Isolation and RNA sequence analysis of cytochrome *b* mutants resistant to funiculosin, a center i inhibitor of the mitochondrial ubiquinol-cytochrome *c* reductase in *Saccharomyces cerevisiae*

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Received 6 February 1990

Funiculosin is a well-known inhibitor of the mitochondrial respiratory chain, probably acting at the ubiquinone reducing site or center i of QH_2-cytochrome *c* reductase. We report here the isolation, mapping and RNA sequence analysis of yeast apo-cytochrome *b* mutants resistant to this inhibitor. Funiculosin-resistance was found to be conferred, in 4 independent isolates, upon replacement of a leucine residue by phenylalanine in position 198 of the cytochrome *b* polypeptide chain.

Funiculosin resistance; Mitochondrial cytochrome *b*; Membrane protein folding; RNA sequencing; Mutational analysis; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

In the mitochondrial respiratory chain, the QH_2-cytochrome *c* reductase (bc_1 complex) transfers electrons from ubiquinol to cytochrome *c*, which is coupled to the electrogenic translocation of protons across the mitochondrial inner membrane [1,2]. The bc_1 complex from *S. cerevisiae* consists of 9 polypeptides, 3 of which have known electron transfer functions (cytochrome *b*, cytochrome c_1 and the Rieske FeS protein). Apo-cytochrome *b* is encoded by the mitochondrial DNA, while all the other subunits are of nuclear origin.

The electron transfer from ubiquinol to cytochrome *c* is best described in terms of a proton motive Q-cycle mechanism [3]. This implies that cytochrome *b* would be responsible for a transmembrane electron transfer between two ubiquinone oxidoreduction centers, these are referred to as center o and center i, and are located near the electropositive and electronegative side of the membrane.

Several organic compounds, most of which are naturally produced by bacteria, inhibit cytochrome *b* oxidoreduction (see [4] for review). Antimycin, funiculosin and diuron are thought to exert their action at center i, while myxothiazol, mucidin (or strobilurin A) and stigmatellin would block center o.

Numerous yeast mutants resistant to antimycin [5,6], diuron [7], myxothiazol [8], mucidin [9–11] and stigmatellin [12] were localized in the split mitochondrial gene coding for apo-cytochrome *b* (gene *COB-BOX*) [8,13–15]. The RNA sequence analysis of more than 30 of them revealed that inhibitor-resistance was conferred upon replacement at a few positions of the cytochrome *b* polypeptide chain [12,16,17]. These results have important implications concerning the cytochrome *b* structure in the membrane and define regions of the protein which might belong to the quinone reaction domains of the bc_1 complex.

We report here the isolation, mapping and RNA sequence analysis of yeast apo-cytochrome *b* mutants resistant to the center i inhibitor funiculosin. Resistance to this inhibitor was found to be conferred upon replacement of a leucine residue by phenylalanine in position 198 of the cytochrome *b* polypeptide chain.

2. MATERIALS AND METHODS

2.1. *Strains*

2.1.1. Wild type strains

KL14-4A, *a his1 trp2 oli1-1 cap1-321 par1-454*; D273-10B/A21, *alpha met oli1-625 ery3-624 par1-626*; D8-22A *alpha his4 ade2*; D225-5A, *alpha ade1 lys2*.

2.1.2. Resistant mutants

Mucidin [10,11]: D225-5A/3D *muc1-771*; K50-15B *ura1 muc3-K50-15B*. D225-5A/101-4B *muc2-772*; diuron [13]: D273-10B/A21 *diu1-724*; antimycin [5]: D8-22A/G25 *ana2-G25*.

2.1.3. *rho*$^-$ strain

The *rho*$^-$ D273-10/A21/R11 has retained from the D273-10B/A21 *rho*$^+$ mitochondrial genome, a segment encompassing the entire *COB-BOX* gene (Slonimski, unpublished results).

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.2. *Media*

YP10: 10% glucose, 1% yeast extract, 1% bacto peptone (Difco); YPGA: 2% glucose, 1% yeast extract, 1% bacto peptone, 10 mg/l of adenine; N3: 2% glycerol, 1% yeast extract, 1% bacto peptone. Solid media contained 2% agar. Inhibitors were added as ethanolic solution to agar-containing media in surfusion at 50°C before solidification to the following final concentrations: diuron, 100 μM; myxothiazol, 1.5 μg/ml; mucidin, 0.3 μg/ml; funiculosin, 5 μg/ml; antimycin, 0.1 μg/ml.

2.3. *Chemicals and genetic methods*

Standard genetic methods were as described in [13]. Antimycin and myxothiazol were purchased from Boehringer. Diuron 3-(3,4-dichlorophenyl)-1,1-dimethylurea, was obtained from E.I. Du Pont de Nemours. Mucidin and funiculosin were gifts from J. Subik (Food Research Institute, Bratislava, Czechoslovakia) and M. Briquet (Laboratoire de l'Hérédité Cytoplasmique, Louvain-la-Neuve, Belgium), respectively.

2.4. *Isolation of the funiculosin-resistant mutants*

A YP10 culture of the wild type strain KL14-4A was plated out for single colonies on YPGA. Individual subclones picked up at random were grown in YP10 and then plated on N3 supplemented with 5 μg/ml of funiculosin at a density of about 5×10^8 cells per plate. Each subclone produced a different amount of funiculosin-resistant colonies after a 5–10-day incubation at 28°C. Some subclones did not give any drug-resistant clone at all. Only one mutant from each parental strain subclone was retained for further analysis. The mutants were subcloned on YPGA and checked for funiculosin resistance.

2.5. *Isolation of mitochondria and respiratory activity measurements*

These were as described in [18].

2.6. *RNA sequencing*

Mitochondrial RNA extraction and RNA sequencing were as described in [17].

3. RESULTS

Spontaneous mutants exhibiting resistance to funiculosin have been isolated from strain KL14-4A on glycerol in the presence of 5 μg/ml of funiculosin, in such a way as to be certain that they resulted from independent mutational events (see section 2 for procedure). The parental strain KL14-4A possesses the long version of the split mitochondrial cytochrome *b* gene (*COB-BOX* gene) which is 7172 bp long and contains 6 exons, the sizes of which range from 14 to 415 bp.

Mutants arising from a mutational event modifying the control of the cell permeability were identified by their increased tolerance to cycloheximide as described in [19]. Among 40 isolates, 36 were resistant up to 1 μg/ml of cycloheximide. Four mutants, KL14-4A/*fun1-210*, KL14-4A/*fun1-226*, KL14-4A/*fun1-232* and KL14-4A/*fun1-236* had a cycloheximide sensitivity similar to the wild type (complete growth inhibition at 0.1 μg/ml).

The genetic mapping of the funiculosin-resistant/cycloheximide-sensitive mutants has been performed as described in [13]. It revealed that all 4 mutations were within the cytochrome *b* gene. Indeed, in crosses between the *fun* mutants and the *rho*$^-$ strain D273-10B/A21/R11 (see section 2) carrying the wild type cytochrome *b* gene, sensitive recombinants were found at a frequency fluctuating between 35 and 45%, indicating the presence of the wild type allele of the *fun* mutations in the cytochrome *b* gene.

Allelism tests have been performed between the *fun* mutations and 5 *COB-BOX* inhibitor-resistant markers whose exact positions were determined previously [12,17]: the mucidin-resistant mutations *muc1-771* (Gly_{137}-Arg, in exon B1), *muc3-K50-15B* (Asn_{256}-Tyr, in exon B5) and *muc2-772* (Leu_{275}-Ser, in exon B6), the diuron-resistant mutation *diu1-724* (Phe_{225}-Ser, in exon B4) and the antimycin-resistant mutation *ana2-G25* (Lys_{228}-Met, in exon B4). The frequency of double-sensitive recombinants was below 0.5% when the funiculosin-resistant mutants were crossed with the diuron- and antimycin-resistant mutants (see Table I). When crossed with the mucidin-resistant mutants, double-sensitive recombinants appeared at a frequency of 2% with *muc2-772* and *muc3-K50-15B* and of 10% with *muc1-771*. A high bias in parental phenotype transmission occurred with *muc2-772* and

Table I

Allelism tests between funiculosin-resistant and various cytochrome *b* inhibitor-resistant mutants

	muc1-771	*ana2-G25*	*diu1-724*	*muc3-K50-15B*	*muc2-772*
fun1-210	22[a]:191[b]:2.6[c]	0:193:0.5	1:200:0.9	6:199:3.1	4:199:31.3
fun1-226	19 :199 :1.9	0:200:0.8	0:198:1.6	4:200:3.2	3:200:26.2
fun1-232	16 :196 :1.8	0:198:0.6	0:199:1.0	3:200:3.2	2:100:23.5
fun1-236	22 :200 :1.7	0:198:0.6	0:198:0.9	4:197:2.4	3:100:31.6

[a] Number of double sensitive recombinants

[b] Total colonies tested

[c] Parental phenotype ratio (Fun^sDrug^s/Fun^sDrug^r)

The funiculosin-resistant mutations were first shown to belong to the cytochrome *b* gene. Their locations within the cytochrome *b* gene were then probed by allelism tests with 5 cytochrome *b* resistant markers of known position (see text for further details). fun, funiculosin; diu, diuron; ana, antimycin; muc, mucidin

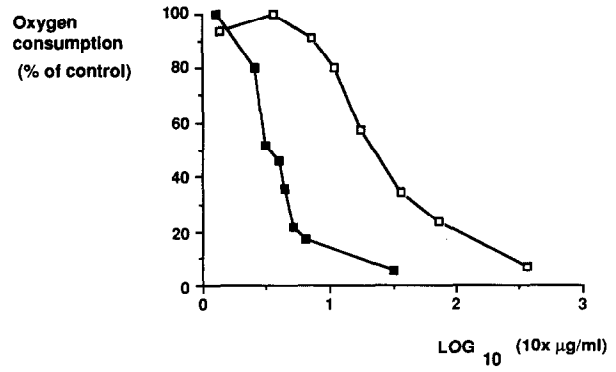

Fig. 1. Effect of funiculosin on NADH oxidation of wild type and funiculosin-resistant mutant mitochondria. The protein concentration was 0.185 mg/ml for strain KL14-4A (■) and 0.33 mg/ml for mutant KL14-4A/*fun1-210* (□). Mitochondria were preincubated in the presence of funiculosin and 0.5 µM CCCP for 4 min before starting the reaction with the addition of NADH to a 2 mM final concentration. Control respiration rate (nmol O_2/min per mg) was 102 for the wild type and 150 for the mutant.

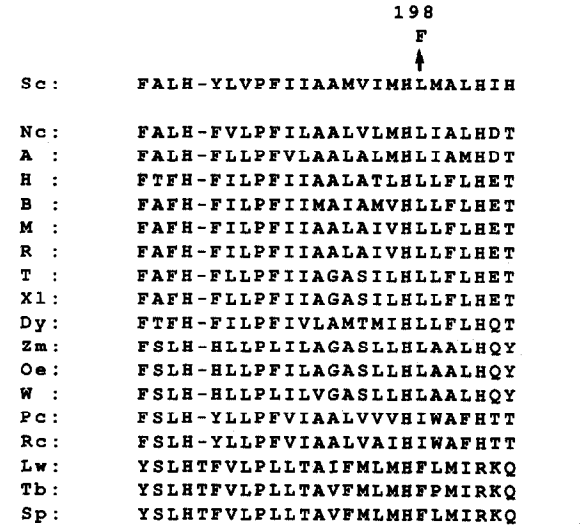

Fig. 2. Amino acid alignments of the wild type cytochrome *b* (b_6 in chloroplasts) region from 18 different species encompassing position 198 involved in funiculosin resistance in *S. cerevisiae*. Sc, *S. cerevisiae*, [37]; Nc, *Neurospora crassa* [38]; A, *Aspergillus nidulans* [39]; H, human [40]; B, bovine [41]; M, mouse [42]; R, rat [43]; Zm, *Zea mais* [44]; Oe, Oenothera [45]; W, wheat [46]; Xl, *X. laevis* [47]; Dy, *Drosophila yakuba* [48]; Pc, *Paracoccus denitrificans* [49]; Rc, *Rhodobacter capsulatus* [50]; Lw, liverwort [51]; Tb, tobacco [52]; Sp, spinach [53].

muc3-K50-15B (ratio between parental phenotypes, Fun^rMuc^s/Fun^sMuc^r, of about 25 and 3, respectively, see Table I). Thus, recombination frequency with these tester markers was largely underestimated.

Surprisingly, no double-resistant recombinant Fun^rMuc^r was present in the crosses between the funiculosin- and the mucidin-resistant mutants (data not shown). One explanation could be that such a recombinant is respiratory deficient. By contrast, some rare double-resistant recombinants Ana^rFun^r and Diu^rFun^r were observed when the funiculosin-resistant mutants were crossed with the antimycin- and diuron-resistant mutants.

The allelism tests indicated that the funiculosin-resistant mutations most likely affect exon B4 of the gene *COB-BOX*. The mutant RNA sequence analysis revealed a unique base change in exon B4 relative to the wild type which replaces Leu_{198} by phenylalanine (TTA-TTT). Considering the agreement between genetic and sequence data, and the fact that the 4 mutants (independent isolates) carry the same base change, demonstrates that this mutation is truly responsible for funiculosin resistance.

Oxygen consumption on isolated mitochondria of mutant KL14-4A/*fun1-210* was much less sensitive to funiculosin than the wild type. The concentration of funiculosin required for a 50% inhibition was 8 times higher for the mutant than for wild type (Fig. 1). No difference in antimycin inhibition could be detected between mutant and wild type (data not shown).

4. DISCUSSION

Four funiculosin-resistant mutations in the cytochrome *b* gene of *S. cerevisiae* have been characterized. These mutants replace a leucine residue by phenylalanine in position 198 of cytochrome *b* polypeptide chain. Funiculosin resistance was expressed in vitro at the level of the mitochondrial respiratory chain, whereas no modification in antimycin, diuron or mucidin sensitivity could be observed. The mutant respiratory activity/growth was nearly the same as the wild type.

Funiculosin-resistant mutants were already localized previously in the cytochrome *b* of *S. cerevisiae* [20]. The mutations mapped to one locus, *FUN1*, in the fourth exon of the gene *COB-BOX* [14]. Unfortunately, these mutants were lost and could not be sequenced.

$Leucine_{198}$ is conserved in all the known mitochondrial cytochrome *bs* (see Fig. 2). It is replaced in the corresponding position by isoleucine in *Paracoccus denitrificans* and *Rhodobacter capsulatus*, and by phenylalanine in cytochrome b_6 of chloroplast, which could explain why the b_6f complex is insensitive to funiculosin.

Molecular bases for resistance to other bc_1 complex inhibitors have been reported recently [12,16,17]. Resistance to the center i inhibitors antimycin and diuron was found to be conferred upon replacement in two regions of the cytochrome *b*, extending from residue 17 to 37 and 225 to 228. Two other regions, from residue 129 to 147 and 256 to 275, were shown to be involved in binding of the center o inhibitors myxothiazol, mucidin and stigmatellin. The present work extends to residue 198, the second center i inhibitor-resistant region.

According to a folding model for cytochrome *b* with 9 transmembrane helices, proposed by Widger et al. [21] and Saraste [22], center i as well as center o inhibitor-resistant mutations would be located on both sides of the membrane (Fig. 3). As already stressed in [12,17], the separation of the two center i and the two center o inhibitor-resistant regions on opposite sides of the membrane seems to be inconsistent with their possible belonging to a common structure. The withdrawal from the membrane of helix IV of the Widger/Saraste model has been proposed from other calculations [23–25]. According to the resulting 8 helix model, the two center i inhibitor-resistant regions would be located on the same side of the membrane (presumed electronegative side), whereas the two center o inhibitor-resistant regions would belong to the opposite side (electropositive) (Fig. 3). Most of the mutations would affect extramembrane segments, the few mutations buried in the membrane being near the interface. This seems to be consistent with two quinone binding sites on opposite sides of the membrane where the inhibitors would exert their action.

Despite their common ability of blocking the same reaction (reoxidation of heme b_{562} by ubiquinone) antimycin, diuron and funiculosin have no evident structural homology in common. They differ also in some of their effects: funiculosin and diuron, for example, do not induce, as does antimycin, a red shift in the reduced spectrum of heme b_{562} [18,55]. This implies some specificity in inhibitor binding which is likely responsible for the mutant alteration and phenotype specificity ([12,16,17], this work). The funiculosin-resistant mutation (leucine$_{198}$ into phenylalanine), for example, does not modify the sensitivity to diuron or antimycin. However, some lines of evidence indicate that the center i inhibitor binding sites might overlap or interact partially with each other. Some mutations interfere with several inhibitors: the antimycin-resistant mutation *anaI-G1a* (glycine$_{37}$ into valine) confers a weak but significant increased tolerance to funiculosin [17,20]; the diuron-resistant mutants are cross resistant in vitro to HQNO [18]. Moreover, antimycin displaces HQNO from its binding site [26,27] and diuron reverses the red shift induced by HQNO [18]. Finally, the sequence data

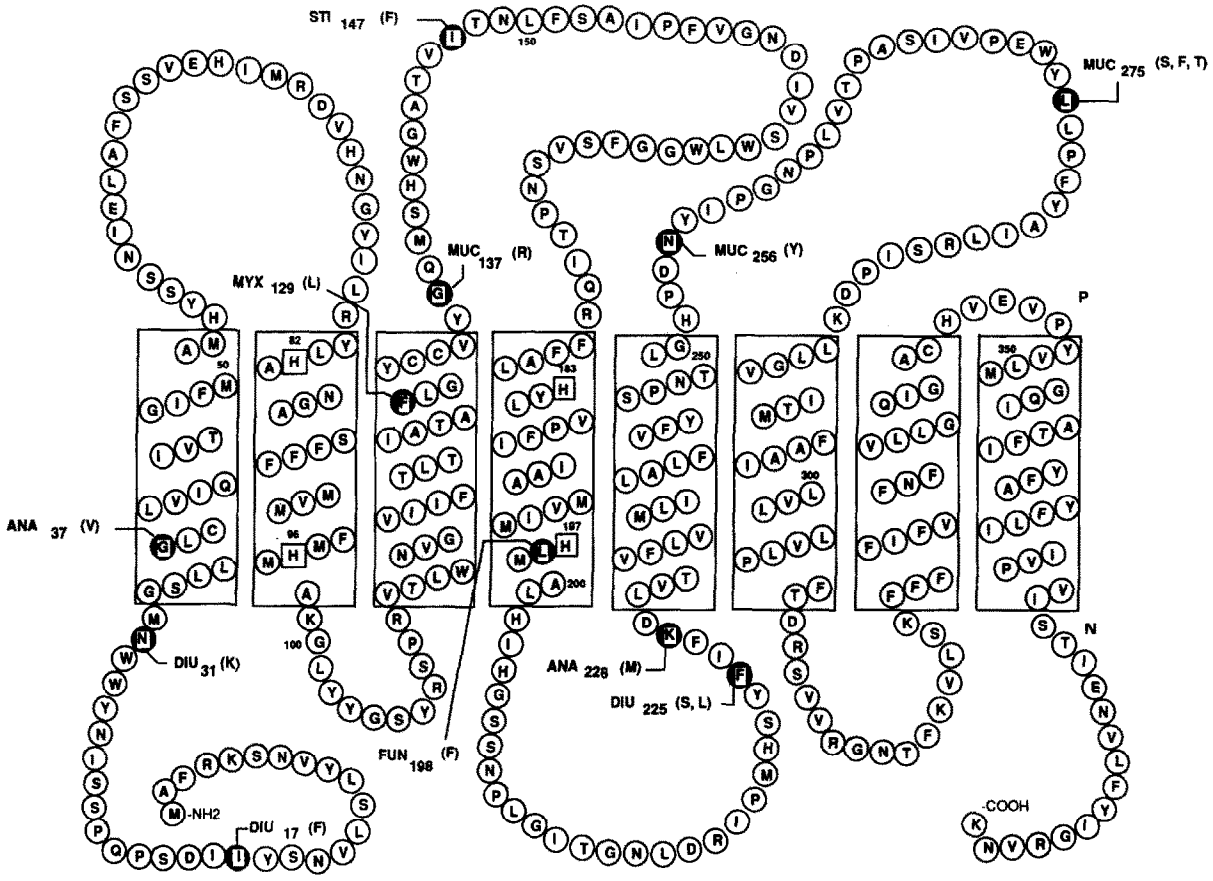

Fig. 3. Cytochrome *b* structure in the inner mitochondrial membrane. The 8 α-helix model is that of Widger et al. [21] and Saraste [22] after the withdrawal of helix IV according to Di Rago and Colson [17]. Amino acids involved in inhibitor-resistant mutants are shown in white surrounded by a black circle: ANA, antimycin; DIU, diuron; MYX, myxothiazol; MUC, mucidin; STI, stigmatellin [12,16,17] and FUN, funiculosin (this work). Numbers in subscript are the amino acid position. P and N represent, respectively, presumed positively and negatively charged sides of the inner mitochondrial membrane.

([12,16,17], this work) reveal that all the corresponding mutations are clustered in two regions which appear to belong to the same side, i.e. the inner side of the mitochondrial membrane.

From the bacterial photosynthetic reaction center and its chloroplastic counterpart, the photosystem II, it might be supposed that this inhibitor-resistant domain could be involved in binding of ubiquinone at center i of the bc_1 complex. It has been known for some time that the QB ubiquinone (plastoquinone) reducing site of the reaction center is blocked by competitive binding of several inhibitors or herbicides, among them diuron [28,29]. Herbicide-resistance was found to be conferred upon replacement in the ubiquinone (plastoquinone) QB binding subunit (subunit L in photosynthetic bacteria [30,31], D1 in photosystem II of chloroplasts [32–34]). From the X-ray structure of the *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* reaction centers, it appears that the mutations belong to the QB binding domain [35,36,54].

It is tempting by analogy, to assume that the center i inhibitor-resistant mutations would belong to a common structure overlapping or interacting with the center i ubiquinone binding site. This view is enhanced by the present work which shows that funiculosin resistance is conferred upon replacement beside histidine$_{197}$, which is thought to be involved in binding of the heme b_{562}, the presumed reductant of ubiquinone at center i.

In the absence of the X-ray structure of the bc_1 complex, this mutational analysis provides, thus, insight into the cytochrome *b* structure in the membrane and defines regions of the protein which might belong to the quinone reaction domains of the bc_1 complex.

Acknowledgements: J.-P.d.R. received an IRSIA fellowship, Belgium. A.-M.C. is a Research Associate to the FNRS, Belgium.

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