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Structure-function relationships of cytochrome *b* by the genetic approach: Intragenic revertants derived from frameshift mutations in the *Saccharomyces cerevisiae* apocytochrome *b* gene

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Introduction

Cytochrome *b* belongs to one of the most widespread protein complexes, the bc_1 complex or Complex III, playing a key role in energy transduction systems (for review see Ref. 1). In 1975 and 1976, the mechanism by which the bc_1 complex links electron transfer to proton translocation across the membrane was proposed by Peter Mitchell and named the protonmotive Q cycle [2,3]. A recent mini-review on the protonmotive Q-cycle has been presented [4]. Two pathways for cytochrome *b* reduction were identified, one pathway, going through centre i at a site in the vicinity of cytochrome b_{562} closer to the inner side of the membrane, is sensitive to antimycin and the other passing through centre o proximal to b_{566} nearer the outer side of the membrane, is myxothiazol sensitive. Mucidin and Strobilurin A are identical and also blocks the myxothiazol sensitive pathway [5]. The spatial organization of these cytochrome *b* redox active centres has been confirmed, the b_{562} heme being close to the middle of the phospholipid bilayer and the b_{566} heme located closer to the outer surface of the membrane [6].

Protonmotive Q-cycle inhibitors are useful tools for the geneticist, since they block the respiratory growth, i.e. growth in the presence of a non fermentable carbon source. Selection for resistance is thus possible and the resulting mutants can be further analysed by genetic mapping for the probable location of the mutation responsible for the resistance phenotype. As early as 1977, 3 segments of the 'yeast cytochrome *b* mitochondrial DNA coding region' were found in *Saccharomyces cerevisiae* to be directly involved in the inhibitor resistance to centre i and centre o inhibitors of the protonmotive Q cycle [7–9].

A decade later, when the appropriate sequencing techniques became available, the sequence analyses of 38 inhibitor-resistance mutations previously mapped to the exons of the *S. cerevisiae* cytochrome *b* split gene were performed by primer elongation with reverse transcriptase using mitochondrial mRNA as template [10–13]. Most resistance mutations were monosubstitutions and in 4 cases disubstitutions were found. Eleven different mutated positions were identified clustered in 4 distinct portions of the gene. Two subregions were found to confer centre i inhibitor-resistance and 2 were found to confer centre o inhibitor-resistance. These 4 subregions are the most conserved of the cytochrome *b* apoprotein; about 20 centre i and centre o inhibitor-resistance mutations were identified in other organisms (mouse, bacteria, yeasts and algae) and were found to be located, also, at the same positions as those found in yeast, or in their close vicinity [14–19]. These pairs of subregions were respectively postulated to be part of centre i and centre o of the protonmotive Q-cycle [1].The positions of the mutated codons were matched with the first cytochrome *b* folding model made available in 1984 [20,21]. Surprisingly, the 2 centre i inhibitor-resistant regions were found on either side of the membrane, and those of centre o, likewise on either side [10–12]. Based on other calculations, an 8-transmembrane alpha-helix model was presented [22–24], where the fourth amphiphilic helix was removed. This model provided a better link between the presumed structure and the function according to the proton-motive Q-cycle mechanism. Indeed, in this

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model the 2 centre i inhibitor-resistant regions were found on the inner side of the membrane and the 2 centre o inhibitor-resistant regions on the outer side [25].

Cytochrome *b* mutants with a deficiency in respiratory growth (i.e. being unable to grow on a non fermentable substrate such as glycerol or ethanol), and carrying a mutation in the apocytochrome *b* gene, have been isolated in Piotr Slonimski's laboratory, sequenced and biochemically characterized ([26]; D. Lemesle-Meunier et al., in preparation). Such deficient mutants were used for the selection of revertants having a structural modification (i.e. an amino-acid change in the apocytochrome *b* sequence) compatible with a partial or total functioning of the bc_1 complex ([27,28]; J.-Y. Coppée and A.-M. Colson, in preparation). Interestingly, all the long distance reversions obtained so far map to the same side of the membrane as for the respiratory deficient mutant from which it has been selected. Moreover, they brought additional data in favor of the functional relationship and possibly spatial proximity between the 2 centre i subregions on the one hand and the 2 centre o subregions on the other.

Some revertants exhibited a thermosensitive phenotype being unable to grow on glycerol at 37°C and presenting weak growth at 28°C. One such revertant carrying a monosubstitution located between the positions of 2 centre o inhibitor-resistant mutations has been used for the selection of secondary thermoresistant revertants [29]. Some double changes presented an increased centre o inhibitor-resistance. Triple amino-acid changes partially compatible with the function were also found. In the present paper, a new class of revertants showing possible interesting structural changes will be described. These revertants derived from 3 frameshift mutations located in 3 distinct subregions of the apocytochrome *b* (Fig. 1). The first (G104) belongs to an extramembranous loop between transmembrane α-helices 2 and 3, at the inner side of the membrane; no mutation, so far, has been described in this part of cytochrome *b*. The second mutation (H204) is located in the extramembranous loop between transmembrane α-helices 4 and 5, which is postulated to be part of the second centre i subregion. This loop is located close to the heme b_{562} histidine ligand in position 197 and it carries centre i inhibitor resistant

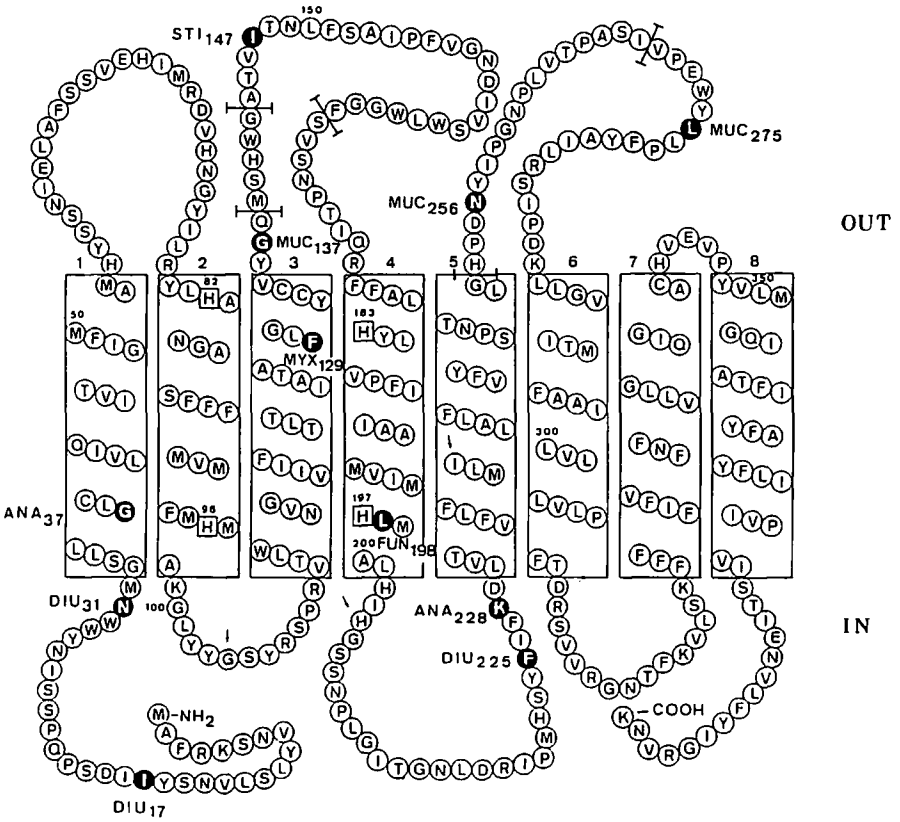

Fig. 1. Cytochrome *b* folding model in the mitochondrial inner membrane. The model represented here is the 8 α-helix folding model. The amino acids involved in inhibitor resistance are shown in white surrounded by a black circle (ANA: antimycin; DIU: diuron; STI: stigmatellin; FUN: funiculosine; MYX: myxothiazol; MUC: mucidin). IN and OUT represent, respectively, the inner and outer sides of the mitochondrial inner membrane. Arrows indicate the positions of the frameshift mutations affecting the cytochrome *b* gene (located here in the correponding amino acids in the cytochrome *b* sequence). The histidines involved in the binding of the 2 haem groups of the cytochrome *b* (His 82–183 and His 96–197) are surrounded by squares; (———) indicates the position of the exon limits of the cytochrome *b*.

mutations. One missense mutation (S206) in the vicinity of H204 and its revertants is also under study in this region (J.-Y. Coppée and A.-M. Colson, in preparation). The third mutant (I239) is located in the sixth transmembrane α-helix at the C terminal end of the second centre i subregion; no mutants so far are known in this portion of cytochrome *b*.

Materials and Methods

The frameshift mutants belong to the collection of Piotr Slonimski (Gif, France) and are derived from the parental strain: 777–3A α *ade1 op1*. Their names are the following: haploid P318 (diploid KP30) is affected at glycine104 (and will therefore be referred to as G104), haploid G1988 (diploid KM308) is affected in histidine 204 (referred to as H204) and haploid M2282 (diploid KM656) is affected in isoleucine 239 (referred to as I 239) (see Table I for further details). Selection, genetic analyses and sequence analyses of frameshift mutants and their revertants were performed as described in [27–29]. Growth curves and NADH- and succinate oxidase activity analyses were done according to [30].

Results and Discussion

Three respiratory growth deficient mutants carrying a frameshift mutation in the cytochrome *b* gene were used for the selection of revertants (Table I). The first mutant carries a mutation in codon 104, which results in a frameshift that leads to a stop codon in 107. The deduced amino-acid changes are G104W-S105F-Y106I-R107stop (Fig. 1). The second mutant carries its frameshift mutation in codon 204 with a stop codon in 208. The deduced amino-acid changes are H204S-G205W-S206F-S207I-N208stop. The third mutant carries its frameshift mutation in codon 239 with a stop codon in 240. The deduced amino-acid change is I239I-L240stop. The 3 mutants are totally unable to grow on glycerol and must carry a truncated apocytochrome *b*.

The characterization of the revertants by sequence analyses revealed the following:

52 reversions were sequenced. 28 were extragenic reversions; they keep the original frameshift mutation and carry a long distant suppressor. They are probable informational suppressors involved in mitochondrial protein synthesis. Those that were genetically analysed are of mitochondrial heredity. They are not interesting for our purpose.

Among intragenic revertants, from 1 to 5 adjacent amino-acid changes were identified. More than one reversion of each type was identified. Two independent reversions at codon 104 resulted in a Y103I change and 6 revertants carried the triple change L101Y-Y102I-Y103I. Nine independent reversions of codon 204 resulted in the H204Y change and 4 revertants carrying the double change H204S-G205C were found. Three independent reversions of codon 239 resulted in 5 adjacent changes: L235T-F236I-M237Y-L238V-I239N. Because the same change was found in several genetically independent reversions, it is less likely that these changes would be polymorphisms. A double mutation can be excluded, because the revertant frequency is about 10^{-7} and the double-mutation frequency would be too low (10^{-14}) to be detected in our screening procedure. None of the revertants was found to return to the wild type sequence.

The respiratory growth of each intragenic revertant was not drastically affected. Based on growth curves in glucose, galactose, ethanol and lactate, the generation time of each revertant was identical (or poorly affected) to that of its parental strain.

None of the intragenic revertants presented temperature sensitive respiratory growth, i.e., absence of growth on nonfermentable substrate on glycerol at 37°C or at 22°C being able to grow at 28°C.

TABLE I

Cytochome b gene intragenic reversions derived from frame shift mutations

Three cytochrome *b* deficient mutants carrying a frameshift mutation were used for the selection of revertants. G104, H204 and I239 represent the positions carrying the frameshift mutation. Intragenic revertants were sequenced. Total indicates the number of revertants sequenced. One-letter code is used for the amino-acid terminology. Codon corresponds to the sequence of amino acids affected by the frameshift mutations and their reversions.

(a) Frameshift in G104

Codon	101	102	103	104	105	106	107	Total
WT	L	Y	Y	G	S	Y	R	
Mut	L	Y	Y	**W**	**F**	**I**	**STOP**	
Rev 1	L	Y	**I**	G	S	Y	R	2
Rev 2	**Y**	**I**	**I**	G	S	Y	R	6
Extragenic revertants								

(b) Frameshift in H204

Codon	204	205	206	207	208	Total
WT	H	G	S	S	N	
Mut	**S**	**W**	**F**	**I**	**STOP**	
Rev 1	**Y**	G	S	S	N	9
Rev 2	**S**	**C**	S	S	N	4
Extragenic revertants						18

(c) Frameshift in I239

Codon	235	236	237	238	239	240	Total
WT	L	F	M	L	I	L	
Mut	L	F	M	L	I	**STOP**	
Rev 1	**T**	**I**	**Y**	**V**	**N**	L	3
Extragenic revertants							4

None of the intragenic revertants presented an in-vivo- or in-vitro-increased resistance to antimycin and diuron.

Cytochrome *b* appeared thus to tolerate important structural changes with little functional effects. From 1 to 5 adjacent amino-acid changes were identified. The 5 adjacent amino-acid changes that carried reversions occurred between amino acids 235 and 239 in the sixth transmembrane α-helix, which is a non conserved region of apocytochrome *b* located downstream to the second centre i subregion. In spite of the important structural change caused by this mutation, no major functional change has been observed, suggesting that this portion is not directly involved in the catalytic activity, and that it has no major role in the biogenesis or stability of the complex.

A triple and a single amino-acid change was found in a non conserved region between amino acids 101 and 104; this region is also not conserved, and does not seem, so far, to play a role in either the biogenesis, stability or catalytic activity of the complex.

Finally, single and double amino-acid changes were found in positions 204 and 205. These amino acids belong to a subregion of apocytochrome *b*, which is presumably part of centre i. The changes described here are natural in plant mitochondrial cytochrome *b* sequences.

One revertant, however, presented a partial functional alteration. It was found to carry the H204Y change in the extramembranous loop, which belongs to the presumed second centre i subregion. In vitro, i.e., on isolated uncoupled mitochondrial membranes, a 35% reduction in the NADH-oxidase activity (186 in the mutant instead of 286 nmol O_2 min^{-1} mg^{-1} protein for the parental strain) has been found in this mutant. Similar reduction of the NADH activity has been observed with revertants from missense mutations proximal to this position, involving amino acids 206 and 208, and also with a long distant reversion of a mutation in 206 located at position 30, which belong to the first presumed centre i region (Jean-Yves Coppée and Anne-Marie Colson in preparation). The bc_1 complex might become rate limiting in this revertant. Therefore, the extramembranous loops near positions 30 and 204-206-208 may be involved in the catalytic activity at centre i.

On the basis of the significant but limited reduction of NADH oxidase activity in revertants with mutational changes affecting the extramembranous loops in positions 30, 204, 206 and 208, which belong to the centre i subregions, we suggest that these amino acids might be directly involved in the catalytic site. Indeed, it is possible that this region would constitute a quinone binding site of the bc_1 complex; the cytochrome b_6 of the chloroplast b_6f complex is divided in 2 subunits in that loop, which might explain the antimycin insensitivity of the b_6 complex and the failure to find stable ubisemiquinone. An effect on the ubisemiquinone EPR signal at centre i should be tested in those structurally modified cytochromes *b*.

The cornerstone of our genetic approach to structure-function relationships of cytochrome *b* is, on one hand, the selection of functional inhibitor-resistant mutants, and, on the other, the selection of functional revertants of respiratory growth deficient mutants. The major interest of this approach is that the screening of mutants covers a large number of mutational events and allows the cell to indicate positions, type of changes, pairs of changes, or multiple changes, which may affect structurally the topology of the protein and its ligand binding sites, but are properly assembled and only partially altered functionally in vivo.

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