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Molecular analysis of revertants from a respiratory-deficient mutant affecting the center o domain of cytochrome *b* in *Saccharomyces cerevisiae*

Thierry Tron, Pascale Infossi, Jean-Yves Coppee and Anne-Marie Colson

Laboratoire de Génétique Microbienne, Place Croix du Sud 4, Université Catholique de Louvain, 1348 Louvain la Neuve, Belgium

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In *bc* complexes, cytochrome *b* plays a major role in electron transfer and in proton translocation accross the membrane. Several inhibitor-resistant and respiratory-deficient mutants have already been used to study the structure-function relationships of this integral membrane protein. We describe here the selection and the molecular analysis of revertants from a thermo-sensitive mit-mutant of known nucleotide changes. Among 80 independent pseudo-wild type revertants screened by DNA-labelled oligonucleotide hybridization, 33 have been sequenced. Eight suppressor mutations, affecting a region critical for both the function and the binding of center o inhibitors (end of helix C) were identified. Two of them were found to be more resistant to myxothiazol.

Mitochondrial cytochrome *b*; Respiratory-deficient mutant; Pseudo-wild-type revertant; DNA hybridization; RNA sequencing; Myxothiazol resistance; *Saccharomyces cerevisiae*

1. INTRODUCTION

Cytochrome *bc* complexes catalyze the electron transfer from ubiquinol to cytochrome *c* or plastocyanine in association with a vectorial proton translocation. Although they differ in their protein composition, all the *bc* complexes contain the same redox proteins [1], i.e. cytochrome *b*, cytochrome c_1 and iron-sulfur protein, which seem to be the only polypeptides involved in oxidoreduction reaction [2,3]. In eucaryotes, all the subunits are nuclearly inherited, except cytochrome *b* which is encoded by the mitochondrial DNA [4]. Cytochrome *b* is an integral membrane protein carrying two haems, b_H and b_L, presumably asymetrically disposed toward the inner and outer sides of the membrane, respectively [5-7]. The two haems are supposed to form a transmembrane electron circuit between two ubiquinone redox sites Q_i and Q_o according to the Q cycle scheme [8].

The structure of such a membrane complex protein is very difficult to investigate, so the use of cytochrome *b* mutants provide a good tool to study the structure-function relationships of this protein. Two kinds of cytochrome *b* mutants carrying missense mutations in the cytochrome *b* gene have been studied: (i) mutants exhibiting a resistance to proton-motive Q cycle inhibitors which interact with the bc_1; DNA sequence analysis of numerous mutants from several species [9-16] has shown that inhibitor resistance was conferred by specific amino acid replacements in discrete regions of the protein. (ii) Mutants unable to grow on respiratory substrates previously mapped in the cytochrome *b* gene, carrying single amino acid substitutions which impair bc_1 complex assembly [17,18] or affect directly the electron transfer chain [17,19,20]. More recently, new mutants have been selected as revertants from respiratory-deficient mutants [21,22]. The analysis of such mutants has revealed interesting features for the understanding of cytochrome *b* function and is very useful in the study of the three-dimensional structure of this protein. In the present work, we describe the selection and the molecular analysis of revertants selected from a thermo-sensitive mutant previously selected as a revertant of a respiratory-deficient mutant [22], G3012 (C_{133}->Y), specifically affected at the haem b_L level [7]. About 80 independent revertants obtained at non permissive temperature (37°C) were screened by oligonucleotide hybridization as described in [21]. Eight different suppressor mutations responsible for the restoration of growth at 37°C were identified by sequencing the cytochrome *b* mRNA in crude mitochondrial RNA preparations from 33 independent revertants. The results will be discussed comparatively to other data from several inhibitor-resistant and respiratory-deficient mutants found in the same region of the protein, a region that seems to be critical for the quinol oxidation and the binding of center o inhibitors.

Correspondence address: A.-M. Colson, Laboratoire de Génétique Microbienne, Place Croix du Sud 4, Université Catholique de Louvain, 1348 Louvain la Neuve, Belgium

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2. MATERIALS AND METHODS

2.1. *Media*

YP 10: 10% glucose, 1% yeast extract, 1% bactopeptone; YPG: 2% glucose, 1% yeast extract, 1% bactopeptone; YPGAL: 2% galactose, 1% yeast extract, 1% bactopeptone; N3: 2% glycerol, 1% yeast extract, 1% bactopeptone. Solid media contained 2% agar.

2.2. *Strains*

KM91 (wt), 777-3A, *α ade* op1 × KL14-4A, a *his1 trp 1* (from the Gif collection). The ts rho$^+$ mit$^-$ cytochrome *b* mutant JDG3012/117 [22] was isolated from the diploid strain KM710: 777-3A/G3012, *α ade* op1 mit$^-$ × KL14-4A/51, a *his1 trp1* rho°. The original mit$^-$ mutation (G3012) was localized by deletion mapping at the end of exon I of the split cytochrome *b* gene [23] and its mitDNA sequence determined (C_{133}->Y)[7].

2.3. *Isolation of the respiratory competent revertants*

Respiratory competent revertants were selected on N3 medium from the diploid mutant strain JDG3012/117 as described in [21] except that the selection was carried out at 37°C (non-permissive temperature) instead of 28°C.

2.4. *Oligonucleotide hybridization*

DNA extracted from revertants as described in [21] were loaded on nylon filters (Amersham), soaked twice for 3 min in 0.5 M NaOH, 1.5 M NaCl, 0.1% SDS then twice for 3 min in 0.5 M Tris-Cl, pH 7.5, 1.5 M NaCl. Filters were dried and exposed for 4 min to UV (305 nm) in order to fix the DNA on the filters, then prehybridized for 30 min at room temperature in 30 ml of mixed 10 × Denhardt, 6 × SSC (Standard Sodium Citrate). The temperature was increased to 80°C and 10 pmol of a ^{32}P end labelled oligonucleotide covering the initial mutation of JDG3012/117 were added. The temperature was gradually decreased to 30°C and hybridization continued overnight. Filters were progressively washed in 6 × SSC at selected temperatures and autoradiographed after each washing.

2.5. *RNA sequencing*

Cytochrome *b* mRNA from crude RNA preparations was sequenced by primer extension with AMV reverse transcriptase as described in [13-15,24].

2.6. *Growth rate measurements*

Growth on glycerol was performed at 28°C and monitored with a Klett densitometer. Generation time was determined from the exponential growth phase.

2.7. *In vivo myxothiazol tolerance test*

The revertants were checked for their ability to grow on glycerol plates in the presence of increased concentrations of myxothiazol ranging from 0.1-1 μg/ml.

3. RESULTS

The rho$^+$ mit$^-$ ts mutant JDG3012/117 has been selected as revertant [22] from the respiratory-deficient mutant G3012 (C_{133}->Y) which has previously been described as being specifically affected at the haem b_L level [7]. The strain JDG3012/117 exhibited a weak growth on glycerol at 28°C and did not grow on glycerol at 37°C. The sequence analysis of this 'thermosensitive revertant' has revealed that the suppressor mutation led to the replacement of alanine 126 by a

Table I

mt DNA sequence alterations of revertants from the thermo-sensitive mit$^-$ mutant JDG3012/117

Type			126					130			133		135			138		Tm °C
	Residues		I	A	T	A	F	L	G	Y	C	C	V	Y	G	Q		
	Wt sequence		ATT	GCT	ACA	GCT	TTT	TTA	GGT	TAT	TGT	TGT	GTT	TAT	GCA	CAG		
	Oligonucleotide							3'T	C	C	A	A	T	A	C	G	T	C 5'		64
	JDG3012/117			T							Y							
				ACT							TAT							56
II	R5	(5)		T							S							
				ACT							TCT							54
	R3	(7)		T							F							
				ACT							TTT							55
	R18	(2)		T							N							
				ACT							AAT							53
	R23	(1)		T							D							
				ACT							GAT							54
	R110	(1)		T							H							
				ACT							CAT							53
I	R11	(3)		T							C							
				ACT							TGT							64
II	R87	(4)		T							Y		I					
				ACT							TAT		ATT					50
III	R82	(1)		T				M			Y							
				ACT				ATA			TAT							56

The mt DNA sequence of JDG3012/117 was determined by Di Rago et al. [22]. Revertants (R, followed by a number) were screened by labelled oligonucleotide hybridization as described in section 2. Oligonucleotide fusion temperatures are given by Tm°C. Hybridization types are given according to [21]: type I (back to wt codon), type II (second mutation included in the probed region), type III (second mutation at a distant site from the probed region). Sequences of revertants were performed as described in [13,15,24]. Numbers in brackets represent the number of independent revertants sequenced having the same suppressor. Mutated amino acids are bold

threonine whereas the original mutation remained unchanged [22].

From this thermo-sensitive strain, about 80 spontaneous independent revertants obtained at non-permissive temperature (37°C) were analysed by a temperature-sensitive oligonucleotide hybridization technique [21]. The original mutated site (residue 133) and its upstream and downstream sequences were probed with an oligonucleotide which is complementary to the wild type mit DNA. Similarly as in [21], three classes of revertants were found (see Table I). Among 80 revertants which were analysed by hybridization, we have found 14 (18%) type I (back to the wild type codon), 35 (45%) type II (second site mutation included in the probed region) and 29 (37%) type III (second site mutation at a distant site from the probed region) revertants. RNA sequences of the probed region (which overlap C_{133}->Y) of 33 revertants have been performed. Results of the sequencing gels are presented in Table I. In 24 revertants, two kinds of compensatory mutations were found: (i) on position 133, all amino acids which can be derived by mononucleotide substitution from the tyrosine codon (TAT) occurred: serine (TCT), phenylalanine (TTT), asparagine (AAT), aspartic acid (GAT), histidine (CAT) and cysteine (TGT, wild type codon) whereas the second mutation of JDG3012/117, a threonine instead of alanine 126, remained unchanged; (ii) acquisition of a third mutation at the level of codon 135 where a valne is replaced by an isoleucine or at the level of codon 130 where a leucine is replaced by a methionine (type III revertant).

Moreover, in nine type III revertants, the presence of the two original mutations of JDG3012/117 (A_{126}->T and C_{133}->Y) was confirmed, whereas the presence of a suppressor mutation was not found in the sequenced region of the mRNA (between positions 120 and 137) (not shown). Genetic analysis by 'petite' deletion mapping of these type III revertants is in progress.

At the level of position 133, serine, phenylalanine, asparagine, aspartic acid and cysteine restored a good efficiency of growth on glycerol ranging from 50% to nearly all of the wild type growth rate (see Table II), whereas histidine allowed only 5% of growth on glycerol. For the two revertants with a third mutation, the efficiency of growth on glycerol was 11% relative to the wild type (Table II).

Revertants which have a good efficiency of growth on glycerol have been tested for their ability to grow in the presence of increasing concentrations of myxothiazol (in vivo myxothiazol tolerance test) (Table II). The presence of an asparagine or an aspartic acid in position 133 leads respectively to a 5-fold and an 8-fold relative increase in resistance comparatively to the wild type whereas strains with other suppressors at this position, i.e. serine, phenylalanine and cysteine, are apparently myxothiazol-sensitive.

Table II

Relative growth rate and level of in vivo myxothiazol tolerance of revertants

Strains	Growth rate (% of wt)	In vivo myxothiazol tolerance (μg/ml)
KM91 (wt)	100	0.1
JDG3012/117 (Y_{133}, T_{126})	2	nd
R5 (S_{133}, T_{126})	62	0.1
R3 (F_{133}, T_{126})	50	0.1
R18 (N_{133}, T_{126})	71	0.5
R23 (D_{133}, T_{126})	56	0.8
R110 (H_{133}, T_{126})	5	nd
R11 (C_{133}, T_{126})	100	0.1
R87 (I_{135}, Y_{133}, T_{126})	11	nd
R82 (Y_{133}, M_{130}, T_{126})	11	nd

Growth on glycerol at 28°C is expressed as the ratio of the revertant's growth rate relative to that of the wild type. In vivo myxothiazol tolerance is expressed in μg of myothiazol per ml of N3 medium necessary to completely impair the growth

nd: not determined

4. DISCUSSION

Herein we describe the isolation and the molecular analysis of a new set of cytochrome *b* mutants. These mutants affect a highly conserved region of the protein which is likely to be critical for the electron transfer, the assembly of the bc_1 complex and the binding of center

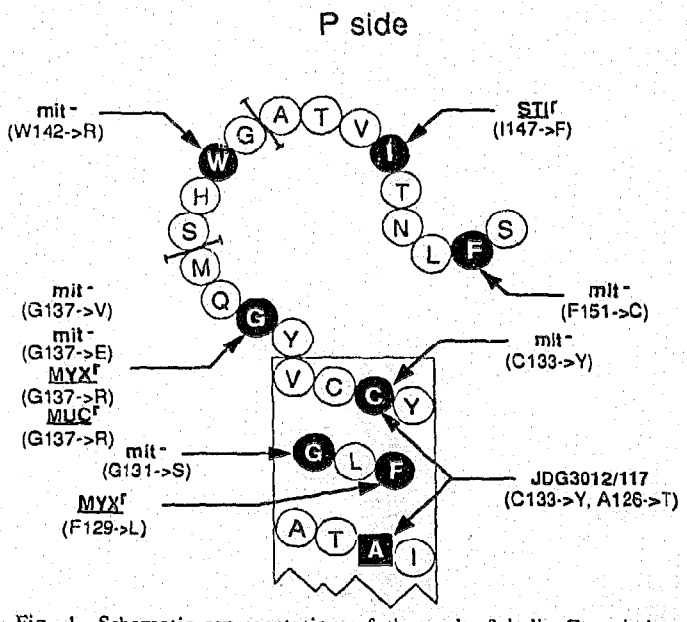

Fig. 1. Schematic representation of the end of helix C and the beginning of the loop c-d from the cytochrome *b* eight helices model [25]. Resistant mutants (underlined). MYX^r, MUC^r, STI^r [15]. Deficient mutants: mit^-131, mit^-142 [17], mit^-133 [7], mit^-137 [20], mit^-151 [18]. Thermo-sensitive mutant: JDG3012/117 [22] (the first suppressor is squared). I——I, indicates the positions of the corresponding exon limits of the split cytochrome *b* gene. P, represents the positive side of the membrane.

o inhibitors. Indeed, as illustrated in Fig. 1, in the current eight helices model [25], the end of helix C and the beginning of the loop c-d form a region where it has been assigned 4 center o inhibitor-resistant mutants and 6 respiratory-deficient mutants, among which two have an impaired bc_1 complex assembly (see legend of Fig. 1).

Eight new local cytochrome *b* structures were obtained from the thermo-sensitive mutant JDG3012/117 which carries two nucleotide changes at codons 133 and 126 (Fig. 2A). From these results, some conclusions can be drawn. First, a true back mutation at position 133 restoring the wild type cysteine (Fig. 2B) isolates the suppressor mutation A_{126}->T and is found to be compatible with a wild type growth (Table II). Second, pseudo-wild type mutations leading to the replacement of tyrosine 133 by S, F, N and D (Fig. 2B) are the same changes as those previously described as suppressors of the original deficient G3012 mutation (C_{133}->Y) [21], except that in our case T_{126} remains present instead of the wild type alanine. This suggests that there is no antagonism between these four suppressors and the mutation T_{126} located about two helical turns below the position 133 (Fig. 2B). This also underlines the specificity of the tyrosine, and more specifically of its hydroxyl group, in the loss of the capacity to grow on respiratory substrates (at least for about 50%). Indeed, the replacement of tyrosine 133 by a phenylalanine, both of amino acids having approximately the same volume, allows

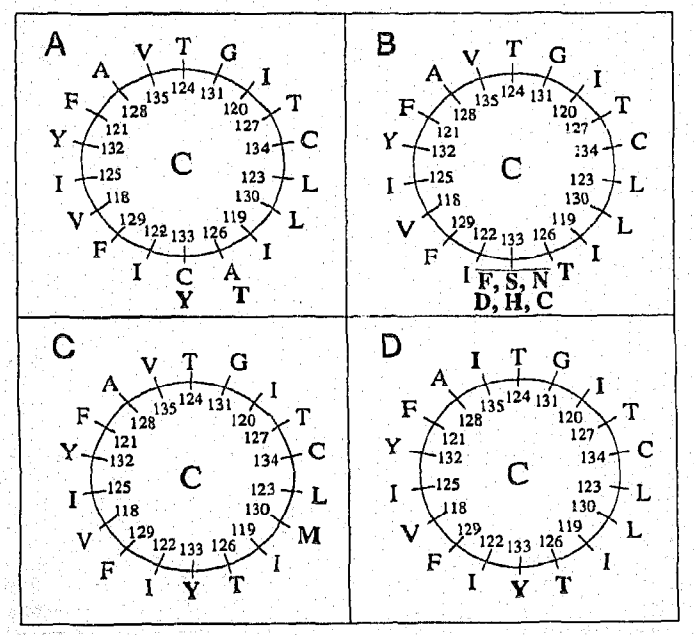

Fig. 2. Helical wheel representations of helix C of the wild type, the mutant and its eight revertants. (A) wild type and mutant (bold) representation, (B) suppressors affecting position 133, (C) suppressor affecting position 130, (D) suppressore affecting position 135.

50% of growth relatively to the wild type. Third, the combination of threonine 126 with a histidine at position 133 is obtained for the first time and is compatible for a weak (5%) growth. This confirms that H_{133} is deleterious for the function, as proposed in [21] because it was the only change that was not found. Thus, at position 133, even if small residues (independently of their hydrophobic or polar character) are more tolerated (60 to about 100% of growth on respiratory substrate) than a large residue like phenylalanine (50%), it seems that the inability to grow on respiratory substrate is specifically due to the presence of tyrosine or histidine at position 133. It is thought that the neighbour invariant tyrosine 132 is involved in the oxidoreduction reaction at center o [21], so Y_{133} and H_{133}, modifying the local topology of the ubiquinol oxidation site, could induce the quinol to react with wrong partnars.

Two revertants carrying a third site mutation which compensate the inability to grow on respiratory substrate of the thermosensitive mutant JDG3012/117 were also analysed. Helical wheel representations of the mutated regions are shown in Fig. 2C and D. Although the relative growth rates of these two revertants are identical (11%), the nature of the suppressors and their locations are very different. Indeed, the suppressor L_{130}->M is located on the same side of helix C as that of the previous mutations Y_{133} and T_{126} (in close spacial vicinity), whereas the suppressor V_{135}->I is located on the opposite side of helix C, one-half turn after Y_{133}. This suggests that these compensatory mutations probably modify the local α-helix structure of the end of helix C, allowing a partial recovery of function even with Y_{133}.

Among revertants exhibiting a good growth on respiratory substrate, i.e. S, F, N, D or C_{133} (coupled with T_{126}), only revertants R18 (N_{133}, T_{126}) and R23 (D_{133}, T_{126}) (cf. Table I) were found to be resistant toward myxothiazol. Position 133 is surrounded by several positions for which resistance mutations toward myxothiazol were found, i.e. residue 129 in yeast [15]. *Rhodobacter capsulatus* [11] and *Chlamydomonas reinhardhii* [9] mutants, residue 137 in yeast [15] and in *Rhodobacter capsulatus* [11] mutants, residue 143 in a mouse mutant [12] and in the natural resistant *Paracentrotus lividus* [26]. These residues are thought to form a part of the myxothiazol binding niche [11,12,15]. This does not mean that position 133 is directly involved in the binding of myxothiazol, but that the introduction of charge a (D) or a polar character (N) and/or a better capacity in hydrogen bonding is likely to modify the myxothiazol cytochrome *b* interacting zone.

We have selected and analysed a new generation of cytochrome *b* mutants affecting a region of the protein which is likely involved at center o of the proton-motive bc_1 complex and so is critical for the function. Further biochemical studies are underway to deepen our

knowledge on the quinol oxidation site and the binding of center o inhibitors.

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REFERENCES

[1] Hauska, G., Hurt, N., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133.
[2] Yang, X., Ljungdahl, P.O., Payne, W.E. and Trumpower, B.L. (1987) in: Bioenergetics: Structure and Function in Energy Transducing Systems (Osawa, T. and Papa, S. eds) pp. 63–70, Japan Sci. Soc. Press, Tokyo/Springer, Berlin.
[3] Yang, X. and Trumpower, B.L. (1988) J. Biol. Chem. 263, 11962–11970.
[4] De Vries, S. and Marres, C.A. (1987) Biochim. Biophys. Acta 895, 205–239.
[5] Ohnishi, T. and Trumpower, B.L. (1980) J. Biol. Chem. 255, 3278–3284.
[6] Berry, E.A. and Trumpower, B.L. (1985) in Coenzyme Q (Lenaz, G. ed.) pp. 365–389, J. Wiley, New York.
[7] Meunier-Lemesle, D., di Rago, J.-P. and Chevillotte-Brivet, P. (1988) in: Fifth European Bioenergetics Conference, Short Reports vol. 5, p. 76, (IUB-IUPAB Bioenergetics Group, eds).
[8] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
[9] Bennoun, P., Delosme, M. and Kück, U. (1990) Genetics, in press.
[10] Coria, R.O., Garcia, M.C. and Brunner, A.L. (1989) Mol. Microbiol. 3, 1599–1604.
[11] Daldal, F., Tokito, M.K., Davidson, E. and Faham, M. (1989) EMBO J. 8, 3951–3961.
[12] Howell, N. and Gibert, K. (1988) J. Mol. Biol. 203, 607–618.
[13] di Rago, J.-P., Perea, J. and Colson, A.-M. (1986) FEBS Lett. 208, 208–210.
[14] di Rago, J.-P. and Colson, A.-M. (1988) J. Biol. Chem. 263, 12564–12570.
[15] di Rago, J.-P., Coppée, J.-Y. and Colson, A.-M. (1989) J. Biol. Chem. 264, 14543–14548.
[16] di Rago, J.-P., Perea, J. and Colson, A.-M. (1990) FEBS Lett. 263, 93–98.
[17] Chevillotte-Brivet, P. and di Rago, J.-P. (1989) FEBS Lett. 255, 5–9.
[18] Lazowska, J., Claisse, M., Gargouri, A., Kotylak, Z., Spyridakis, A. and Slonimski, P.P. (1989) J. Mol. Biol. 205, 275–289.
[19] Meunier-Lemesle, D., Chevillotte-Brivet, P. and Pajot, P. (1980) Eur. J. Biochem. 111, 151–159.
[20] Tron, T. and Lemesle-Meunier, D. (1990) Curr. Genet., in press.
[21] di Rago, J.P., Netter, P. and Slonimski, P.P. (1990) J. Biol. Chem. 265, 3332–3339.
[22] di Rago, J.P., Netter, P. and Slonimski, P.P. (1990) J. Biol. Chem. 265, 15750–15757.
[23] Jacq, C., Lazowska, J. and Slonimski, P.P. (1980) in: The Organisation and Expression of the Mitochondrial Genome (Kroon, A.M. and Saccone, C. eds) pp. 139–152, North-Holland, Amsterdam.
[24] Perea, X. and Jacq, C. (1985) EMBO J. 4, 3281–3288.
[25] Brasseur, R. (1988) J. Biol. Chem. 263, 12571–12575.
[26] Degli Esposti, M., Gheli, A., Butler, G., Roberti, M., Mustich, A. and Cantatore, P. (1990) FEBS Lett. 263, 245–247.