The EMBO Journal vol.8 no.13 pp.3951 - 3961, 1989

Mutations conferring resistance to quinol oxidation (Q_z) inhibitors of the cyt bc_1 complex of *Rhodobacter capsulatus*

Fevzi Daldal, M.K.Tokito, E.Davidson[1] and M.Faham

Department of Biology, Plant Science Institute, University of Pennsylvania, Philadelphia, PA 19104, USA

[1]Present address: Institute for Structural and Functional Studies, 3401 Market Street, Philadelphia, PA 19104, USA

Communicated by B.Chance

Several spontaneous mutants of the photosynthetic bacterium *Rhodobacter capsulatus* resistant to myxothiazol, stigmatellin and mucidin—inhibitors of the ubiquinol:cytochrome *c* oxidoreductase (cyt bc_1 complex)—were isolated. They were grouped into eight different classes based on their genetic location, growth properties and inhibitor cross-resistance. The *petABC* (*fbcFBC*) cluster that encodes the structural genes for the Rieske FeS protein, cyt *b* and cyt c_1 subunits of the cyt bc_1 complex was cloned out of the representative isolates and the molecular basis of inhibitor-resistance was determined by DNA sequencing. These data indicated that while one group of mutations was located outside the *petABC*(*fbcFBC*) cluster, the remainder were single base pair changes in codons corresponding to phylogenetically conserved amino acid residues of cyt *b*. Of these substitutions, F144S conferred resistance to myxothiazol, T163A and V333A to stigmatellin, L106P and G152S to myxothiazol + mucidin and M140I and F144L to myxothiazol + stigmatellin. In addition, a mutation (*aer126*) which specifically impairs the quinol oxidase (Q_z) activity of the cyt bc_1 complex of a non-photosynthetic mutant (R126) was identified to be a glycine to an aspartic acid replacement at position 158 of cyt *b*. Six of these mutations were found between amino acid residues 140 and 163, in a region linking the putative third and fourth transmembrane helices of cyt *b*. The non-random clustering of several inhibitor-resistance mutations around the non-functional *aer126* mutation suggests that this region may be involved in the formation of the Q_z inhibitor binding/quinol oxidation domain(s) of the cyt bc_1 complex. Of the two remaining mutations, the V333A replacement conferred resistance to stigmatellin exclusively and was located in another region toward the C terminus of cyt *b*. The L106P substitution, on the other hand, was situated in the transmembrane helix II that carries two conserved histidine residues (positions 97 and 111 in *R.capsulatus*) considered to be the axial ligands for the heme groups of cyt *b*. The structural and functional roles of the amino acid residues involved in the acquisition of Q_z inhibitor resistance are discussed in terms of the primary structure of cyt *b* and in relation to the natural inhibitor-resistance of various phylogenetically related cyt *bc*/*bf* complexes.

Key words: cyt bc_1 complex/cyt *b* mutants/membrane proteins/photosynthetic bacteria/$Q_{z(o)}$ inhibitors resistant mutants

Introduction

The ubiquinol:cytochrome *c* oxidoreductase (or the cyt bc_1 complex) is a membrane-bound, redox-driven proton pump present in mitochondria of eukaryotes and in many prokaryotes, including photosynthetic bacteria. A structurally and functionally similar complex, cyt b_6f, is also present in plant chloroplasts (for recent reviews see Dutton, 1986; Prince, 1986; Cramer *et al.*, 1987; Yang *et al.*, 1987; Malkin, 1988). During respiration and photosynthesis, these evolutionarily well-conserved, energy-transducing complexes catalyze electron transfer from the lipid-soluble quinol derivatives ubiquinol and plastoquinol, to the water-soluble electron acceptors, cytochrome *c* and plastocyanin. Concomitant with this electron transfer, protons are translocated vectorially across the cellular membrane, and the overall process contributes to the establishment of a proton motive force necessary for ATP synthesis (Mitchell, 1966).

The subunit composition of cyt bc_1 complexes depends on their origins (e.g. see Ljungdahl *et al.*, 1987). They always contain two *b*-type cytochromes (b_H and b_L) of different properties, carried by a single polypeptide of ~40 kd, a *c*-type cytochrome of ~30 kd and a 2Fe2S cluster containing protein of ~20 kd. In cyt b_6f complexes of chloroplasts the cyt *b* subunit is split into two components, cyt b_6 and subunit IV, which are homologous to the N- and C-terminal parts respectively, of bacterial or mitochondrial cyt *b* subunits (see reviews cited above). Additional subunits have also been observed in purified cyt bc_1 or b_6f complexes, and have been implicated in the binding of quinone and the biogenesis of the complex (Tzagaloff *et al.*, 1986; Yu and Yu, 1987).

The structural genes of the three redox-active subunits of the cyt bc_1 complexes have been isolated, and their nucleotide sequences determined from several bacterial species including *Rhodobacter capsulatus* (Gabellini and Sebald, 1986; Davidson and Daldal, 1987a), *Rhodobacter sphaeroides* (Davidson and Daldal, 1987b), *Paracoccus denitrificans* (Kurowski and Ludwig, 1987), *Bradyrhizobium japonicum* (Thony-Meyer *et al.*, 1989) and from the cyanobacterium *Nostoc* (Kallas *et al.*, 1988a,b). In *R.capsulatus* these three genes appear clustered as an operon, named *fbc* (Gabellini *et al.*, 1985) or *pet* (Daldal *et al.*, 1987), with the 5′ to 3′ order of the genes being *petA*(*fbcF*) (Rieske FeS protein), *petB*(*fbcB*)(cyt *b*) and *petC*(*fbcC*)(cyt c_1).

A cyt bc_1 complex is thought to contain two distinct catalytic domains on each side of the membrane (for reviews

Table I. Phenotype and genetic linkage of the Q_z Inh^R mutants of *R.capsulatus* and the base-pair and deduced amino acid changes corresponding to the mutations they carry

Group	Representative strain	Phenotype (*genotype*)	Co-trans. freq.[a] (*ins171*)	AA and bp change[b]	Cyt *b* domain
I	MXT101 SR18mxt22[c] SR18muc4 SR5mxt1	Ps^+, Myx^R, Muc^R (*myx101*)	9	G152S ($G_{1773}A$)	Q_zI
II_a	MXT102 MXT110 MXT112	Ps^+, Myx^R, Stg^R (*myx102*)	10	F144L ($T_{1749}C$)	Q_zI
II_b	MXT103 SR18muc1	Ps^+, Myx^R (*myx103*)	9	F144S ($T_{1750}C$)	Q_zI
III	STG1 STG4 STG6	Ps^+, Stg^R (*stg*1)	21	V333A ($T_{2317}C$)	Q_zII
IV	STG3	Ps^+, Stg^R (*stg*3)	8	T163A ($A_{1806}G$)	Q_zI
V	STG5	Ps^+, Stg^R (*stg*5)	0	not in *pet*	–
VI	STG10	Ps^+, Myx^R, Stg^R (*stg*10)	8	M140I ($G_{1739}A$)	Q_zI
VII	MUC21 MUC1 MUC2	Ps^+, Myx^R, Muc^R (*muc*21)	7	L106P ($T_{1636}C$)	?
–	R126	Ps^- (*aer*126)	9	G158D ($G_{1792}A$)	Q_zI

[a]The co-transduction frequency indicates the average percentage of Inh^S colonies found among at least 200 Kan^R transductants tested for each mutant using the insertion *ins171* :: *kan* linked to the cyt bc_1 cluster.
[b]The numbers indicate the position of the given basepair in the *pet*(*fbc*) operon (Davidson and Daldal, 1987a).
[c]SR18mxt22, SR18muc4, SR5mxt1 and SR18muc1 mutations were obtained directly using the plasmids SR18-404 or SR5-404 which carry a wild-type copy of the *pet*(*fbc*) operon.

see Crofts and Wraight, 1983; Rich, 1984, 1986; Prince, 1986). The quinol oxidation site (called Q_z in bacterial and Q_o in mitochondrial systems) is on the outer positive side of the membrane. It converts a quinol molecule to a quinone by transferring an electron to the Rieske FeS center and another to the lower potential cyt *b* heme (b_L). The electron accepted by the cyt b_L is subsequently transferred to cyt b_H (Glaser and Crofts, 1984; Robertson and Dutton, 1988) which then reduces a quinone trapped at the quinone reduction site (called Q_c in bacterial, Q_i in mitochondrial systems) located in the vicinity of the inner negative face of the membrane.

Several inhibitors are known to affect the reactions catalyzed at the $Q_{z(o)}$ and $Q_{c(i)}$ sites of the cyt bc_1 complex (for a review see von Jagow and Link, 1986). Myxothiazol, mucidin (or strobilurin A; von Jagow *et al.*, 1986) and stigmatellin (von Jagow and Ohnishi, 1985) interfere with the elecron transfer between ubiquinol, Rieske FeS protein and cyt b_L at the Q_z site. Although stigmatellin also affects the photosystem II in chloroplasts (Oettmeier *et al.*, 1985) its effect on quinol oxidation is similar in both cyt bc_1 and b_6f complexes (Nitschke *et al.*, 1989). On the other hand, myxothiazol has virtually no inhibitory effect on chloroplast cyt b_6f complex (Rich, 1984). Inhibitors such as UHDBT and UHNQ act on the electron flow further downstream from the Q_z site, between the FeS protein and the cyt c_1, and inhibitors like antimycin, funiculosin and HQNO affect the electron flow from cyt b_H to quinone at the Q_c site.

To date, mutants resistant to different classes of inhibitors have been isolated in both the budding (Subik, 1975) and the fission (Lang *et al.*, 1975) yeast, and in mouse (Howell *et al.*, 1983) mitochondria. We have also reported the isolation of similar mutants from the photosynthetic bacterium *R.capsulatus* (Daldal *et al.*, 1986). In the present study, the selection, analysis and determination of the molecular basis of several inhibitor-resistant (Inh^R) mutants of *R.capsulatus* are described. The differences in the amino acid sequence of a small region of cyt *b* from bacteria, mitochondria and chloroplasts are correlated with the natural inhibitor sensitivity or resistance of various cyt *bc*/*bf* complexes. In a separate study, the nature and extent of the functional effects of these mutations are characterized extensively (D.E.Robertson, F.Daldal and P.L.Dutton, in preparation).

Results

Isolation of R.capsulatus mutants resistant to Q_z inhibitors

Myxothiazol, mucidin (strobilurin A) and stigmatellin that affect the quinol oxidation (Q_z) site of the cyt bc_1 complex inhibited photosynthetic growth of *R.capsulatus* strain MT1131 on MPYE rich, or on RCV minimal, media at final concentrations of $\sim 10^{-6}$, $\sim 10^{-5}$ and $\sim 10^{-6}$ M respectively. They also arrested the respiratory growth of strain M6G, a quinol oxidase-minus derivative of *R.capsulatus* (Daldal, 1988), constrained to grow via the cyt bc_1–cyt $oxidase_{410}$-dependent branch of respiration. On the other hand, antimycin (and its derivatives), funiculosin and HQNO, which affect the quinone reduction (Q_c) reaction, showed no effect on photosynthetic growth of MT1131 or on respiratory growth of M6G at final concentrations of up

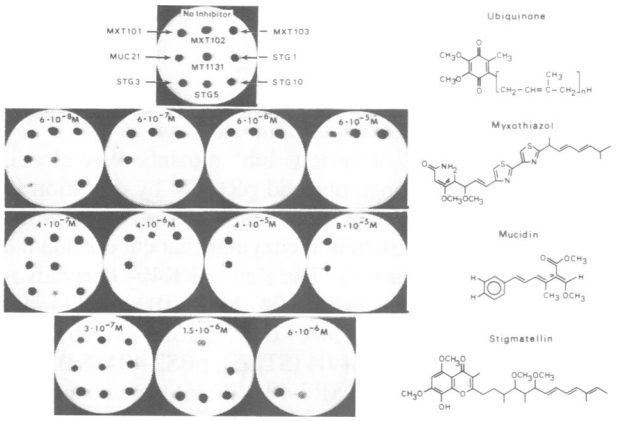

Fig. 1. The degree of inhibitor-resistance of various mutants to different inhibitors. In each case chemoheterotrophically grown cells were spotted onto MPYE medium containing different inhibitors at various concentrations indicated on top of the plates, and were incubated for 48–72 h under photosynthetic growth conditions.

to 10^{-4} M, 20 and 50 μg/ml respectively. Similarly, UHDBT (Bowyer *et al.*, 1982) and UHNQ (and its derivatives with shorter 3-alkyl chains) (Matsuura *et al.*, 1983), which interrupt the electron transfer for the Rieske FeS protein to cyt c_1, did not induce any growth inhibition at final concentrations of up to 20 μg/ml. The effects of the Q_z and Q_c inhibitors on photosynthetic growth of *R.sphaeroides* strains Ga and 2.4.1 were also similar to those observed with MT1131.

Spontaneous mutants resistant to myxothiazol, mucidin and stigmatellin at final concentrations of 5×10^{-6}, 5×10^{-5} and 4×10^{-6} M respectively were selected under photosynthetic growth conditions on MPYE using MT1131 as the parental strain. For each inhibitor 10–20 independent derivatives were retained for further studies.

Physiological characterization of the Qz Inh^R mutants

To detect different possible classes among the Inh^R mutants their cross-resistance to various Q_z inhibitors were determined (Table I). Mutants exclusively resistant to myxothiazol (Myx^R) (MXT103) or to stigmatellin (Stg^R) (STG1 and STG3) were readily distinguished from those resistant to both myxothiazol and mucidin (Myx^R, Muc^R) (MXT101 and MUC21) or to myxothiazol and stigmatellin (Myx^R, Stg^R) (MXT102 and STG10).

The degree of inhibitor resistance of different categories of mutants was determined by the 'spot-test technique' (see Materials and methods) and an example of the data is shown in Figure 1. The comparative levels of resistance for various mutants were as follows: for Myx^R, (MXT103, MXT102) > MXT101 > (MUC21, STG10) > (MT1131, STG3) > (STG1, STG5); for Stg^R, STG3 > (STG5, STG10) > (STG1, MXT102) > (MT1131, MUC21, MXT103, MXT101); and for Muc^R, (MUC21, MXT101) > (MXT102, MXT103) > STG3 > (MT1131, STG10) > (STG1, STG5). Interestingly, several mutations also induced increased sensitivity to some inhibitors whilst conferring increased resistance to others; for instance, the Stg^R mutants STG1 and STG5 were more sensitive to myxothiazol and mucidin than the wild-type parent MT1131 (Figure 1).

The global physiological effect of Inh^R mutations was assessed by measuring the photosynthetic and chemoheterotrophic growth rates of various mutants. In the absence of inhibitor, photosynthetic and chemoheterotrophic growth rates of the mutants were comparable to those of the parental

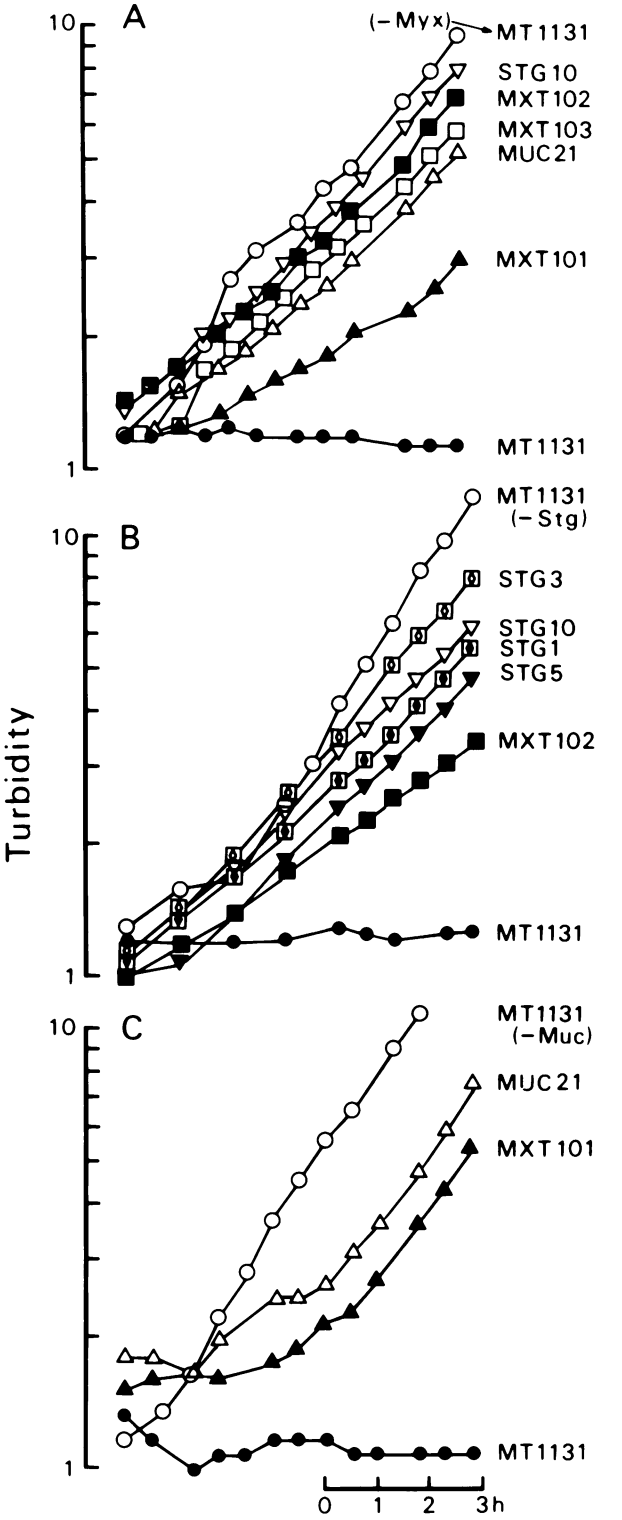

Fig. 2. Photosynthetic growth of various mutants in the presence of different Q_z inhibitors. Photosynthetically grown cells were inoculated onto MPYE medium containing 5×10^{-6} M myxothiazol (**A**), 1.5×10^{-6} M stigmatellin (**B**) and 4×10^{-5} M mucidin (**C**) and the turbidity of the cultures was monitored as described in Materials and methods. MT1131(–Myx), MT1131(–Stg) and MT1131(–Muc) indicate the control growth curves obtained without inhibitors under the same growth conditions.

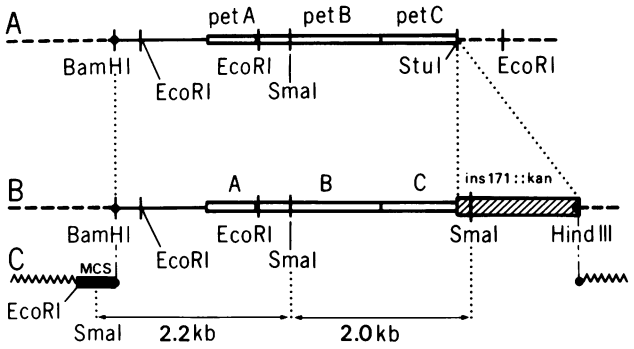

Fig. 3. (**A**) Restriction map of the *petABC*(*fbcFBC*) cluster on the chromosome of the wild-type *R.capsulatus* strain MT1131. The dotted line corresponds to the chromosomal DNA outside the *Bam*HI and *Stu*I sites around the *pet*(*fbc*) cluster. (**B**) The *ins171* :: *kan* insertion (dashed box) was introduced via GTA crosses (see Results) next to the *petC*(*fbcC*) gene at the *Stu*I site yielding Kan^R derivatives of the Inh^{R}R mutants. (**C**) These latter strains were used to clone out the chromosomal *Bam*HI−*Hin*dIII fragments containing Inh^R *pet*(*fbc*) operons by selecting for Kan^R provided by the insertion *ins171* :: *kan*. When necessary, the 2.2 and 2.0 kb *Sma*I fragments were subcloned into the phage M13 derivative mp10 for DNA sequence determination. MCS and wavy line correspond to the 'multiple cloning site' and the vector pRK404 (Ditta *et al.*, 1985) respectively.

strain MT1131 (data not shown). With the exception of MXT101 (with myxothiazol) and MXT102 (with stigmatellin), the presence of inhibitors also did not grossly alter the photosynthetic growth of various Inh^R mutants while completely arresting that of MT1131 (Figure 2).

Mapping of the Q_z Inh^R mutations

To determine whether inhibitor resistance is due to mutations in the *pet*(*fbc*) operon, spontaneus Inh^R mutants were mapped with respect to the silent insertion *ins171* :: *kan* linked to the *pet*(*fbc*) operon (Daldal *et al.*, 1987) (Figure 3). The 'gene transfer agent', a transducing phage-like particle specific to *R.capsulatus* species (Yen *et al.*, 1979), produced by the strain R121-I171 carrying the *ins171* :: *kan* was used as donor and Kan^R derivatives of the Inh^R mutants were selected chemoheterotrophically. They were then tested for their inhibitor-sensitivity under photosynthetic growth conditions. All of the Myx^R and the Muc^R mutants were found linked to *ins171* :: *kan* with co-transduction frequencies of 7−10% (Table I). A similar co-transduction frequency (~9%) was also observed for the *aer126* mutation of R126 which specifically impairs the Q_z site of the cyt bc_1 complex (Robertson *et al.*, 1986). On the other hand, three distinct classes of mutations were detected among the Stg^R mutants. The first group of mutants (i.e. STG3) showed a co-transduction frequency of ~8%, a number similar to that observed with the Myx^R and the Muc^R mutants. A second group of mutants (i.e. STG1) were more tightly linked to *ins171* :: *kan*, with a co-transduction frequency of ~20%, indicating that they were located closer to the 3′ end of the *pet*(*fbc*) operon than the first group of mutants (Figure 3). Finally, the third group of mutants (i.e. STG5) were not linked to *ins171* :: *kan* suggesting that they were not located within the cyt bc_1 cluster (Table I). These results, together with inhibitor cross-resistance patterns (Figure 1), indicated that at least eight different classes of mutants (I = MXT101 and 104; II_a = MXT102, 107, 109, 110, 111 and 112; II_b = MXT103, 105, 108, 114, 115 and 116: III = STG1, 2, 4, 6, and 14; IV = STG3, 7, 8, 11, 12 and 13; V = STG5, 9, 15 and 16; VI = STG10; VII = MUC21, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16) existed in our Inh^R mutant collection.

Molecular basis of the Q_z Inh^R mutations

The *pet*(*fbc*) operons of various Inh^R mutants were cloned into the broad host range plasmid pRK404 by digestion of the chromosomal DNA of their Kan^R derivatives with the *Hin*dIII and *Bam*HI restriction enzymes that cut outside the *pet*−*kan* cluster (Figure 3). The Kan^R pRK404 derivatives pSR18-404 (wild-type), pBE1-404 (MXT101), pSR24-404 (MXT102), pSR26-404 (MXT103), p1SH-404 (STG1), p3SH-404 (STG3), pMK2-404 (STG5), pBS2-404 (STG10), pMF1-404 (MUC21) and pSR7-404 (R126) were conjugated back to a cyt bc_1^- mutant, MT-CBC1, and the trans-conjugants obtained were tested for photosynthetic growth in the presence and absence, of Q_z inhibitors. With the exception of pMK2-404 (STG5) and pSR7-404 (R126), they all yielded Tet^R, Kan^R merodiploids that grew photosynthetically and had Inh^R phenotypes identical to those of their haploid parents (Table I). As expected, the plasmid pSR7-404 was unable to complement MT-CBC1 since it carried the defective cyt bc_1 cluster of the non-photosynthetic parent R126. Interestingly, the plasmid pMK2-404 complemented the photosynthetic growth defect of MT-CBC1 but did not confer on it any resistance to stigmatellin. This result, in agreement with the earlier mapping data (Table I), demonstrated that the *stg5* mutation must be located outside the *pet*(*fbc*) operon. The nature and the location of this class of mutations are currently unknown.

The mutant *pet*(*fbc*) operons were sequenced either directly from the plasmids, or after cloning the appropriate *Sma*I fragments [containing the *petA*(*fbcF*) and the N-terminal part of *petB*(*fbcB*) or the C-terminal part of *petB*(*fbcB*) and the *petC*(*fbcC*) (Figure 3) into a phage M13mp10 derivative. In many cases, an entirely sequenced restriction fragment which carried the mutation determined by sequencing was exchanged with its counterpart present on the plasmid pSR18-404 containing a wild-type copy of the cyt bc_1 cluster. The reconstructed plasmids were crossed back to MTCBC1 to confirm that the mutation defined by sequencing was indeed the basis of the inhibitor-resistance observed. The data, recapitulated in Table I, indicated that Inh^R phenotypes were due to single basepair changes in *petB* (*fbcB*), encoding cyt *b*. Resistance to myxothiazol was conferred by the $T_{1750} \rightarrow C$ (F144S), to stigmatellin either by the $A_{1806} \rightarrow G$ (T163A) or the $T_{2317} \rightarrow C$ (V333A), to both myxothiazol and mucidin by the $T_{1636} \rightarrow C$ (L106P) or the $G_{1773} \rightarrow A$ (G152S) and to both myxothiazol and stigmatellin by either the $G_{1739} \rightarrow A$ (M140I) or a $T_{1749} \rightarrow C$ (F144L) basepair substitutions. Furthermore, in the case of the non-photosynthetic mutant R126 the $G_{1792} \rightarrow A$ mutation yielded a glycine to an aspartic acid replacement at position 158 of cyt *b*. Interestingly, two different basepair-substitutions, $T_{1750}C$ and $T_{1749}C$, at the same codon of *petB*(*fbcB*) yielded two different amino acid substitutions (F144S and F144L) which provided resistance to either myxothiazol exclusively, or to both myxothiazol and stigmatellin together (Figure 1; Table I).

The Q_z Inh^R mutations described here are not distributed randomly. Six of these are located in a very small segment of cyt *b*, between the amino acid residues 140 and 163. Secondary structure prediction analyses (Rao and Argos,

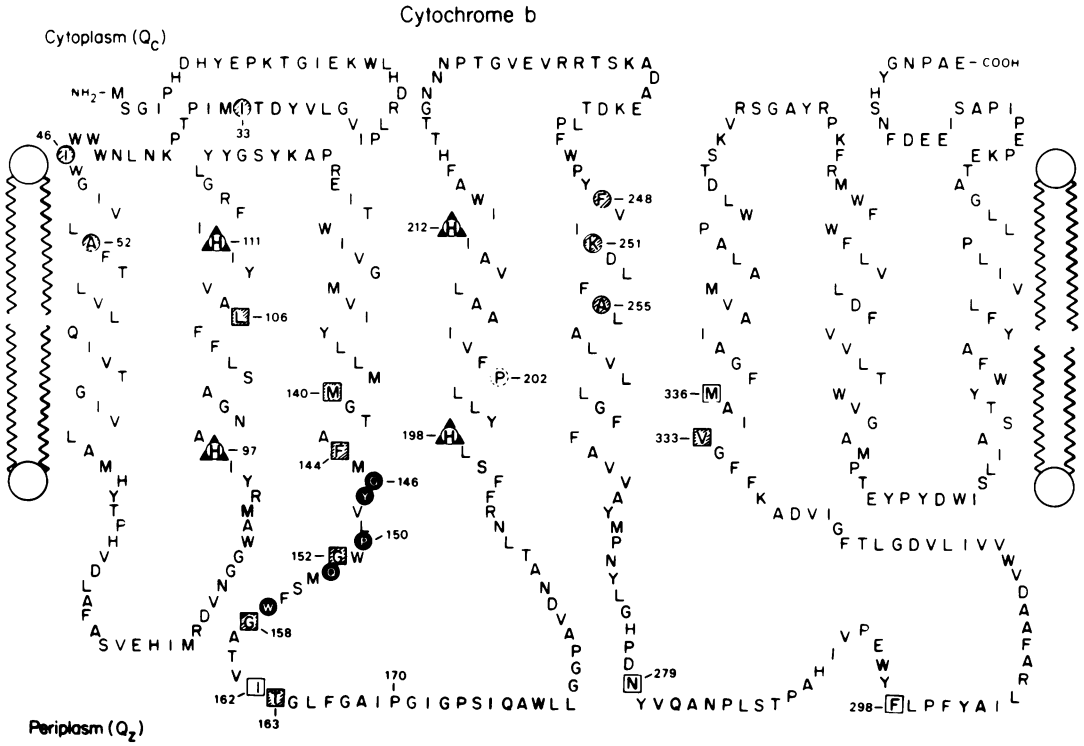

Fig. 4. Location of the inhibitor-resistance mutations on a cyt *b* model displaying eight transmembrane helices from *R.capsulatus*. Q_c and Q_z refer to the quinone reduction and quinol oxidation sites of the cyt bc_1 complex. The four triangles containing H are the universally conserved histidine residues thought to be the axial ligands for the cyt b_L (H97 and H198) and the cyt b_H (H111 and H212); hatched circles denote *S.cerevisiae* (di Rago and Colson, 1988) and mouse (Howell and Gilbert, 1988) cyt *b* mutations conferring resistance to $Q_{c(i)}$ inhibitors; open and hatched rectangles indicate the $Q_{z(o)}$ Inh^R mutations observed in *S.cerevisiae* (di Rago *et al.*, 1989) or mouse (Howell and Gilbert, 1988) mitochondrial and in bacterial cyt *b* (this work) respectively. The white-over-black residues are those that are well conserved in the Q_zI region, and the dotted circle is the P202 residue of the helix IV. The numbering of the amino acid residues is for *R.capsulatus* cyt *b*.

1986; Brasseur, 1988) indicate that this region, called Q_zI, is located between the putative transmembrane helices III and IV of cyt *b* on the outer side of the membrane-lipid bilayer (Figure 4). The clustering in the Q_zI region of several Inh^R mutations around the non-functional *aer126* mutation suggests that this region may be involved in the formation of the inhibitor-binding/quinol-oxidation (Q_z) domain of the cyt bc_1 complex. Of the two remaining mutations the V333A substitution was found in another region located toward the C-terminal end of cyt *b* which is homologous to the subunit IV of the cyt b_6f complex of chloroplast. Finally, the L106P substitution that confers resistance to myxothiazol and mucidin was located in the middle of helix II which contains the two universally conserved histidine residues (H97 and H111 in *R.capsulatus*) thought to be the axial ligands of the two heme groups (b_L and b_H) of cyt *b* (Figure 4). The overall distribution of the Q_z Inh^R mutations obtained in this study indicates that at least seven different amino acid residues at three distinct parts of cyt *b* may be mutated to affect the resistance or the sensitivity of the cyt bc_1 complex to Q_z inhibitors.

Discussion

Effects of cyt bc_1 inhibitors on growth of R.capsulatus

The primary aim of this work was to obtain mutant cyt bc_1 complexes that are functionally perturbed but still properly assembled so that their analyses can yield information about the location and structure of the functional sites of this complex. Knowing that photosynthetic growth of *R.capsulatus* requires a functional cyt bc_1 complex (Daldal *et al.*, 1987), various inhibitors of this complex were tested for their effect on growth of this bacterium. Only myxothiazol, mucidin and stigmatellin, inhibitors that affect the quinol oxidation (Q_z) step, were bacteriostatic for photosynthetic growth of *R.capsulatus* and *R.sphaeroides*, and allowed the isolation of Inh^R mutants. More than 45 spontaneous mutants with various resistance patterns were characterized at the molecular level, and among them the presence of at least seven different classes of cyt *b* mutations were found (Table I). The genetic data obtained from the analysis of this collection of mutants indicated that the Q_z inhibitors are specific for the cyt bc_1 complex *in vivo*. With the exception of the class V mutations (i.e. STG5) all of the Inh^R mutations isolated thus far mapped to *petB(fbcB)* encoding the cyt *b* subunit.

Antimycin, funiculosin and HQNO which inhibit *in vitro* electron transfer from the cyt b_H to a quinone molecule at the quinone reduction (Q_c) site do not affect the photosynthetic growth of *R.capsulatus* or *R.sphaeroides*. In contrast, they are potent inhibitors of respiration in eukaryotic cells, and mitochondrial mutations providing resistance to these substances have now been analyzed at the molecular level in budding (di Rago and Colson, 1988) and in fission (Weber and Wolf, 1988) yeast and in mouse cells (Howell and Gilbert, 1988). Why the Q_c inhibitors have no effect *in vivo* on photosynthetic bacteria is puzzling. One possibility is that although they are effective on chromatophores (which are inside-out vesicles) *in vitro*, they

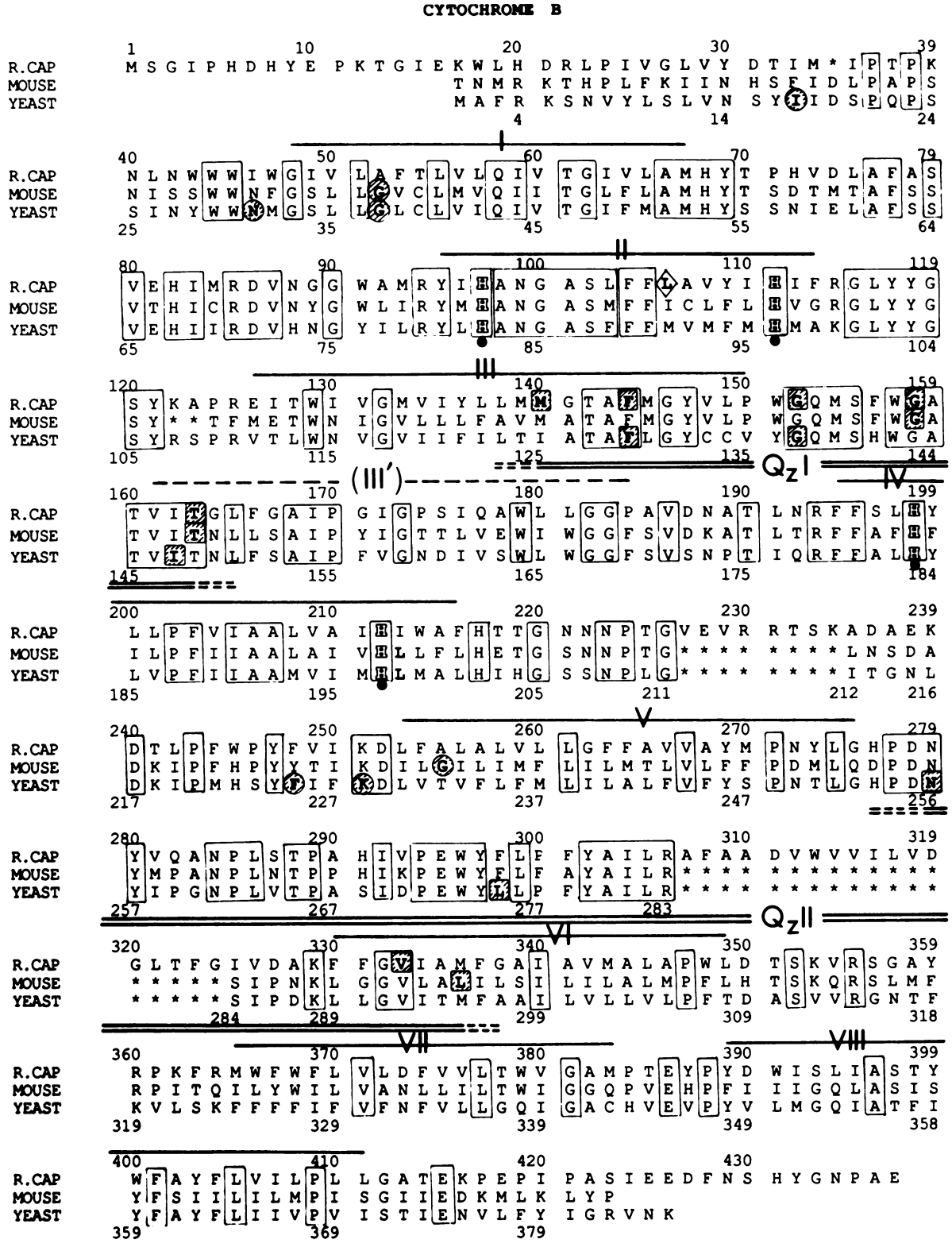

Fig. 5. Comparison of the $Q_{c(i)}$ and $Q_{z(o)}$ Inh^R mutations of yeast (di Rago and Colson, 1988; di Rago *et al.*, 1989) and mouse (Howell and Gilbert, 1988) mitochondrial with those of *R.capsulatus* (this work) cyt *b*. The aligment of the cyt *b* sequences of these three species is as in Hauska *et al.* (1988) and the numbering above and under each line is for *R.capsulatus* and for *S.cerevisiae* residues respectively. The hatched circles and small squares indicate the amino acid residues highlighted by the $Q_{c(i)}$ and $Q_{z(o)}$ Inh^R mutations respectively; the rectangles indicate conserved residues between the three organisms, and the outlined H correspond to the axial ligands for the two heme groups of cyt *b*. Putative transmembrane helices of *R.capsulatus* cyt *b* are indicated approximately by a bar containing a Roman numeral above the sequence and the amphiphilic helix III' (helix IV in Widger *et al.*, 1984) is shown as a dotted line. The double line under the sequence indicate the tentative limits of the Q_zI and the Q_zII regions of *R.capsulatus* cyt *b*.

Table II. Mitochondrial cyt *b* mutations conferring resistance to $Q_{i(c)}$ inhibitors and hypothetical bacterial cyt *b* mutations predicted to convey inhibitor-sensitivity

Position Rc/Sc	Organisms		
	S.cerevisiae	Mouse	*R.capsulatus*
33/17	I → F : Diu^R	[**F → I : Diu^S**][a]	I
46/31	N → K : Diu^R	N	[**N → N : Diu^S**]
52/37	G → V : Ana^R	G → V : Ana^R	[**A → G : Ana^S**]
248/225	F → L,S : Diu^R	Y	F
251/228	K → I : Ana^R	K	K
255/232	[**T → G : $HQNO^s$**]	G → D : $HQNO^R$	[**A → G : $HQNO^S$**]

[a]Predicted mutations and their corresponding phenotypes are shown between brackets in bold characters. Data related to *S.cerevisiae* and mouse mitochondrial cyt *b* mutations are from di Rago and Colson (1988) and Howell and Gilbert (1988) respectively. Position numbers for cyt *b* residues are given for both *R.capsulatus* (Rc) and *S.cerevisiae* (Sc) and are separated by /.

may not reach efficiently the Q_c site in growing cells because of either their limited diffusion across the membrane or their biodegradation. However, it has been found that antimycin A can decrease the light-dependent membrane potential in intact resting cells of strain N22 (Myatt *et al.*, 1987). This observation suggests that the blockage of the Q_c site may be bypassed by a pathway insensitive to Q_c inhibitors.

Another possibility stems from a comparison of the primary structure of bacterial and mitochondrial cyt *b* (Figure 5). The decreased affinity of the Q_c inhibitors to *R.capsulatus* cyt bc_1 complex may also derive from the presence of natural substitutions in the bacterial cyt *b* at residues homologous to those conferring resistance to $Q_{i(c)}$ inhibitors in mitochondria (Figure 5; Table II) (di Rago and Colson, 1988; Howell and Gilbert, 1988; Weber and Wolf, 1988). Of these six positions, only three—I33/17, F248/225 and K251/228—are conserved in *R.capsulatus*. The N46/31, G52/37 and G255/232 residues have been substituted naturally by isoleucine, alanine and alanine respectively (Figure 5 and Table II). Considering that a single mutation alone may be responsible for resistance *in vivo* to a given $Q_{i(c)}$ inhibitor, then *R.capsulatus* cyt bc_1 complex may be naturally resistant to diuron, antimycin and HQNO because of the presence of the N46/31I, G52/37A and G255/232A natural substitutions respectively (Table II). Similarly, yeast and mouse mitochondrial cyt bc_1 complexes may be resistant to HQNO and diuron because of the G255/232T and I33/17F natural substitutions respectively. The reverse replacements of I46/31 → N, A52/37 → G and A255/232 → G may then increase the sensitivity of the bacterial cyt bc_1 complex to diuron, antimycin and HQNO respectively. However, although the I33/17F substitution has been observed in yeast to provide resistance to diuron (di Rago and Colson, 1988) the remaining N46/31I, G52/37A or G255/232A substitutions are currently unknown to provide resistance to any $Q_{i(c)}$ inhibitors. Furthermore, an alanine residue at the position 37 of *Schizosaccharomyces pombe* (52 or *R.capsulatus*) cyt *b* does not prevent the isolation of antimycin- or diuron-resistant mutants by its substitution with either a valine or a glycine (Weber and Wolf, 1988).

Location and nature of the Q_z Inh^R mutations

The mutations providing resistance to Q_z inhibitors are not uniformly distributed. All of the Inh^R mutations affecting the cyt bc_1 complex are located exclusively in the cyt *b* subunit, and no Inh^R mutation was found in the Rieske FeS protein either in bacterial (this work) or in mitochondrial systems studied so far (Howell and Gilbert, 1988; di Rago *et al.*, 1989; Ljungdahl *et al.*, 1989). Interestingly, several Stg^R mutants also confer resistance to UHDBT *in vitro* (D.E.Robertson, F.Daldal and P.L.Dutton, in preparation), suggesting that some cyt *b* mutations may indirectly affect the properties of the FeS protein.

Table III. Bacterial and mitochondrial cyt *b* mutations conferring resistance to $Q_{z(o)}I$ inhibitors

Position Rc/Sc	Organism	Amino acid substitution	Phenotype	Cyt bc_1 domain
140/125	bacteria	M → I	Myx^R, Stg^R	$Q_{z(o)}I$
144/129	bacteria	F → L	Myx^R, Stg^R	$Q_{z(o)}I$
	yeast	F → L	Myx^R	
	bacteria	F → S	Myx^R	
152/137	bacteria	G → S	Myx^R, Muc^R	$Q_{z(o)}I$
	yeast	G → R	Myx^R, Muc^R	
158/143	bacteria	G → D	Ps^-	$Q_{z(o)}I$
	mouse	G → A	Myx^R	
162/147	yeast	I → F	Stg^R	$Q_{z(o)}I$
163/148	bacteria	T → A	Stg^R	$Q_{z(o)}I$
	mouse	T → M	Stg^R	
279/256	yeast	N → Y	Myx^R, Muc^R	$Q_{z(o)}II$
298/275	yeast	L → S,F,T	Myx^R, Muc^R	$Q_{z(o)}II$
333/292	bacteria	V → A	Stg^R	$Q_{z(o)}II$
336/295	mouse	L → F	Stg^R	$Q_{z(o)}II$

Data related to yeast and mouse mitochondrial cyt *b* mutations are from di Rago *et al.* (1989) and Howell and Gilbert (1988) respectively. Position numbers for cyt *b* residues are given for both *R.capsulatus* (Rc) and *S.cerevisiae* (Sc) and separated by /.

The distribution of the Q_z Inh^R mutations within the cyt *b* subunit is also not random. The Q_z Inh^R mutations that are characterized to date in *R.capsulatus*, and in *Saccharomyces cerevisiae* (di Rago *et al.*, 1989) and mouse (Howell and Gilbert, 1988) mitochondria are listed in Table III, and their distributions in cyt *b* are compared in Figure 5. In bacteria, as is also the case in mitochondria, these mutations are located mainly in two distinct regions of cyt *b*. In *R.capsulatus* the first of these regions, called Q_zI, may be defined as the sequence between residues 140 and 163 where several Myx^R, Muc^R and Stg^R mutaions are located. The second region, which may be called Q_zII by analogy to Q_zI, contains the bacterial mutation V333A as well as several Myx^R, Muc^R and Stg^R mutations observed in mitochondria (Table III). In *R.capsulatus* it may include residues

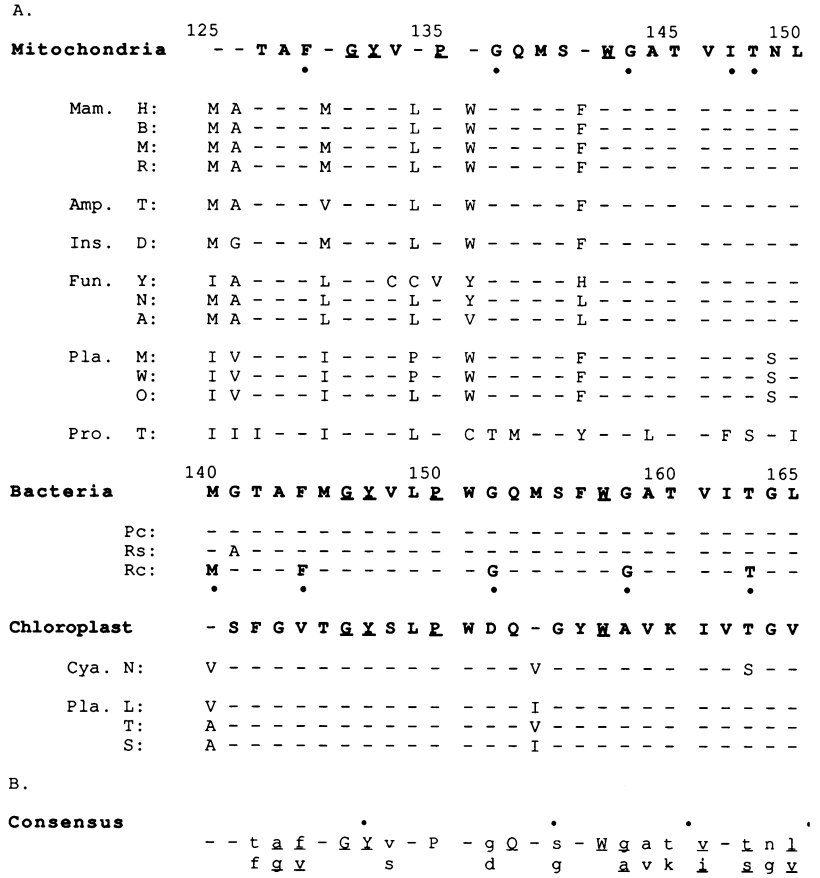

Fig. 6. (**A**) Amino acid identity in the $Q_{z(o)}I$ region of cyt *b* from various *bc/bf* complexes of mitochondria, bacteria and chloroplasts. The amino acid sequences are taken from Hauska *et al.* (1988) and the numbering of the positions is according to that of *S.cerevisiae* (mitochondria) and *R.capsulatus* (bacteria); (−) indicates a residue identical to the average sequence shown in bold characters for mitochondria, bacteria and chloroplast. The dots correspond to the positions where the $Q_{z(o)}I$ Inh^R mutations were observed, and the well-conserved residues are underlined. Abbreviations are as follows: Mam., mammals; H, human; B, bovine; M, mouse; R, rat; Amp., amphibian; T, toad; Ins., insect; D, *Drosophila melanogaster*; Fun., fungi; Y, *S.cerevisiae*; N, *Neurospora crassa*; A, *Aspergillus nidulans*; Pl., plants; M, maize; W, wheat; O, oenothera; Pro., protozoa; T, *Trypanosoma brucei*; Pc, *Paracoccus denitrificans*; Rs, *Rhodobacter sphaeroides*; Rc, *Rhodobacter capsulatus*; Cya., cyanobacteria; N, *Nostoc*; L, liverwort; T, tobacco; and S, spinach. (**B**) A consensus sequence for the $Q_{z(o)}I$ region of cyt *b*. Well-conserved residues are shown in capital letters and are underlined if they are absolutely conserved. The lower-case letters indicate the positions where only one other amino acid substitution is observed and they are underlined if this substitution is conservative.

corresponding to the yeast mutation N279/256Y towards the N-terminal and the mouse mutation L336/295F towards the C-terminal ends of cyt *b*. This latter region has pronounced homology to the subunit IV of cyt b_6f complexes which may bind plastoquinone (Doyle *et al.*, 1989).

Six of the eight bacterial Q_z Inh^R mutations are confined to the Q_zI region, and four of them have also been observed in yeast (F144/129L and G152/137R; di Rago *et al.*, 1989) and in mouse (G158/143A and T163/148M; Howell and Gilbert, 1988) mitochondria (Table III; Figure 5). Furthermore, the Stg^R yeast mutation I162/147F is located next to the T163/148A substitution which confers the same resistance to the mouse and the bacterial cyt bc_1 complexes. On the other hand, the M140/125I mutation, which provides moderate levels of resistance to myxothiazol and stigmatellin (Figure 1; Table I) is unique to bacteria, but a similar substitution is naturally present in yeast cyt *b* (Figure 5). In this region of cyt *b* the T163 is the most highly conserved residue among those that are targets to mutations yielding inhibitor-resistance (Figure 6). It is present in almost all cyt *b*, including plant chloroplast cyt b_6, with the only two exceptions being the trypanosomal cyt *b* and the cyanobacterial (*nostoc*) cyt b_6 where it is replaced by a serine. The F144 and G158 residues are also highly conserved in all known mitochondrial (including the trypanosomal) and bacterial cyt *b* sequences but not in cyt b_6 of chloroplast origins, where they are substituted by a valine and an alanine respectively. The G152 residue, on the other hand, is present in all mitochondrial and bacterial cyt *b* sequences with the unique exception of trypanosomal cyt *b* where it is replaced by a threonine. However, it is substituted by a non-conservative aspartic acid residue in cyt b_6 of chloroplast origin (Figure 6). Finally, of the five Q_z Inh^R mutations located in the Q_zI region, the M140 residue is the least conserved (Figure 6). Comparison of various cyt *b* sequences from different sources (Hauska *et al.*, 1988) indicates that this site may also be occupied by an isoleucine in cyt *b* of mitochondria and by either an alanine or a valine in cyt b_6 of chloroplasts.

The F144, G152 and G158 residues, which are well conserved in both bacterial and mitochondrial cyt *b* proteins but not in chloroplast cyt b_6 (Figures 5 and 6), are targets for mutations providing resistance to myxothiazol and mucidin. These inhibitors are potent on both bacterial and mitochondrial cyt bc_1 complexes but they are without effect on cyt b_6f complexes of chloroplasts (Rich, 1984; von

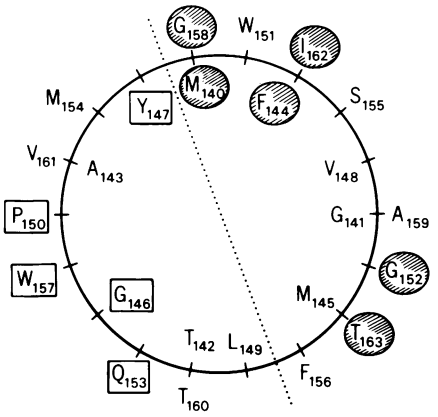

Fig. 7. A helical wheel diagram representing the two hypothetical contiguous helices on each end of the conserved proline 150 residue in the Q_zI region of *R.capsulatus* cyt *b*. The amnio acid residues from 140 to 150 and from 150 to 163 are represented in the inside and in the outside of the circle, respectively. The rectangles on the left and the ellipsoids on the right indicate the amino acid residues that are well conserved and those that confer resistance to Q_z inhibitors respectively.

Jagow and Link, 1986). It was recently observed that a glycine to an alanine replacement at the position equivalent to G158/143 in mouse mitochondrial cyt *b* provides resistance to myxothiazol (Howell and Gilbert, 1988). Similarly, in *R.capsulatus* the identical substitution, either engineered via site-directed mutagenesis or selected as a spontaneous revertant of the G158D mutation also provides resistance to this inhibitor (our unpublished data). Furthermore, the systematic replacement of the *R.capsulatus* residues G152 and F144 by other amino acid residues has revealed that an aspartic acid and a valine at these sites respectively, confers resistance to myxothiazol (our unpublished data). Considering that the G158/143A, G152/137D and F144V substitutions confer resistance to myxothiazol either in mouse mitochondrial (Howell and Gilbert, 1988) or in bacterial cyt bc_1 complexes, the molecular basis of the natural resistance of cyt b_6f complexes to myxothiazol may be the natural presence of the G158A, G152D and F144V substitutions in cyt b_6. In agreement with this idea, the T163 residue, which is conserved both in mitochondrial and bacterial cyt *b* and chloroplast cyt b_6, is associated with resistance to stigmatellin, a quinol oxidation inhibitor that affects both the cyt bc_1 and b_6f complexes *in vitro* (Oettmeier *et al.*, 1985; Nitschke *et al.*, 1989).

The Q_zI region of cyt b

The Q_zI region of cyt *b* has an interesting primary structure (Figure 6). The amino acid residues at positions G146, Y147, P150,Q153 and W157 are well conserved in all cyt *b* sequences from mitochondria to chloroplasts. Furthermore, many other sequence positions (T/F142, A/G143, F/V144, V/S148, G/D152, S/G155, G/A158, A/V159, T/K160, V/I161, I/V162, T/S163, N/G164 and L/V165) are substituted only by one or two other, often conservative, amino acid residues. Although there are several differences in the primary sequences of the Q_zI region (positions 140, 141, 145, 149, 151 and 156) of mouse, yeast and *R.capsulatus* (Figure 6), interestingly the same positions confer resistance to the same inhibitors. This observation suggests that the Q_zI region may have a phylogenetically conserved overall architecture. Secondary structure prediction analyses (Rao and Argos, 1986; Brasseur, 1988) indicate that the Q_zI region is between the putative transmembrane helices III and IV, in close proximity to the universally conserved histidine residues 97 and 198 (Figure 4). We have noticed that if the amino acid residues extending from 140 to 163 were to be modeled as a hypothetical helix 'nicked' by the conserved proline at position 150 instead of a 'helix−linker−helix' structure (Brasseur, 1988), then the residues M140, F144, G152, G158, I162 and T163 that are targets for mutations providing resistance to Q_z inhibitors may be accommodated on one side of this 'nicked helix' structure while the well-conserved residues G146, Y147, P150, Q153 and W157 may be on the other side (Figure 7). The nature of the local architecture of the Q_zI region is currently unknown.

Inh^R mutations not located in the Q_zI region of cyt b

Of the two remaining Inh^R mutations the V333A substitution is in the Q_zII domain located toward the C-terminals of cyt *b*, next to the absolutely conserved G332 residue (Hauska *et al.* 1988). In cyt *b* models with eight transmembrane helices, this region is located between the putative transmembrane helices V and VI and on the same side of the membrane as the Q_zI domain (Rao and Argos, 1986; Brasseur, 1988). The Q_zII region is currently less well defined than Q_zI because of the limited number of known mutations in this region and the lesser overall conservation among various cyt *b* proteins (Hauska *et al.*, 1988).

In *R.capsulatus* a third mutation, L106P, located in the middle of the helix II that carries the two universally conserved histidines (H97 for cyt b_H and H111 for cyt b_L) was also observed to provide resistance to both myxothiazol and mucidin (Table III). This mutation of unusual nature and location appears to affect, perhaps indirectly, both the Q_z and the Q_c sites of the cyt bc_1 complex (D.E.Robertson, F.Daldal and P.L.Dutton, in preparation). No known cyt *b* protein carries a proline residue at this position, although the conservative isoleucine, methionine, phenylalanine and valine substitutions are naturally present in cyt *b* of various organisms (Hauska *et al.*, 1988). The existence of Inh^R mutations in a region quite distinct from the Q_zI and Q_zII domains raises the possibility that other secondary inhibitor-binding regions remote from the quinol binding site may exist in cyt *b* and that the cyt bc_1 complex may bind quinol and some of the inhibitors without mutual exclusion, as observed by Brandt *et al.* (1988).

It is currently not known whether the mutations providing resistance to inhibitors indicate the residues directly involved in the binding and oxidation of the quinol by the cyt bc_1 complex. In the case of the bacterial photochemical reaction center of known three-dimensional structures (Deisenhofer *et al.*, 1985; Chang *et al.*, 1986; Allen *et al.*, 1987), it is now clear that atrazine and terbutryn resistance mutations directly affect the residues constituting the quinone binding pockets of this complex (Gilbert *et al.*, 1985; Sinning and Michel, 1987; Bylina *et al.*, 1989). Whether this may also be the case for the cyt bc_1 complex is unknown. However, the loss of quinol oxidation ability as well as the acquisition of resistance observed by various substitutions at positions like G158 (our unpubished data) suggests that some of the residues highlighted by Inh^R mutations may be directly

involved in the formation of the quinol oxidation site of the cyt bc_1 complex.

A major shortcoming of the analysis of Inh^R mutants is that inhibitor-resistance cannot be obtained by destruction of determinants essential for chemical catalysis or for assembly of a multi-subunit complex. The specific residues defined by Inh^R mutations may reflect the minor differences between the biomimetic compounds and the natural substrates or products. If so, then their analysis may only reveal globally the areas of importance for inhibitor-binding and/or quinol oxidation. The accurate definition of the residues essential for catalysis will ultimately require a three-dimensional structure for the cyt bc_1 or b_6f complex, which is presently unavailable. Nevertheless, in its absence the ongoing molecular genetic analyses of the Q_z Inh^R mutations indicate the presence of at least two essential regions; Q_zI and Q_zII, predominantly related to quinol oxidation catalyzed by the cyt bc_1 complex. The specific roles of the amino acid residues in these regions now need to be examined in detail to define better their contributions to quinol oxidation and inhibitor-resistance.

Materials and methods

Media, strains, plasmids and growth conditions

Escherichia coli strains were grown in LB broth or M9 synthetic medium, and *R.capsulatus* strains were cultured by respiration (aerobic, dark) or by photosynthesis (anaerobic, light) on MPYE rich or on RCV minimal medium supplemented with antibiotics as described earlier (Daldal *et al.*, 1987). Plates containing myxothiazol, stigmatellin and mucidin were prepared by mixing exactly 25 ml of MPYE medium containing 2% of agar (Difco) with the desired amount (determined spectroscopically according to von Jagow and Link, 1986) of inhibitor dissolved in ethanol.

The photosynthetic growth rate of Inh^R mutants was measured in MPYE liquid medium by monitoring the turbidity with a Klett–Summerson colorimeter equipped with a red filter as described earlier (Daldal, 1988). The degree of inhibitor-resistance was estimated semi-quantitatively by spotting ~1500–3000 chemoheterotrophically grown cells embedded in 5 μl of MPYE soft agar (0.7%) to MPYE plates containing various concentrations of inhibitors. The strain HB101 [F^- *proA2, leu, hsdS20* (r_B^-, m_B^-) *recA13, ara14, lacY1, galK2, rpsL20, xyl5, mtl, supE44*, λ^-] was used as an *E.coli* recipient and the plasmids were related either to pBR322 or pRK404 (Ditta *et al.*, 1985). All *R.capsulatus* mutants were derived from MT1131 (*crtD121*, Rif^R), a 'green' derivative of SB1003 (Marrs, 1981).

Genetic procedures, recombinant DNA techiques and DNA nucleotide sequence determination

Spontaneous *R.capsulatus* mutants resistant to cyt bc_1 inhibitors were selected under photoheterotrophic growth conditions using BBL anaerobic jars equipped with BBL H_2 + CO_2 gas packs (cat. no. 70304). The mutation frequency observed under these conditions was in the order of 10^{-6}, and to ensure the independence of the mutants, only one colony per selection plate was retained for further analysis. *Escherichia coli* transformation and triparental matings involving pRK404 derivatives were as described earlier (Daldal *et al.*, 1987). Gene transfer agent (GTA)-mediated crosses used strain R121-I171, a derivative of the GTA overproducer strain R121 (Yen *et al.*, 1979) that carries the insertion *ins171*::*kan* located at the last codon of *petC*(*fbcC*) encoding cyt c_1 (Daldal *et al.*, 1987).

Recombinant DNA techniques were performed as reported earlier (Daldal, 1983) or according to Maniatis *et al.* (1982) and single-strand (using the phage M13 derivative mp10) or double-strand (using the plasmids pBR322 and pRK404 derivatives) DNA sequencing used either the Klenow fragment of DNA polymerase or the commercial 'Sequenase' version of T7 polymerase in the presence of ^{32}P- or ^{35}S-labeled dATP according to the instructions provided by UBS Corp (Cleveland, OH). Besides the universal sequencing primer for the phage M13, the following custom-made synthetic oligonucleotides derived from the *petABC*(*fbcFBC*) operon (numbers indicating the start positions in the *pet*(*fbc*) operon; Davidson and Daldal, 1987a) were also used as primers.

pd4: (5′T_{1188}GCCCCTGCCACGGC3′)
pd5: (5′T_{1471}TGCCTTCACGCTGG3′)
pd6: (5′C_{1721}ATCTATCTGCTGAT3′)
pd7: (5′C_{2051}TGGCCGTATTTCGT3′)
pd8: (5′T_{2272}CGATGGGCCTGACTT3′)
pd9: (5′C_{2489}TGGATCTCGCTCAT3′)
pu9: (5′G_{2767}TACTTGCCGAAGAT3′)
pu8: (5′C_{2566}TCGGTCGCGCCCAGA3′)
pu7: (5′A_{2310}GAACTTCGCATCGA3′)
pu6: (5′G_{2069}ATCACGAAATACGGC3′)
pu5: (5′A_{1817}AACAGGCCGGTGAT3′)
pu4: (5′T_{1582}TCACGTCGCGCATG3′)

Chemicals

Myxothiazol, antimycin A and its derivatives (A1, A2, A3, and A4) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) were purchased from Boehringer Mannheim and Sigma respectively. 5-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was obtained from Dr B.L.Trumpower (Dartmouth University, NH). Stigmatellin, 3-undecyl-2-hydroxy-1,4-naphthoquinone (UHNQ) and its derivatives with shorter alkyl side-chains (3-alkyl-2-hydroxy-1,4-naphthoquinone (alkyl-HNQ), and mucidin were gifts from Drs G.Hofle (Gesellschaft fur Biotechnologische Forschung, Braunschweig, FRG), P.L.Dutton (University of Pennsylvania, PA) and V.Musilek (Institute of Microbiology, Prague, Czechoslovakia) respectively. All enzymes and chemicals used in molecular cloning and DNA sequencing were of molecular biology grade, obtained from commercial sources and used as instructed. Radiolabeled dNTPs were from Amersham.

Acknowledgements

We are grateful to Drs A.-M.Colson and N.Howell for providing us with manuscripts of their work before publication. This work was supported by PHS grant 38237 from the National Institutes of Health. One of us (F.D.) is grateful to Dr B.L.Marrs who originally isolated and generously gave us the myxothiazol-resistant derivatives of SB1003, most probably similar to those reported here.

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Received on June 23, 1989; revised on September 7, 1989