Role of Arg-166 in Yeast Cytochrome c$_1^*$

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A systematic screen for dominant-negative mutations of the CYT1 gene, which encodes cytochrome c$_1$, revealed seven mutants after testing $-10^4$ Saccharomyces cerevisiae strains transformed with a library of mutagenized multicopy plasmids. DNA sequence analysis revealed multiple nucleotide substitutions with six of the seven altered Cyt1p having a common R166G replacement, either by itself or accompanied with other amino acid replacements. A single R166G replacement produced by site-directed mutagenesis demonstrated that this change produced a nearly nonfunctional cytochrome c$_1$, with diminished growth on glycerol medium and diminished respiration but with the normal or near normal level of cytochrome c$_1$ having an attached heme group. In contrast, R166K, R166M, or R166L replacements resulted in normal or near normal function. Arg-166 is conserved in all cytochromes c$_1$ and lies on the surface of Cyt1p in close proximity to the heme group but does not seem to interact directly with any of the physiological partners of the cytochrome bc$_1$ complex. Thus, the large size of the side chain at position 166 is critical for the function of cytochrome c$_1$ but not for its assembly in the cytochrome bc$_1$ complex.

The cytochrome bc$_1$ complex, also known as complex III of the respiratory chain or ubiquinol:cytochrome c oxidoreductase, is an oligomeric complex found in the inner mitochondrial membrane of eukaryotes and in the plasma membrane of bacteria (1–4). This complex transfers electrons from ubiquinol to cytochrome c and couples this transfer to a proton gradient across the inner mitochondrial or bacterial plasma membrane by a mechanism known as the proton motive Q cycle (2, 5–7). The prokaryotic and eukaryotic cytochrome bc$_1$ complexes contain three essential catalytic subunits having the following characteristic prosthetic groups: cytochrome b with two b-type hemes; cytochrome c$_1$ with a c-type heme; and the so-called Rieske protein that contains a high potential [2Fe-2S] cluster.

In addition to the three catalytic subunits, mitochondrial cytochrome bc$_1$ complexes from vertebrates (8) and the yeast Saccharomyces cerevisiae (9, 10) contain eight and seven additional subunits, respectively. X-ray crystallographic atomic structures have been determined for the soluble fragment of the Rieske protein at 1.5-Å resolution (11) and for entire mitochondrial cytochrome bc$_1$ complexes from bovine, chicken (12–14), and S. cerevisiae (15) at 2–3-Å resolution. The cytochrome bc$_1$ complexes are dimers with each monomer of the yeast complex consisting of the following 10 protein subunits (with the orthologous bovine subunits or synonyms shown in parentheses): Cyt1p (cytochrome c$_1$); COB (cytochrome b); Rip1p (ISP, Rieske protein); Cor1p (Core 1, SU1); Qcr2p (Core 2, SU2); Qcr6p (SU8); Qcr7p (SU6); Qcr8p (SU7); Qcr9p (SU10); and Qcr10p (SU11). In addition, the subunit SU9 is present in the vertebrate but not in the yeast cytochrome bc$_1$ complex. Furthermore, the protein prepared for determining the atomic structure of the yeast complex lacked Qcr10p (15). (The overall structure and components of the bovine cytochrome bc$_1$ complex are presented in Fig. 1.) SU11 of the bovine complex, orthologous to Qcr10p, forms a transmembrane helix that is bound on the outside of the complex to the helices of the Rieske protein and SU10 (Qcr9p) (14). Removal of SU10 did not affect enzymatic activity (8), but it may be important for the correct assembly of the complex (10). The similar overall structure of the yeast compared with the vertebrate complexes suggests that Qcr9p is associated with the complex in the same way (15). Furthermore, the relative positions and orientations of heme groups and the distances between the iron positions indicate that the yeast and vertebrate complexes are essentially the same, although the exact position, length, and conformation of connecting loops varied (15).

Cytochrome c$_1$ is responsible directly for the electron transfer reaction with cytochrome c by catalyzing the oxidation of ubiquinol and reduction of cytochrome c (16, 17). Like all other physiological partners, cytochromes c and c$_1$ interact with each other through electrostatic forces (18).

Although cytochrome c$_1$ is a mitochondrial protein, it is encoded by a nuclear gene, CYT1, translated in the cytosol, and subsequently imported into mitochondria, a process involving cleavage of a leader sequence (19, 20). In yeast, the cleavage of a 61-amino acid amino-terminal region from the 309-amino acid-long precursor results in a 248-amino acid-long mature form.

We have undertaken a genetic investigation of the functional requirements of amino acid residues of yeast cytochrome c$_1$ and of possible critical interactions between cytochromes c$_1$ and c. Although mutational analysis of cytochromes c$_1$ from yeast (17, 21–23) and Rhodobacter sphaeroides and Rhodobacter capsulatus (7, 24–27) has been used in several studies, we have elected to isolate and characterize dominant-negative mutants. The characterization of altered cytochromes c$_1$ generated by “random” mutagenesis may reveal functional requirements that are difficult to predict even with a detailed knowledge of the structure of the protein. However, the vast majority of nonfunctional proteins generated by random mutagenesis are

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1 S. L. Hatch, D. A. Pearce, F. Sherman, and G. McLendon, unpublished result.
defective for trivial reasons such as missense mutations that affect folding or assembly and nonsense mutations that produce truncated proteins. On the other hand, interesting nonfunctional but stable proteins can be detected by the dominant-negative genetic test (28). For example, if the overexpression of a cyt1-x mutation inhibits the function of the normal CYT1+ chromosomal gene, then the cyt1-x allele most likely encodes a nonfunctional cytochrome c1, which is competing with the normal form.

The systematic screen for dominant-negative mutations of the CYT1 gene carried out in this study revealed that the R166G replacement caused a nearly nonfunctional cytochrome c1, with diminished growth on glycerol medium and diminished respiration but with the normal or near normal level of cytochrome c1 having an attached heme group. Furthermore, the small size of the side chain was responsible for the defect because R166K, R166M, or R166L replacements resulted in normal or near normal function of cytochromes c1.

**EXPERIMENTAL PROCEDURES**

**Numbering of Amino Acid Positions of Cytochrome c1**—The amino acid positions of yeast cytochrome c1 are assigned in this paper according to the full-length precursor having 309 amino acid residues. For example, Arg-166 of yeast cytochrome c1 corresponds to Arg-102 of bovine cytochrome c1.

**Media and General Methods**—Standard YPD (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose) and synthetic media (Standard YPD 2 (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% (v/v) glycerol) and YPG (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% (v/v) glycerol)) and synthetic media constituents were obtained from Difco or Roche Molecular Biochemicals. All other chemicals used were from Sigma. Agarose was purchased from Roche Molecular Biochemicals.

DNA sequencing of segments containing CYT1 was carried out with the oligonucleotides OL.ZA01–OL.ZA04 (Table I) using the ABI PRISM dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaQ DNA polymerase (Big Dye).

**Yeast Strains and Plasmids**—The yeast strains used in this study along with their complete and partial genotypes are presented in Table II. The normal CYT1 strain B-7553 described by Dumont et al. (34) served as the parental strain for generating the cyt1-Δ::TRP1 mutant B-9737 by the one-step gene replacement procedure. A 1.1-kilobase KpnI-SpeI segment containing the CYT1 gene in the plasmid pAB1192 was replaced with an 829-base pair KpnI-SpeI segment containing the TRP1 gene, resulting in a plasmid denoted pAB1193. B-7553 was transformed with a fragment from pAB1193 encompassing cyt1-Δ::TRP1, and the desired disruptant was confirmed by PCR analysis.

The plasmids used in this study are listed in Table III, and some are described below.

**Dominant-negative Mutants**—A library of 1.5-kilobase CYT1 segments containing BamHI and SacI sites and random alterations was generated from pAB1097 by error-prone PCR with oligonucleotides OL.ZA01 and OL.ZA03. PCRs were carried out separately with 0.05, 0.10, 0.15, and 0.20 mM MnCl2. All reactions contained the appropriate amounts of DNA (200–600 ng), MgCl2 (1.5–6.0 mM), oligos (4.0–6.0 pmol), dNTPs, buffer, and AmpliTaq DNA polymerase. The libraries of PCR segments were inserted in pAB1198, and the resulting plasmids were amplified in XL1-Blue.

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**Table I**

| Oligonucleotide number | Sequence |
|------------------------|----------|
| OL.ZA01                | GGGGGTCGGGATCCATAGACTAATCTAACG |
| OL.ZA02                | GGGGGTCGGGATCCATAGACTAATCTAACG |
| OL.ZA03                | GGGGGTCGGGATCCATAGACTAATCTAACG |
| OL.ZA04                | GGTATCCCGATCGCATGATGATGATGATGAC |
| OL.ZA05                | GGTATCCCGATCGCATGATGATGATGATGAC |
| OL.ZA06                | GCCGAGCTATGGGGCCGGCAGGGACAC |
| OL.ZA07                | ACGAAGCAGAACAGGCTGCAATTGGCTGCAATACAAAGT |
| OL.ZA08                | ACGAAGCAGAACAGGCTGCAATTGGCTGCAATACAAAGT |
| OL.ZA09                | ACGAAGCAGAACAGGCTGCAATTGGCTGCAATACAAAGT |
| OL.ZA10                | ACGAAGCAGAACAGGCTGCAATTGGCTGCAATACAAAGT |
| OL.ZA14                | TCCATTGCAATGGCTAAGGCTTGTAGTGAAC |

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2 The abbreviations used are: YPD, yeast extract peptone dextrose medium; YPG, yeast extract peptone glycerol medium; PCR, polymerase chain reaction.
ent error-prone PCR conditions, were pooled and used to transform the B-7553 strain; the resulting transformants were plated on SD–uracil plates. Approximately 104 colonies from the SD–uracil plates were replica-plated on SG–uracil, SD–uracil, YPG, and YPD plates for the detection of dominant-negative mutants. A transformant was considered to be a dominant-negative mutant if it exhibited diminished growth on SG–uracil, if the corresponding Ura+ strain lacking the plasmid had normal growth on YPG, and if reintroduction of the plasmid in pAB1198 again resulted in a transformant with diminished growth.

**Oligonucleotide-directed Mutagenesis—**Oligonucleotide-directed mutagenesis was carried out by the procedure described by Kunkel et al. (37) using the plasmid pAB2306 (Table III), E. coli strain C3236 (dut1 ung1 thi1 relA1/pCJ105(CmR)) (38), and the oligonucleotides OL.205–OL.214 (Table I). The E. coli strain XL1-Blue (supE44 hsdR17 recA1 endA1 gyrA8 thi1 relA lac−) was used for the amplification and storage of plasmids. The site-directed change was confirmed by DNA sequencing of the CYT1 region.

**Low Temperature Spectroscopic and Spectrophotometric Analysis of Intact Cells—**The yeast strains were grown on the surface of YP1%S (1% Bacto-yeast extract, 2% Bacto-peptone, and 1% sucrose) plates at 23 °C for 4 days, 30 °C for 3 days, or 37 °C for 2 days, which are slightly modified conditions of our standard procedure (39). The levels of cytochromes c1 and c2 were estimated in intact cells at −196 °C by spectroscopic visual examination (40) and by absorbance recordings using an Aviv model 14 spectrophotometer as described by Hickey et al. (41).

**Rates of Respiration—**Oxygen uptake was measured polarographically with a commercially available Teflon-covered Clark electrode, the Yellow Stone Instruments oxygen monitor (Yellow Springs, OH), as described previously (42), using 3-mL solutions of 44 mM KH2PO4, 1 mM glucose, and various amounts of washed yeast cells obtained from cultures grown to late stationary phase in YPD medium. [QO2] is expressed as microliters of oxygen consumed per hour per milligram of yeast, dry weight.

### Yeast Cytochrome c1

#### TABLE II

**Yeast strains**

| Strain number | Complete genotype |
|---------------|-------------------|
| B-7553        | MATa CYC1 cyg-5Δ:CYH2 cyh2 leu2−3,112,12 his3Δ102 trp1-289 can1−100 CYT1 |
| B-9737        | MATa CYC1 cyg-5Δ:CYH2 cyh2 leu2−3,112,12 his3Δ102 trp1-289 can1−100 cyg1Δ:TRP1 |

#### TABLE III

**Description of plasmids**

| Plasmid number | Description |
|----------------|-------------|
| pAA625         | Also designated pRS116; a 4.9-kb CEN6 URA3 plasmid having BamHI and SalI cloning sites (35) |
| pAB1097        | Also designated pMH101; A 5.9-kb plasmid derived from pUC13 having a 3.2-kb BamHI-SalI segment with CYT1 (21) |
| pAB1198        | Also designated YEp436; a 13.3-kb 2 μ plasmid having LEU2-d and URA3 markers (36) |
| pAB2306        | pAA625 (CEN6 URA3) with a 3.2-kb segment having CYT1 inserted at BamHI and SalI cloning sites; used for generating site-directed mutants |
| pAB2580        | pAB1198 (2 μ URA3) having a PCR-generated 1.5-kb segment with CYT1 inserted at BamHI and SalI cloning sites; used for generating dominant-negative mutants |
| pAB2657–pAB2663 | Domain-negative mutants (Table V) |
| pAB2665–pAB2672 | Site-directed mutants (Table VI) |

* kb, kilobase.

#### TABLE IV

**Growth of dominant-negative (B-13412–B-13418) and site-directed (B-13445–B-13452) mutants**

| Strain number | YPD | YPG | SD – uracil | SG – uracil |
|---------------|-----|-----|-------------|-------------|
|               | 22 °C | 30 °C | 37 °C | 22 °C | 30 °C | 37 °C | 22 °C | 30 °C | 37 °C |
| B-7553        | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-12705       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-12707       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13412       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13413       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13414       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13415       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13416       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13417       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13418       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-9737        | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13435       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13436       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13445       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13446       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13447       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13448       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13449       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13450       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13451       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| B-13452       | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
RESULTS AND DISCUSSION

Dominant-negative Mutants—We undertook an extensive screen for dominant-negative mutations of the CYT1 gene that encodes cytochrome \( c_1 \) with the aim of identifying amino acid residues that are critical for function but not for stability or incorporation into the cytochrome \( bc_1 \) complex. For example, residues that are on the surface of the complex and are required for interaction with their physiological partners would be expected to be revealed with dominant-negative mutations. Because such altered cytochromes \( c_1 \) are expected to be stable, they should be particularly amenable to biochemical studies.

In this study, we have used a library of multicopy plasmids containing a CYT1 segment that was mutated randomly by error-prone PCR. The plasmids, which are derivatives of pAB1198 (Table III), are maintained at a high copy number in the strain B-7555 (Table II) because of the URA3 marker and the 2 \( \mu \) origin of replication. In addition, the LEU2-d markers can be used to produce an even higher copy number on medium lacking leucine.

The screen is based on the lack of utilizing a nonfermentable carbon source, glycerol, because of competition of an altered nonfunctional form for the wild-type cytochrome \( c_1 \). Because manifestation of dominant-negative mutations depends on the presence of the plasmid, the desired mutants can be differentiated conveniently from other glycerol-negative mutants such as commonly occurring \( p^- \) mutations.

Approximately \( 10^4 \) transformants containing the library of mutagenized plasmids were screened for diminished growth on synthetic medium lacking uracil in a plasmid-dependent manner. A total of 181 colonies with some degree of diminished growth was uncovered, but only 12 were almost completely negative. The CYT1 region of seven of these was subjected to DNA sequencing. The seven strains were designated B-13412–B-13418; the corresponding plasmids were designated pAB2657–pAB2663; and the corresponding altered alleles were designated cyt1-101–cyt1-107 (Tables IV and V).

The growth of the strains under various conditions is presented in Table IV, and as an example, the growth of B-13415 on SG medium is presented in Fig. 2.

The sequences of the CYT1 region of the pAB2657–pAB2663 plasmids, presented in Table V, revealed multiple base pair substitutions with many of the changes common to more than one plasmid. The two plasmids pAB2658 and pAB2659 were identical, and all seven plasmids contained the neutral change L36L (CTC \( \rightarrow \) CTC) and the radical change R166G (AGA \( \rightarrow \) GGA), respectively. The plasmid pAB2663 contained 14 base pair substitutions including the formation of a UAG nonsense codon at amino acid position 227. The multiple and common base pair substitutions suggest that the altered PCR products may be related clonally, a result that would be expected if the DNA fragments were derived from common molecules because of low amounts of starting material. Thus, it is unclear which if any of the multiple directed changes occurred independently.
Yeast Cytochrome c₁

Properties of mutants constructed by site-directed mutagenesis

| Strain number | Plasmid | Allele | Oligonucleotide | Codon change | Amino acid changes | Cytochrome c₁ | Growth on glycerol medium | Qₒ₂ |
|---------------|---------|--------|-----------------|--------------|-------------------|--------------|-------------------------|-----|
| B-9737        | pAB625  | No insert |                 |              | 0 0              | 0 0        | R166G                  | 0.5 |
| B-13436       | pAB2306 | CYTI   |                 |              | 0 0              | R166L       | + +                     | 8.9 |
| B-13445       | pAB2254 | cyt1-201| OL.Z07          | AGA → GGA    | 0 0              | R166M       | + +                     | 50.4|
| B-13446       | pAB2266 | cyt1-202| OL.Z08          | AGA → TCG    | 0 0              | R166K       | + +                     | 5.53|
| B-13447       | pAB2267 | cyt1-203| OL.Z09          | AGA → ATG    | + +              | R166K       | + +                     | 5.53|
| B-13448       | pAB2268 | cyt1-204| OL.Z10          | AGA → AAA    | + +              | R166K       | + +                     | 5.53|
| B-13449       | pAB2269 | cyt1-205| OL.Z06          | ACC → GCC    | + +              | R166K       | + +                     | 5.53|
| B-13450       | pAB2270 | cyt1-206| OL.Z05          | TCG → TGG    | + +              | R166K       | + +                     | 5.53|
| B-13451       | pAB2271 | cyt1-207| OL.Z06, OL.Z05  | TCG → TGG, ACC → GCC | S49L, T63A | + +                     | 5.53|
| B-13452       | pAB2272 | cyt1-208| OL.Z14          | AGA → TAG    | R227End           | 0 +         |                          |     |

The results clearly established that at least the R166G replacement was responsible for the dominant-negative phenotype. This amino acid replacement occurred in six of the seven sequenced plasmids, pAB2657–pAB2662, and was the only amino acid change in the two plasmids pAB2660 and pAB2661. Furthermore, the only plasmid lacking the R166G replacement, pAB2663, contained the UAG nonsense mutation at amino acid position 227. The drastic nature of premature chain termination suggests that the R227End change in pAB2663 is responsible for the dominant-negative effect.

Site-directed Mutants—To confirm and extend these findings and to determine whether other replacements may confer a dominant-negative phenotype, the following changes were introduced in the single-copy CEN6 plasmid by oligonucleotide-directed mutagenesis (Table VI): R166G; R166M; R166L; R166K; S49L; T63A; S49L and T63A; and R227End. Strain B-9737 (cyt1-Δ ura3) was transformed with each of the plasmids, and the transformants, B-13445–B-13452, were examined for growth on a variety of media at various temperatures and for the levels of the cytochromes aₐ, b, c₁, and c. The growth of B-13445 (R166G) and B-13452 (R227End) was diminished greatly on YPG medium, and the growth of B-13446 (R166L), B-13446 (R166K), and B-13451 (S49L, T63A) was diminished only slightly on SG – uracil medium (Tables IV and VI, Fig. 3). These results confirm that the R166G and R227End changes are responsible for the cytochrome c₁ defects and presumably for the dominant-negative phenotypes. Furthermore, the T63A replacement in pAB2657, pAB2658, and pAB2659 and the S49L replacement in pAB265T are apparently innocuous. The detrimental effect of the R166G replacement was substantiated further from the diminished respiratory rate of strain B-13445, which was equivalent to the strain B-13435 lacking cytochrome c₁ (Table VI). Nevertheless, B-13445 (R166G) contained the nearly normal level of cytochrome c₁, as indicated by the α-peak in the low temperature (−196 °C) spectrophotometric recording (Fig. 4).

In contrast to the R166G replacement, the R166M, R166L, and R166K replacements at most only caused minor diminution of function as indicated by the normal or nearly normal level of growth on YPG medium (Table IV). This finding suggests that the large size but not the charge of the Arg-166 side chain is critical for maintaining the normal function of cytochrome c₁.

Yeast Arg-166 (Vertebrate Arg-102)—The importance of the yeast Arg-166 (or vertebrate Arg-102) along with the adjacent
Ala-165 residue is reflected by the phylogenetic conservation of these residues in cytochrome $c_1$ from all species including higher and lower eukaryotes and prokaryotes. As shown in Fig. 5, of the almost 250 residues, Ala-165 and Arg-166 represent two of the 32 absolutely conserved residues in all cytochromes $c_1$.

Insight into the function of Arg-166 is provided by considering its position in the cytochrome $bc_1$ complex and the proposed models of electron transfer. As shown in Fig. 1, Arg-166 is located on the surface of cytochrome $c_1$ in close proximity to the exposed pyrrole C corner of the heme group but not adjacent to any of the other components of the complex. The surface location of Arg-166 is consistent with proper assembly and stability of the altered R166G cytochrome $c_1$. Zhang et al. (13) suggested that electron transfer into cytochrome $c_1$ occurs through the D propionate and out of cytochrome $c_1$ through the C corner of the heme to cytochrome $c$. On the other hand, in vitro protection and cross-linking experiments suggested that at least two different regions of cytochrome $c_1$, encompassed by vertebrate positions 63–81 and 167–174, are folded together to form the cytochrome $c$ binding site (43, 44). It remains to be seen if the R166G cytochrome $c_1$ still binds cytochrome $c$ but is unable to transfer electrons. In this regard, an R166G replacement is not expected to disrupt the $a$-helical structure in this region, and it is unclear how R166G but not R166K, R166M, or R166L replacements could effect binding to cytochrome $c$.

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