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FEBS Letters 339 (1994) 1–6

FEBS 13622

FEBS LETTERS

Analysis of revertants from respiratory deficient mutants within the center *N* of cytochrome *b* in *Saccharomyces cerevisiae*

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Received 22 October 1993; revised version received 27 December 1993

Abstract

Four modified cytochrome *b*'s carrying mononucleotide substitutions affecting center *N* residues were analysed. The mutant carrying a G33D change does not incorporate heme into the apocytochrome *b* and fails to grow on non-fermentable carbon sources. Out of 85 genetically independent revertants derived from this mutant, 82 were true back-mutants restoring the wild type sequence (D33G). The remaining three replaced the aspartic acid by an alanine (D33A) indicating that small size residues are best tolerated at this position which is consistent with the perfect conservation of the G33 during evolution. This glycine may be of crucial importance for helix packing around the hemes. The replacement of methionine at position 221 by lysine (M221K) produced a non-functional cytochrome *b* [(1993) J. Biol. Chem. 268, 15626–15632]. Non-native revertants replacing the lysine 221 by glutamic acid (K221E) or glutamine (K221Q) expressed a selective resistance to antimycin and antimycin derivatives having a modified dilactone ring moiety. Cytochrome *b* residues in 33 and in 221 seemed to contribute to the quinone reduction (Q_N) site of the cytochrome bc_1 complex. Possible intramolecular interactions between the N-terminal region and the loop connecting helices IV and V of cytochrome *b* are proposed.

Key words: Mitochondrial cytochrome *b*; Respiratory deficiency mutation; Non-native reversion; Antimycin resistance; *Saccharomyces cerevisiae*

1. Introduction

In the mitochondrial respiratory chain, the cytochrome bc_1 complex (or cytochrome *c* reductase) catalyses the oxidation of ubiquinol by cytochrome *c* coupled to a vectorial translocation of protons across the mitochondrial inner membrane [1]. In *Saccharomyces cerevisiae*, it consists of at least ten different polypeptide subunits, three of which carry redox prosthetic groups: cytochrome *b*, cytochrome c_1 and the Rieske iron sulfur protein [2]. The cytochrome *b* plays a central role in the energy-transducing activity of the bc_1 complex by transferring electrons through the membrane from an electropositive ubiquinol oxidation proton ejector (Q_P) site to an electronegative ubiquinone reduction proton acceptor (Q_N) site [3].

Extensive mutational studies have allowed the identification of the axial ligands of the cytochrome *b* hemes and of several residues which are important for the function of the cytochrome bc_1 complex [4–8]. However, it should be noted that most of the mutations identified up to now affect the Q_P site whereas only a few mutations modifying the Q_N site have been found. Much information has been gained on the structural characteristics of the ubiquinol oxidation site but little is known on the quinone reduction site. We report here the isolation and characterization of four different functionally modified cytochrome *b* mutants carrying a single amino acid replacement in regions involved in the binding of antimycin, a Q_N site inhibitor. One of these mutants (G33D) was selected for the loss of the ability to grow on respiratory substrates. Three others (D33A, K221E and K221Q) were obtained as pseudo-revertants from the G33D mutant and from another respiratory deficient mutant (M221K) that has been described previously [9]. The results support the possible involvement of the G33 in helix packing around the hemes [10] and suggest intramolecular interactions between the N-terminal region and the loop connecting helices IV and V of the current 8-helix cytochrome *b* folding model [11–13].

2. Materials and methods

2.1. Media

YG: 1% yeast extract, 1% bactopeptone, 3% glycerol; YPGAL: 1% yeast extract, 1% bactopeptone, 3% galactose; YPD10: 1% yeast ex-

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SSDI 0014-5793(94)00006-H

tract, 1% bactopeptone, 10% glucose; ETOH: 1% yeast extract, 1% bactopeptone, 3% ethanol. Solid media contained 2% agar.

2.2. Chemicals

Antimycin was purchased from Boehringer Mannheim.

2.3. Strains

The two respiratory-deficient mutants and the wild type strain are from the Gif Collection. The wild type, KM 91, results from the cross between KL14-4A (*a his1 trp2 rho*^{0}) and 777-3A (*α ade1 op1 rho*$^+$) [14,15]. The original rho$^+$ mit$^-$ respiratory deficient mutants, M4410 and M7622 were isolated from the haploid strain 777-3A and carried the nuclear *op*$_1$ mutation [16]. They were crossed with KL14-4A (*a his1 trp2 OP1 rho*^{0}) in order to restore the nuclear genetic background allowing ATP/ADP translocation. The mtDNA sequence of M4410 has been determined by Lemesle-Meunier [9].

2.4. Oligonucleotides

The sequence of the primers and their positions on the split cytochrome *b* gene ('long version' of the gene [17–19]) are:

P1: 4516–4537: 5′-GTA TGA ATG CAT TGG AAT TCT-3′
P2: 5′-ATA TTT TAT AAA TAA ATA ATA ATA ATA TGG-3′
P3: 329–350: 5′-CCT ACA TTTCAT AAT AGT ACT C-3′
P4: 4383–440: 5′-CCC TCT AAT CCA GAG ATT CTTTGC G-3′
P5: 4579–4604: 5′-GAA TAG AAT ACA AAT AAT GCT AAA AT-3′

The position of the primers are given according to their first and last base pair numbers on the cytochrome *b* gene except for primer P2 which is just upstream the cytochrome *b* gene.

2.5. Isolation of revertants

As described in [5].

2.6. Total DNA extraction

Cells grown in 1 ml YPGAL overnight at 28°C were centrifuged 5 min at 2,000 × *g*. The pellet was treated with a suspension containing 400 μl 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 400 μl phenol, and glass pearls. The suspension was shaken vigorously for 5 min and centrifuged for 20 min. The aqueous phase was treated with an equal volume of phenol, centrifuged and extracted with an equal volume of chloroform. The nucleic acids were ethanol precipitated and resuspended in 10 mM Tris-HCl pH 7.5, 1 mM EDTA. For symmetrical PCR amplification 500 ng of total DNA were used.

2.7. Symmetrical and asymmetrical PCR

These were carried out according to McCabe [20]. For asymmetrical PCR, 20 ng of double stranded DNA were used. The couple P2-P3 was used for symmetrical amplification of DNA of the revertants from KM708 and the couple P4-P5 for symmetrical amplification of DNA of the revertants from KM108.

2.8. DNA Sequencing

Sequencing was carried out on asymmetrical PCR products using Sequenase kit (USB, Cleveland, Ohio, USA) with [^{35}S]dATP (Amersham). The respiratory deficient mutant M7622 (G33D) was sequenced by reverse transcription of the cytochrome *b* mRNA with avian myeloblastosis virus reverse transcriptase (Amersham Corp.) as described by di Rago and Colson [21].

2.9. Isolation of mitochondria and growth rates determinations

As described in [22].

2.10. Respiratory activities measurements

As described by Briquet and Goffeau [23].

3. Results

3.1. Genetic location and sequence determination of the mit$^-$ mutant M7622

The mit$^-$ mutant M7622 was derived from a strain (see Section 2) possessing the long version of the split mitochondrial cytochrome *b* gene which is 7,172 base pairs long and contains six exons [17–19]. According to the *cob-box* physical and genetic maps, the mutation should be located close to the 5′ end of the cytochrome *b* gene [17–19]. This was confirmed by sequence determination which revealed a unique nucleotide substitution in exon B1 leading to the replacement of the glycine in position 33 (GGT) by an aspartic acid (GAT). The perfect agreement between genetic mapping and sequencing data allows us to conclude that the detected apocytochrome *b* alteration is truly responsible for the mutant phenotype.

The mutant M7622 exhibited a stringent respiratory-deficient phenotype with total absence of growth on non-fermentable substrates even after prolonged incubation

Table 1
Summary of mtDNA sequence alterations, growth rates, respiratory activities and inhibitor sensitivities of the mit$^-$ mutants M221K and G33D and their revertants

Mutation	Number of revertants	T_m (°C)	Growth rate GAL	Growth rate ETOH	NADH oxidase activity	Succinate oxidase activity	I_{50} antimycin (pg/ml)	I_{50} Compound A (pg/ml)	I_{50} Compound B (pg/ml)	I_{50} Compound C (pg/ml)
Wild type	–	58	1	1	100	100	180	1.9	5.6	76
M221-K	–	55	–	–	0	0	–	–	–	–
M221-Q	8	49	1	1.2	61	95	480	7.2	12.8	226
M221-E	48	49	1.1	1.1	80	100	500	5.3	20	180
G33-D	–	–	–	–	0	0	–	–	–	–
G33-A	1	–	1	1	63	100	170	3.9	26	207

The mtDNA sequence of mutant M221K was determined by Lemesle-Meunier et al. [9]. NADH and succinate oxidase activities were assayed from mitochondria at 28°C in a Clark electrode and are expressed relative to the wild type KM91 (NADH and succinate activities were respectively 196 and 14 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ for strain KM91). The presence of a secondary mutation near the original mutated site was detected by oligonucleotide hybridization [5]. The fusion temperatures for the wild type, the respiratory deficient mutant M221K and its revertants are indicated under T_m (melting temperature (°C)). Growth in galactose (YPGAL) and ethanol (ETOH) media is expressed as the ratio of the mutant's growth rate in exponential phase relative to that of the wild type. The I_{50} values are the inhibitor concentrations (in pg/ml) required to reduce by 50% the NADH oxidase activity.

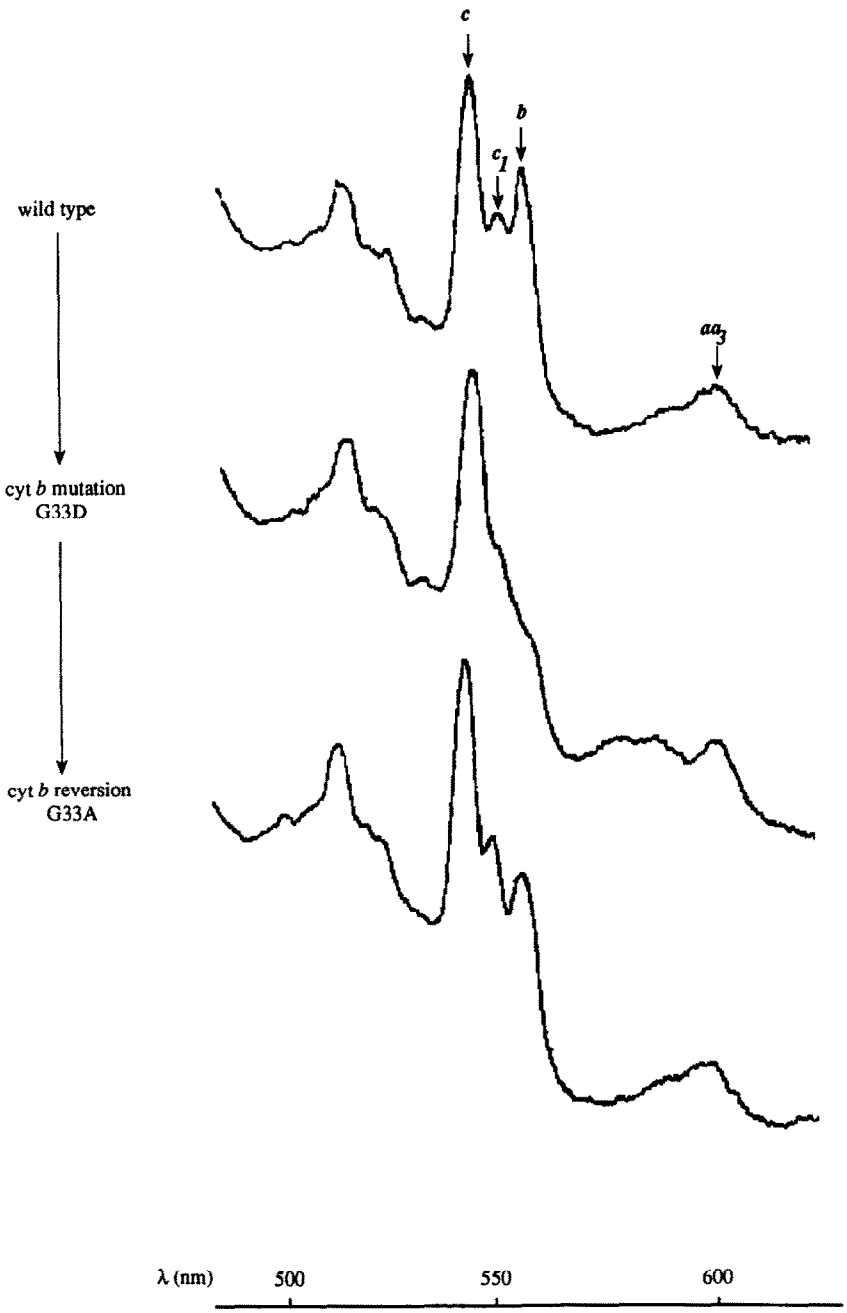

Fig. 1. Cytochrome absorption spectra of whole cells from the wild type (KM91), the respiratory deficient mutant G33D and its pseudo revertant. The spectra were recorded from whole cells at liquid nitrogen temperature after reduction of cytochromes by dithionite. Arrows indicate the positions of the a band absorption maxima of cytochrome *c*, cytochrome c_1 and cytochromes *a* and a_3.

periods (up to three weeks). Spectral analysis of whole cells grown on galactose revealed the complete absence of dithionite-reducible cytochrome *b* whereas cytochrome *c* and aa_3 were clearly detectable (Fig. 1). Mitochondria extracted from the mutant were devoid of NADH and succinate oxidase activities.

3.2. *Isolation and characterization of revertants from the mutants M4410 (M221K) and M7622 (G33D)*

The mutant M4410 has been shown to be the result of a single mutation at codon 221 (M221K/ATG-AAG) [9]. This mutation gives an assembled but inactive bc_1 complex resulting in total lack of growth on non-fermentable carbon sources. A total of 92 spontaneous revertants were selected (the revertant frequency was estimated to be $5 \cdot 10^{-8}$). The true wild type revertants were screened out by temperature sensitive hybridization with primer P1 as described in [5]. As a result, 36 appeared to be true wild type revertants having the same melting temperature as the wild type (i.e. 58°C), 56 showed a lower melting temperature than the respiratory deficient mutant (i.e. 49°C) and therefore were supposed to carry a

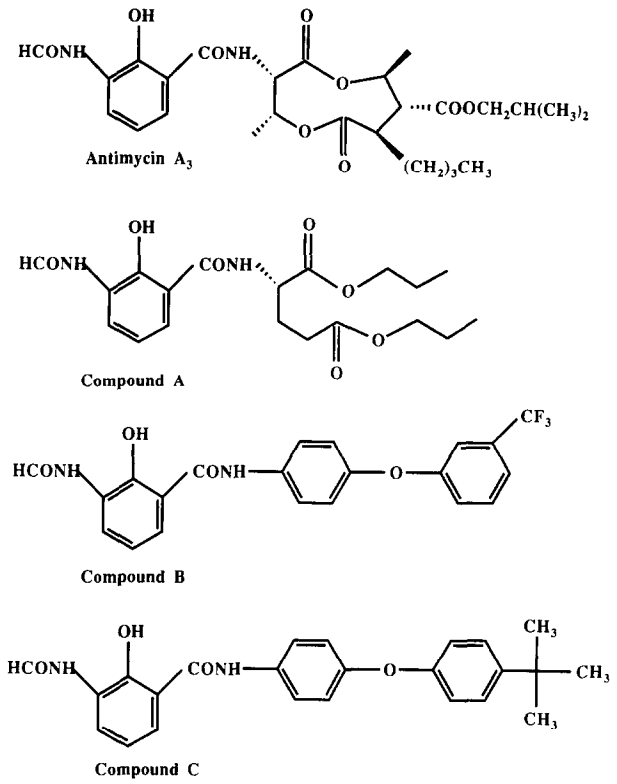

Fig. 2. Chemical structure of natural antimycin and synthetic derivatives. The structure of natural antimycin A was illustrated taking antimycin A3 as an example. Compounds A, B and C correspond to compounds 17, 7 and 6, respectively, in [25].

reversion in the close vicinity of the deficient mutation (Table 1). All the pseudo-wild type revertants were sequenced as described in Section 2. DNA amplification was first carried out on total DNA using primers P4 and P5. The amplified double stranded DNA was used for asymmetrical PCR amplification using only primer P4. The resulting single stranded DNA was sequenced by the chain termination method using primer P5 as template. The sequence results are presented in Table 1. Most of the revertants (48 revertants) carried E (GAG) and 8 revertants Q (CAG) instead of K (AAG) in position 221. These revertants showed growth on respiratory substrates which is comparable to that of the wild type; also they exhibited normal succinate respiration and only slightly reduced NADH oxidase activity (Table 1).

A total of 85 genetically independent revertants were selected from the mutant G33D (the revertant frequency was estimated to be $5 \cdot 10^{-7}$). All the revertants from this mutant were sequenced with primer P3 (after the symmetrical PCR amplification with the primers P2-P3 and the asymmetrical amplification with P2). The sequencing revelaed that all the revertants were true wild type revertants except three which replaced D (GAT) in position 33 by A (GCT). These revertants exhibited a significantly reduced NADH oxidase activity but grew on respiratory carbon sources (glycerol and ethanol) at the same rate as the wild type (see Table 1). Spectral analysis of these revertants revealed a significant reduction of cytochrome *b* whereas cytochromes *c* and aa_3 were not affected (Fig. 1).

We tested the in vitro resistance of each revertant to natural antimycin and three synthetic antimycin derivatives (compounds A–C) which possess the natural salicylic acid but not the dilactone ring structure (Fig. 2). These synthetic inhibitors have been shown to be good mimics of natural antimycin [25]. The I_{50} values (defined as the inhibitor concentration allowing 50% of the control respiratory activity) are shown in Table 1. The revertants carrying the substitution K221E or K221Q were resistant to all the inhibitors tested. On the other hand, the revertant possessing A instead of G in position 33 was specifically resistant to the three antimycin derivatives but sensitive to antimycin.

4. Discussion

Four different cytochrome *b* mutants with amino acid changes in positions 33 (G33D and G33A) and 221 (M221Q and M221E) were isolated and characterized. The mutant G33D screened for respiratory deficiency was found to lack NADH and succinate oxidase activities and was devoid of spectrally detectable cytochrome *b*. The characterization of revertants selected from this mutant led to the identification of a pseudo-reversion corresponding to the replacement of the G33 by A. No other pseudo-reversions were found although a large number of revertants were anlaysed. Thus only a small size residue such as alanine seems to be tolerated at position 33. According to the present cytochrome *b* folding model, the G33 is buried in the membrane near the N-terminal end of the transmembrane helix I. In recent compilations of about 800 cytochrome *b* sequences from various origins, it appeared that the G33 has been perfectly conserved during evolution indicating important steric constraints for the position 33 [26].

Three other glycine residues located in the bilayer are also perfectly conserved. These are G117 and G131 in helix III and G47 in helix I. The respiratory deficient mutation G131S prevents the assembly of cytochrome *b* [27]. Given to its location close to the heme *b*565, it was proposed that the G131 is important for helix packing around the hemes [6]. This has been further substantiated by the observation that the 4 conserved glycines in helices I and III are spaced by 13 residues similarly to the 4 heme liganding histidines in helices II and IV [10]. The present work gives experimental arguments that the G33 is indeed necessary for heme packing. The addition of a single methyl group in position 33 leads to substantial decrease in cytochrome *b* content and the bulkier aspartic acid leads to complete absence of heme incorporation into the apocytochrome *b* (Fig. 1).

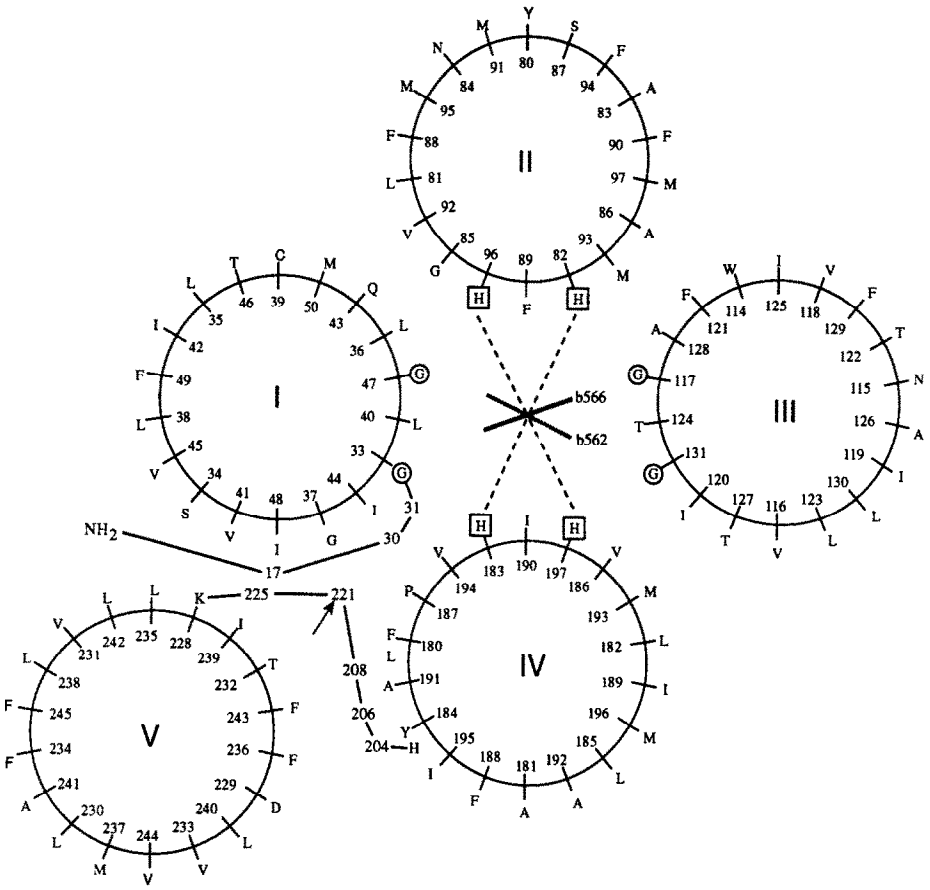

Fig. 3. Helical wheel representation of the trans-membrane *a* helices I, II, III and IV of cytochrome *b*. The numbers indicate the position of amino acids in the yeast cytochrome *b* sequence. The histidines involved in the binding of the two haems of cytochrome *b* are surrounded by squares and the four invariant glycines by circles. The M221 is indicated by an arrow.

The NADH and succinate oxidase activities are nearly totally abolished when the non-polar M221 is replaced by the positively charged lysine [9]. Also the antimycin-binding site is lost. Restoration of the wild type phenotype is obtained when the K221 is replaced by the negatively charged E or by Q. Therefore the respiratory deficiency observed in M221K might be due to the presence of a positive charge. According to the current cytochrome *b* folding model, the position 221 is located on the electronegative side of the mitochondrial inner membrane, in the loop linking helices IV and V. M221 is not conserved among species [26]. It is present in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* but is replaced by Phe in all other organisms (except maize where serine is found).

Since the two deficiency mutations are close to mutations conferring resistance to antimycin and diuron (in positions 31 and 225 (for the diuron resistance) and in position 228 (for the antimycin resistance)), it was determined whether our new alterations also confer resistance to antimycin and its derivatives. The natural salicylic acid moiety (hydroxy group in position 2 and formylamino group in position 3) is supposed to be essential to the inhibitory activity [28]. In contrast, the natural dilactone ring structure is not essential [25]. This moiety might regulate close fitting of the salicylic acid moiety to the binding site not only by increasing the hydrophobicity of the molecule but also by some steric factors [25,29]. Three synthetic antimycin derivatives used in this study have been shown to be good mimics of natural antimycin [25]. One could assume that the salicylic acid moiety of these inhibitors interacts to the binding site in the same manner to that of natural antimycin.

Irrespective of wide structural variations in the dilactone ring moiety, the reversions K221E and K221Q conferred resistance to both natural and synthetic antimycin derivatives (Table 1). It is therefore likely that the region surrounding position 221 interacts to the salicylic acid moiety of antimycin. The reversions K221E and K221Q also conferred resistance to diuron (data not shown), in accordance to the previous results [21,30]. The revertant D33A was specifically resistant to all synthetic antimycin derivatives, but not to natural antimycin. These results suggest that while position 33 is not directly involved in the interaction with the dilactone ring of natural antimycin, it interacts with the portion corresponding to dilactone ring moiety of synthetic inhibitors.

A tentative topology of the N-terminal part of cyto-

chrome *b* and of the loop linking the trans-membrane α helices IV and V relative to the catalytic core of the protein described by Tron et al. [10] is proposed in Fig. 3. We have taken into account the following observations:

(i) Mutations conferring specific resistance to HQNO and funiculosin have been identified in positions 232 in mouse [32] and 198 in yeast [33]. Reversions from respiratory deficiency mutations in 204 and 206 have been identified in positions 204, 205, 206 and 208 [34]. They confer resistance to funiculosin and to HQNO (for position 208) (Brasseur, G., and Brivet-Chevillotte, P., personal communication) and would therefore be located close to positions 198 and 232. The association of these amino acids would also bring together positions 202, 206 and 229 which could be implicated in proton conduction at the Q_N site [31].

(ii) We can also bring together positions 17 and 225 (where mutations conferring specific resistance to diuron have been identified [21,30]) as well as positions 37 and 228 (where mutations conferring specific resistance to antimycin have been identified [21]).

(iii) The positions 30 and 206 should also be relatively proximal since a respiratory deficient mutation in 206 can be compensated by a reversion in 30 [34].

(iv) Positions 31 and 221 could be close to each other because these are the only positions where mutations confer resistance both to antimycin (and to its three derivatives) and to diuron. These positions should be located near the catalytic core of the protein.

Amino acid changes affecting inhibitor resistance at center *N* were identified not only among mutants which have been selected for inhibitor resistance but also among revertants of deficient mutants. It is noteworthy that a double nucleotide change of the wild type sequence would be necessary to obtain the mutations in the revertants. This probably explains why these alterations have not been identified during the characterization of the inhibitor resistant mutants. Interestingly, these positions are clustered within the two predicted center *N* subregions which involve the loop between helices IV and V and the N-terminal end of helix I at the inner side of the mitochondrial inner membrane.

Acknowledgements: This work was supported by an EEC-SCI Contract No. 0010-C, an FRFC Contract No. 2.4536.88, the Ligue Nationale Française contre le Cancer. A.M.C. is Research Associate to the Belgian Science Foundation.

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