described previously (11). Excess 80 mM cold methionine and 4 μg/ml puromycin were added (0 time) and samples taken after 30 and 90 min of chase. Equivalent amounts of total cellular proteins were separated by SDS-PAGE on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and exposed to X-ray film. The mitochondrially translated ribosomal protein Var1p, subunits 1 (Cox1p), subunit 2 (Cox2p) and 3 (Cox3p) of COX, cytochrome b (Cyt. b), and subunit 6 (Atp6p) and subunit 8 and 9 (Atp8/9) of the oligomycin-sensitive ATPase are identified in the margin.
Fig. 4. Steady-state levels of COX subunits in mutants and transformants. Mitochondria were prepared from the following strains grown at 24°C on rich galactose medium (YPGal): W303-1B (W303), the wild type parental strain; W303ΔCYC1 (ΔCYC1), the cyc1 null mutant; W303ΔCYC1,7 (ΔCYC1,7), the cyc1, cyc7 double mutant; W303ΔCYC1,7/ST7 (ΔCYC1,7/ST7), the double mutant with a chromosomally integrated copy of CYC1, W303ΔSCO1 (ΔSCO1), a sco1 null mutant; W303ΔCOX15 (ΔCOX15), a cox15 null mutant; W303ΔMSS51 (ΔMSS51), a mss51 null mutant. Total mitochondrial proteins (20 µg for detection of cytochrome c and 40 µg for detection of COX subunits) were separated by SDS-PAGE electrophoresis on a 12% polyacrylamide gel. The proteins were transferred to nitrocellulose and reacted with antibodies to cytochrome c (Cyt. c) and COX subunits 1 (Cox1p), 2 (Cox2p), 3 (Cox3p), 4 (Cox4p), and 5 (Cox5p). The antibody-antigen complexes were visualized with the Super Signal kit.

Fig. 5. Cytochrome c accessibility to proteinase K. Mitochondria were prepared by the method of Glick and Pon (21) from wild type (W303) and the cyc1-166 mutant W303ΔCYC1,7/ST11 (ΔCYC1,7/ST11) grown in YPGal to early stationary phase at 24°C. The mitochondria (Mt) and mitoplasts (Mp) were incubated in the presence of 100 µg/ml proteinase K for 60 min on ice. The reaction was stopped by addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM and the mitochondria and mitoplasts were recovered by centrifugation at 100,000 × g av. The pellets were suspended in, 0.6 M sorbitol, 20 mM Heps, pH 7.5 and proteins were precipitated by addition of 0.1 volume of 50% trichloroacetic acid and heated for 10 min at 65°C. Mitochondrial and mitoplast proteins from wild type and the transformant (40 µg) were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose and probed with antibody against Sco1p, α-ketoglutarate dehydrogenase (αKGD), cytochrome b2 (Cyt b2), and cytochrome c (Cyt. c). Proteins were visualized with the Super Signal kit.
Fig. 6. Cytochrome \( c \) and COX stability in wild type and mutant cells at 37\(^\circ\)C. A. The wild type W303-1B (W303) and the \( \text{cyc1-166} \) mutant W303\( \Delta \text{CYC1,7/ST11} \) (\( \Delta \text{CYC1,7/ST11} \)) were grown to early stationary phase at 24\(^\circ\)C in YPGal. The cultures were adjusted to 2 \( \times 10^{-5} \) M cycloheximide and 1/4 of each culture was harvested (0 time). Equal volumes of the remaining 3/4 of the cultures were collected after for 1, 4, and 14 hours incubation at 37\(^\circ\)C. Mitochondria were prepared and assayed for COX (dashed bars) and NADH cytochrome \( c \) reductase (open bars). The relative amount of cytochrome \( c \) (solid bars) in mitochondria was determined from Western blots as in Fig. 1B. Mitochondrial cytochrome spectra were obtained as in Fig. 2, except that the protein concentration was 4 mg/ml during the extraction. C. The steady-state levels of COX subunits 1 (Cox1p), 2 (Cox2p), 5 (Cox5p), and cytochrome \( c \) were analyzed as in Fig. 4. The COX subunits were visualized with the SuperSignal substrate kit (Pierce, Rockford, IL). Cytochrome \( c \) was visualized by a secondary reaction with \( ^{125}\text{I} \)-protein. The values of cytochrome \( c \) shown in the bar graph in part A were obtained from this gel after quantification in a PhosphorImager (Molecular Dynamics). D. The \( \text{cyc1-166} \) mutant W303\( \Delta \text{CYC1,7/ST11} \) was grown in YPGal at 24\(^\circ\)C and 37\(^\circ\)C to early stationary phase. Mitochondria were prepared and extracted at a protein concentration of 5 mg/ml. The spectra were recorded as in B.

Fig. 7. Phenotype of \( \text{cyc3} \) mutants and transformants. Mitochondria were prepared from the \( \text{cyc3} \) null mutant \( \text{aW303ACYC3} \) (\( \Delta \text{CYC3} \)), from \( \text{aW303ACYC3/ST5} \) (\( \Delta \text{CYC3/ST5} \)), the mutant transformed with \( \text{CYC7} \) on a high copy plasmid, and \( \text{aW303ACYC3/ST13} \) (\( \Delta \text{CYC3/ST13} \)), the mutant transformed with \( \text{CYC1} \) on a high copy plasmid. A. Mitochondrial cytochromes were extracted and spectra recorded as described in the legend to Fig. 2. B. Total mitochondrial proteins (\( 20 \mu g \) for detection of cytochrome \( c \) and \( 40 \mu g \) for detection of COX subunits) were separated by SDS-PAGE on a 12\% polyacrylamide gel. Following transfer to nitrocellulose the Western blot was probed with antibodies to COX subunits 2 (Cox2p), 3 (Cox3p), and 5 (Cox5p), and to cytochrome \( c \) (Cyt. \( c \)). The antibody-antigen complexes were visualized as described in the legend to Fig. 1.
Fig. 8. Apocytochrome c in mitochondria of cyc3 mutants transformed with CYC1 and CYC7 on high copy plasmids. Mitochondria were prepared by the method of Glick and Pon (21) from the cyc1-l66 mutant W303ΔCYC1,7/ST11 (ΔCYC1,7/ST11) grown in YPGal to early stationary phase at 24°C. Mitochondria were also prepared from the cyc1 null mutant W303ΔCYC1 (ΔCYC1) and aW303ΔCYC3/ST5 (ΔCYC3/ST5) and aW303ΔCYC3/ST13 (ΔCYC3/ST13), the cyc3 null mutant transformed with CYC7 and CYC1, respectively on high copy plasmids. The mitochondria were incubated in the presence of 100 µg/ml proteinase K for 60 min on ice. The digestions were stopped with PMSF and further treated as described in the legend to Fig. 5. Proteinase K treated and untreated samples (50 µg protein) were separated by SDS-PAGE, transferred to nitrocellulose and treated with antiserum to cytochrome c followed by a secondary reaction with 125I-protein A. The relative amounts of cytochrome c were quantitated in a PhosphorImager (Molecular Dynamics). AU, arbitrary absorbance units.
Barrientos et al., Fig 3
Barrientos et al., Fig 4
Barrientos et al., Fig. 5

**W303**

| Prot. K | Mt  | Mp  |
|---------|-----|-----|
| -       | +   | -   |

- α-KGD
- Cyt. b₂
- Sco1p
- Cyt. c

**ΔCYC1,7/ST11**

| Prot. K | Mt  | Mp  |
|---------|-----|-----|
| -       | +   | -   |

- α-KGD
- Cyt. b₂
- Sco1p
- Cyt. c
Barrientos et al, Fig. 7

A

\[ A = 0.01 \]

\( \Delta \text{CYC3} \)

\( \Delta \text{CYC3/ST5} \)

\( \Delta \text{CYC3/ST13} \)

nm

500 550 600 650

B

W303  \( \Delta \text{CYC3} \)  \( \Delta \text{CYC3/ST5} \)  \( \Delta \text{CYC3/ST13} \)

Cyt.c

Cox2p

Cox3p

Cox5p
Barrientos et al., Fig 8

|          | ΔCYC1,7/ST11 | ΔCYC1 | ΔCYC3/ST5 | ΔCYC3/ST13 |
|----------|--------------|-------|-----------|------------|
| PK       | -            | +     | -         | +          |
| AU       | 114          | 11    | 3         | 4          |

Cyt. c
Cytochrome oxidase assembly does not require catalytically active cytochrome c
Antoni Barrientos, Danielle Pierre, Johnson Lee and Alexander Tzagoloff

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Cytochrome oxidase assembly does not require catalytically active cytochrome c.

Antoni Barrientos, Danielle Pierre, Johnson Lee, and Alexander Tzagoloff

The following errors escaped our notice and may have caused problems in the interpretation of the results presented in the paper.

Page 8885, 1st paragraph, left column, line 4: “upper panel” should be “lower panel.”

Page 8885, 1st paragraph, left column, line 10: “lower panel” should be “upper panel.”

Legend to Fig. 6, line 8: “COX (dashed bars)” should be “COX (open bars).”

Legend to Fig. 6, line 9: “reductase (open bars)” should be “reductase (dashed bars).”

Page 8886, 1st paragraph, left column, lines 1 and 2: Fig. 3B should be Fig. 5.

Vol. 278 (2003) 16176–16182

Polymerization of calsequestrin. Implications for Ca\(^{2+}\) regulation.

HaJeung Park, Si Wu, A. Keith Dunker, and ChulHee Kang

Page 16178, left column, line 11: ΔC27 should be changed to ΔN2.

Page 16178, left column, line 30: 100 mg/ml should be changed to 100 μg/ml.

Vol. 278 (2003) 30813–30820

Voltage dependence of the Ca\(^{2+}\)-activated cation channel TRPM4.

Bernd Nilius, Jean Preenen, Guy Droogmans, Thomas Voets, Rudi Vennekens, Marc Freichel, Ulrich Wissenbach, and Veit Flockerzi

Page 30820, lines 13 and 17: The citation should be (12), instead of (6).