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Functional mapping reveals the importance of yeast cytochrome *b* C-terminal region in assembly and function of the bc_1 complex

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Abstract Genetic and molecular analyses have been undertaken for four respiratory deficient mutants (mit$^-$). The four mutations affect the C-terminal region of apocytochrome *b*. The frameshift (L263STOP) and non-sense (Q338STOP) mutations give rise to a truncated apocytochrome *b*. The mutant G337R conserves only 32% of its NADH oxidase activity which suggests that the presence of a positively charged amino acid in the transmembranous helix 7 of cytochrome *b* alters, either directly or indirectly, the bc_1 function, without affecting its assembly. The mutation G352V has a 65% loss of cytochrome *b* spectral content and prevents all of the mitochondrial respiratory activity. This leads us to believe that the glycine, conserved in position 352, may play a crucial role in bc_1 complex function.

Key words: Cytochrome *b*; *Saccharomyces cerevisiae*; Respiratory deficient mutant; C-terminal region

1. Introduction

The ubiquinol cytochrome *c* oxidoreductase (also called bc_1 complex), an oligomeric transmembranous complex, catalyses the reduction of the *c*-type cytochrome of the mitochondrial respiratory chain. It contains at least 3 catalytic subunits: cytochrome *b*, the Fe-S protein and cytochrome c_1.

Two spectrally distinguishable hemes of different redox potentials, the b_{560} and b_{566} (b_{562} and b_{565} in yeast) are non-covalently bound to the apocytochrome *b*. They form the core of the bc_1 complex and are responsible not only for the electron transfer between the ubiquinol and cytochrome c_1 via the Fe-S protein at center P, but also for the transmembranous electron transfer to ubiquinone and ubisemiquinone at center N, according to the Q-cycle mechanism [1].

Furthermore, only apocytochrome *b* is coded by the mitochondrial genome while the other subunits of the complex are of nuclear inheritance. Mutations affecting the mitochondrial gene *cob* (also called *cob-box*), coding for the apocytochrome *b* in yeast (*Saccharomyces cerevisiae*), had provided powerful arguments to predict cytochrome *b* secondary structure. The location of the mutations responsible for resistance towards inhibitors of the two different classes had suggested a revision of the former nine helices model for the structure of cytochrome *b* (for review see [2]). Furthermore, the location of long distant suppressors of respiratory deficient mutations had provided predictions about the 3-D structure of cytochrome *b* [2,3]. However, the identification of respiratory deficient mutations as well as the alignment of several species' cytochrome *b* amino acid sequences had also contributed to the discovery of some residues which can be involved in cytochrome *b* function [3,4]. Moreover, the crystallization of beef heart mitochondrial cytochrome-bc_1 complex [5] will now enable us to steer our attention toward the relation between the structure and function of the bc_1 complex.

Thus, to obtain some insights into the structure-function relationship of the bc_1 complex, analysis of mutations located in the *cob* gene and exhibiting a respiratory deficient phenotype has been undertaken. The mutants were selected by Meunier et al. [6] using $MnCl_2$ as a mitDNA mutagen, and after genetic analysis only mutants exhibiting mutations near the 3′-terminal of the cytochrome *b* gene were analysed. This region which is poorly conserved has not yet been extensively studied. Four novel respiratory deficient mutations were characterized: the frameshift mutation at position 263 and the nonsense mutation at position 338 have shown a truncated apocytochrome *b* which is unable to assemble with the rest of the bc_1 complex. The two other mutations are missense mutations which involve the replacement of the glycine at position 337 by a positively charged amino acid and the change of, a highly conserved, glycine at position 352 by a valine. Growth rate, cytochrome spectral analysis, identification of the apocytochrome *b* band on polyacrylamide gel electrophoresis and in vitro respiratory activity have been examined. These data together with the sequencing data will be discussed in order to relate function to structure in the bc_1 complex.

2. Materials and methods

Strains: All mutants used in this work (L57, L79, L215 and L55) were isolated from the intronless haploid strain BM1/1-16 *a ade1 op1 rho$^-$ mit$^+$* [6]. Genetic locations were made by test crosses with strains devoid of mitochondrial DNA: KL14-4A *a his1 trp2 rho^{0}OP1* [7] and with specific *rho$^-$* strains: PI1 and L29 which encompass DNA exons 4–6 and 1–2, respectively.

Media: Strains were grown in N0: 2% glucose, 1% yeast extract (Gibco), 1% peptone (Gibco); N3: 2% glycerol, 1% yeast extract (Gibco), 1% peptone (Gibco); GAL: 2% galactose, 1% yeast extract (Gibco), 1% peptone (Gibco) and GAL min: 0,7% yeast nitrogen base (Difco), 2% galactose.

For solid media, 2% of agar was added.

Test-crosses: The crosses were performed as described by Kotylak and Slonimski [8].

DNA sequencing: Double and single stranded DNA amplification was produced using the method described by McCabe [9]. The product of single stranded DNA amplification was sequenced as previously described in the Pharmacia Biotech catalogue (^{17}Sequencing Kit, 1996).

Growth rate: Strains were first grown in N0 liquid medium (described above) for 24 h, then cultures were inoculated in N3 liquid

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medium. Growth was followed photometrically with a Klett-Summerson colorimeter equipped with a red filter.

Isolation of mitochondrial particles: The mitochondria were prepared as described by Meunier and Colson [10].

Spectral analysis: Cytochrome spectral analysis was performed as described by Rickwood et al. [11] using mitochondrial particles, with dithionite as reductant and ferricyanide as an oxidant.

Respiratory activity measurements were carried out as previously described [19].

Identification of mitochondrially translated polypeptides: All strains used in this study were obtained from strain BM1/1-16 [6] which contains the nuclear *op1* marker. Since this marker interferes with efficient labelling of mitochondrial translation product in vivo, the mutants were first mated to a *rho*^{0} strain KL14-4A which contains the dominant *OP1*$^+$ wild-type allele and the resulting diploids label normally. The in vivo labelling and mitochondrial isolation were performed according to the protocol described by Meunier et al. [6].

3. Results

3.1. Genetic location and molecular analysis of the mit$^-$ mutations

The mutants were isolated from an intronless mit$^+$ (BM1/1-16) as described by Meunier [6] using $MnCl_2$ as a mitDNA mutagen.

In order to circumscribe the location of mit$^-$ mutations, strains were first crossed with KL14-4A *rho*^{0} giving rise to a progeny which failed to grow on respiratory medium (glycerol medium, see Section 2), which means that mutations were of mitochondrial inheritance. The mutants were then crossed with two *rho*$^-$ strains (L29 and PI1, Section 2) and only mutants for which the *rho*$^-$ PI1 (which cover exons 4–6) could restore the wild-type ability to grow on respiratory medium were kept to be analysed. Thus, DNA sequencing of 5 mutants was undertaken (Table 1), which revealed that mutant L57 exhibited a (−T) leading to replacement of leucine in position 263 by a 'nonsense' codon. The substitution of thymine in place of cytosine led to a nonsense codon instead of glutamine at position 338 (L215 and L214 mutants) and the substitution of adenosine in place of guanosine led to the replacement of glycine by arginine in position 337. The last mutation changed the glycine to a valine in position 352 located at cytochrome *b* helix 8 (Fig. 1).

Table 1
Nucleotide sequence of the DNA regions affected by the mutations studied in this work

Sequences					
WT	G_{260} GGT	N_{261} AA	P_{262} CCT	L_{263} TTA	V_{264} GTA
L57	G_{260} GGT	N_{261} AAT	P_{262} CCT	**STOP** TAG −T	
WT		L_{336} TTA	G_{337} GGA	Q_{338} CAA	
L79		L_{336} TTA	R_{337} AGA	Q_{338} CAA	
L215		L_{336} TTA	G_{337} GGA	**STOP** TAA	
WT	L_{350} TTA	M_{351} ATG	G_{352} GGA	Q_{353} CAA	
L55	L_{350} TTA	M_{351} ATG	*V_{352}* GTA	Q_{353} CAA	

The different sequences are compared to the wild-type sequence, and the changed bases are indicated as (−T) or (+T) where deletions or insertions are introduced. The changed amino acids are written in italics.

3.2. Phenotypical characteristics

To characterise further the four mit$^-$ mutants, their phenotypes were checked for growth rate, NADH oxidase activity, cytochrome spectral properties and the pattern of mitochondrial translation products.

The mit$^-$ mutants were, by definition, unable to grow on respiratory medium, thus all the mutants carrying a nonsense codon (Table 1) lack spectrally detectable cytochrome *b* as well as a mitochondrial translation product (Table 2, Figs. 2 and 3) which, in the wild-type mit$^+$ migrates with an apparent molecular weight of 31 000 in polyacrylamide gel electrophoresis. This observation supports the results of previous studies on *cob* mutants which had been derived from different strains in other laboratories [12–16]. The missing polypeptide is almost certainly the apoprotein of the cytochrome *b*, which is consistent with the lack of functional cytochrome *b* in the mutant. In addition, a 'new' band of lower apparent molecular weight was shown for these mutants (Fig. 2). The molecular weight of the wild-type apocytochrome *b* calculated from the amino acid sequence was estimated as 43 620. However, that of the possibly mutant apocytochrome *b* was estimated as 38 180 for the L215 mutant (Q338STOP) which would give rise to an eventual apoprotein of 337 amino acids. An estimated molecular weight of 29 636 for the L57 mutant (L263STOP) would produce an eventual apocytochrome *b* with 262 amino acids, according to the position of a 'STOP' codon. The comparison between the calculated values and those estimated by electrophoretic system showed a collinear correlation. These results indicate that a frameshift and nonsense mutations mapped in the structural gene are responsible for the appearance of shorter polypeptides, which therefore would be apocytochrome *b* fragments.

The L79 mutant (G337R) presented 49% of the wild-type growth rate with a poor NADH oxidase activity (32% of the wild-type strain, Table 2). Its spectral cytochrome *b* content, expressed as the ratio (cyt. *b*)/(cyt. c_1+cyt. *c*), was not significantly affected (75% of the wild-type strain, Fig. 3). In addition, the mutant L79 (G337R) mitochondrial translation product migrates with an apparent molecular weight of 31 000 in dodecyl sulfate/polyacrylamide gels, similar to the wild-type mit$^+$ (Table 2, Fig. 2).

The L55 mutant (G352V) also retained 40% of the wild-type spectral cytochrome *b* content, but almost no NADH oxidase activity was detected for this mutant which means that the substitution of a conserved glycine, at position 352, by a bulky valine is responsible for a specific defect of the NADH oxidase activity rather than assembly.

4. Discussion

One mutant which grew slowly and 3 mutants which failed to grow on respiratory medium were analysed in this work. The test crosses with a strain of opposite mating type, carrying the *OP1*$^+$ wild type and devoid of mitDNA have shown that their respiratory deficient phenotype is due to a mutation of mitochondrial inheritance. The crosses with specific *rho*$^-$ clones demonstrated that all mutations were located within the 3′-terminal region of the *cob-box* gene. The DNA sequencing revealed one frameshift mutation (L57) mapped in *cob-*

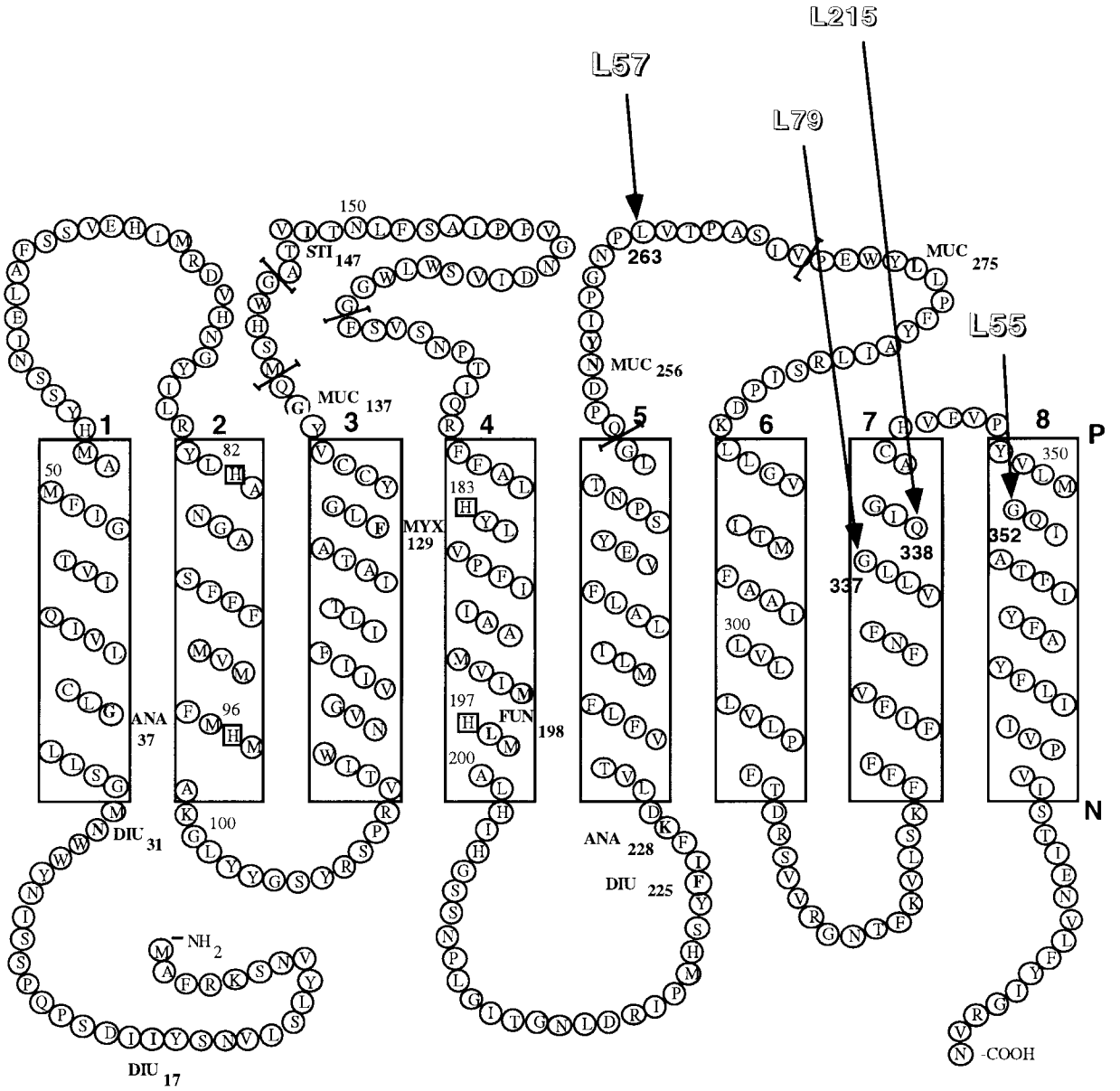

Fig. 1. Cytochrome *b* folding model with eight spanning α helices in the mitochondrial inner membrane [18]. ANA (antimycin), DIU (diuron), STI (stigmatellin), FUN (funiculosin), MYX (myxothiazol) and MUC (mucidin) indicate the amino acids involved in the inhibitor resistance. The histidines in squares are suggested to be the axial ligands of the two hemes. N and P indicate the electronegative and electropositive sides of the membrane. The amino acids affected by the mutations studied in this work are denoted by arrows, and the names of the mutants exhibiting the mutations are L57, L79, L215 and L55, respectively.

box exon 5, one nonsense mutation in *cob-box* exon 6 (L215) and two substitutions in *cob-box* exon 6; one changing the amino acid glycine to a positively charged, arginine, in position 337 located at transmembranous helix 7 of the cytochrome *b*, near the electropositive side of the mitochondrial inner membrane; the second substitution changing a highly

Table 2
Phenotypes of the mutants

Strains	Growth rate (%)	NADH oxidase activity (%)	(cyt. *b*)/(cyt. c_1+cyt. *c*) (%)	Apocytochrome *b*
Wild-type strain	100	100	100	+
L57	0	0	0	−
L79	49	32	75	+
L215	0	0	0	−
L55	0	2	40	+

All the growth rate, the NADH oxidase activity (in the presence of 2 mM NADH and 5 μM CCCP) and the ratio (cyt. *b*)/(cyt. c_1+cyt. *c*) are expressed as the percentage of the revertant values relative to those of the wild type. The presence or absence of apocytochrome *b* (normal band) is denoted by (+) or (−), respectively. For the wild type, the value of the growth rate is 0.26 h^{-1}, that of the NADH oxidase activity is 355 nmol O_2/min per mg and the value of the ratio (cyt. *b*)/(cyt. c_1+cyt. *c*) is 0.7.

conserved glycine [4] by a bulky valine in position 352 in helix 8 of the eight helices cytochrome *b* model.

Neither the frameshift nor the nonsense mutations revealed a cytochrome *b* spectral absorbancy nor the wild-type mitochondrial translation product which was in agreement with their non-functional bc_1 complex. However, the mitochondrial translation product showed a new band with a lower molecular weight (Fig. 2). The comparison of the possible apocytochrome *b* molecular weights, calculated from amino acid sequences and electrophoresis data showed a linear correlation, which suggests that a smaller band appearing in these mutants pattern would represent the apocytochrome *b* remaining fragment, devoid of its C-terminal region, and probably unable to bind the *b* hemes, since no cytochrome *b* spectral absorbancy was detected for the two mutations (L57 and L215). These results reveal the importance of the three transmembranous helices, at the C-terminal region of the cytochrome *b* eight helices model, in the assembly of a functional bc_1 complex, which is consistent with data previously presented by Di Rago et al. [17] who had worked with a nonsense mutation at position 335 and a frameshift mutation at position 349, the two mutants revealing an unassembled bc_1 complex similarly to our two mutants (this work).

However, the mutant L79 (G337R) exhibited a decreased respiratory activity leading to a decreased growth rate, without affecting the cytochrome *b* spectral properties. According to cytochrome *b* alignments [4], the glycine in position 337 has never been changed by either a positively or a negatively charged amino acid. In addition, no charged residue has been predicted to be in the transmembranous helices of the cytochrome *b* eight helices model. This suggests that the arginine in the transmembranous helix 7 of the cytochrome *b* eight helices model would affect the chemical properties of the helix and perhaps its topology, thus altering either directly or indirectly the bc_1 complex function.

The most important information revealed in this work is supplied by the mutant L55 (G352V). This mutant consisted of a substitution of a bulky valine in place of a highly conserved glycine, in position 352, located at helix 8 of the cytochrome *b* eight helices model. This glycine is also conserved in chloroplast subunit IV and located at two amino acids from its C-terminal end according to the cytochrome *b* alignment [4]. The substitution (G352V) led to a decreased spectral cytochrome *b* content with no detectable respiratory activity. Since all reversions isolated by Di Rago et al. [17] from the frameshift mutation at position 349 affected the region flanked between residues 345 and 354 keeping the glycine at position 352. This leads us to believe that the glycine conserved in this region also plays a crucial role in the function of the bc_1 complex and the b_6f complex.

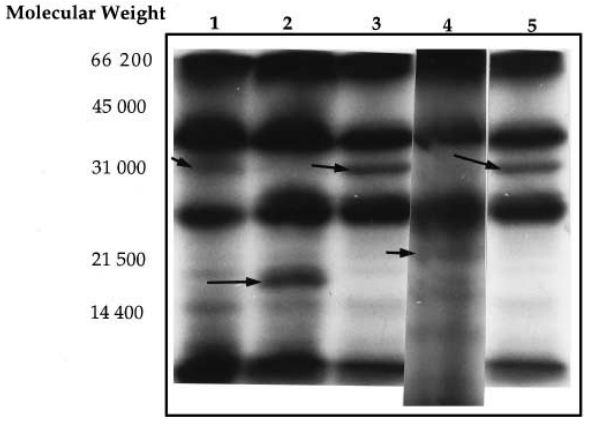

Fig. 2. Electrophoresis of mitochondrially translated products. Lanes: 1, wild-type strain; 2, L57 (L263STOP); 3, L79 (G337R); 4, L125 (Q338STOP); 5, L55 (G352V). Arrows indicate the corresponding band for apocytochrome *b*. The molecular mass is given in Da and corresponds to the low molecular mass migration standard stained with Coomassie G-250.

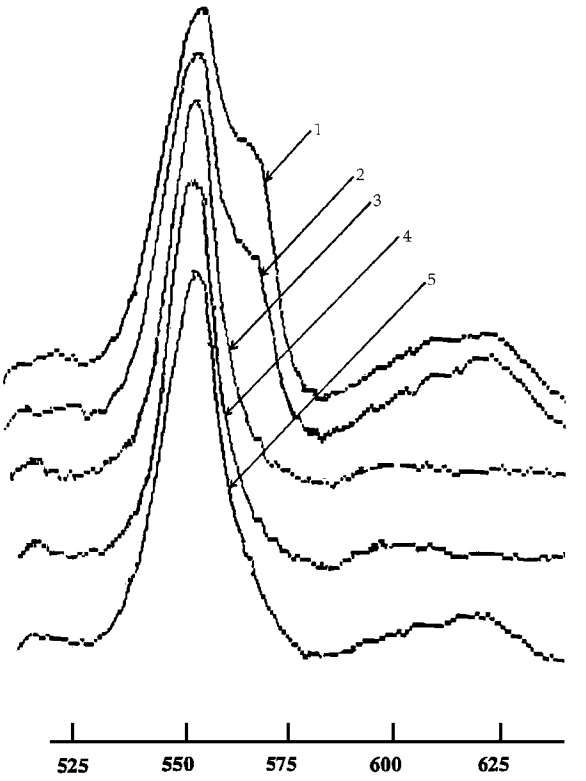

Fig. 3. Cytochrome absorption spectra of mitochondrial particles. The mitochondrial particles were isolated from wild type (1), L79 (G337R) (2), L57 (L263STOP) (3), L215 (Q338STOP) (4), and L55 (5). The spectra were recorded from mitochondrial particles (2.3–2.9 mg) after reduction of cytochromes by dithionite.

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