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Minireview

Random Mutant Generation and Its Utility in Uncovering Structural and Functional Features of Cytochrome *b* in *Saccharomyces cerevisiae*

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The generation of random mutations in the mitochondrial cytochrome *b* gene of *Saccharomyces cerevisiae* has been used as a most fruitful means of identifying subregions that play a key role in the bc_1 complex mechanism, best explained by the protonmotive Q cycle originally proposed by Peter Mitchell. Selection for center i and center o inhibitor resistance mutants, in particular, has yielded much information. The combined approaches of genetics and structural predictions have led to a two-dimensional folding model for cytochrome *b* that is most compatible with current knowledge of the protonmotive Q cycle. A three-dimensional model is emerging from studies of distant reversions of deficient mutants. Finally, interactions between cytochrome *b* and the other subunits of the bc_1 complex, such as the iron–sulfur protein, can be affected by a single amino acid change.

KEY WORDS: Cytochrome *b* structure and function; random mutants; yeast; resistance mutants; revertants of deficient mutants.

INTRODUCTION

My fascination for the respiratory chain was born in 1971 when I realized the congruency of three concepts: (1) respiratory inhibitors interfere with the electron transport chain; (2) the respiratory chain in eukaryotes is part of an intracellular organelle, the mitochondrion, which has its own heredity; (3) yeasts, unicellular eukaryotes, are able to grow from the energy produced either by respiration or by fermentation.

The yeast *Saccharomyces cerevisiae* appears as a model organism for a genetic approach to the structure and function of respiratory chain components. It can grow on glucose by fermentation and it relies on a functional respiratory chain for growth on respiratory substrates such as glycerol or ethanol. Any mutation or inhibitor dramatically affecting the normal functioning of the respiratory chain results in an inability to grow by respiration on glycerol or ethanol. To me, a geneticist, selection for respiratory chain inhibitor resistance thus became the most straightforward approach toward understanding the structure–function relationships of respiratory chain components.

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RESULTS AND DISCUSSION

Inhibitors and Proton/Electron Coupling Mechanism of the bc_1 Complex

The mechanism by which electron transfer is coupled to proton translocation at the bc_1 complex level is now known and best explained by the proton motive Q cycle, as originally proposed by Peter Mitchell (Mitchell, 1976). This outstanding investigator postulated the existence of two distinct sites on opposite sides of the membrane for interactions between quinones and the complex. He named them center i and center o. The appropriateness of this model was demonstrated in studies using specific inhi-

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bitors for each center (von Jagow and Engel, 1981; Rich, 1984; von Jagow and Link, 1986). Two pathways were identified for cytochrome *b* reduction. One is antimycin A sensitive, and goes through center i at a site near cytochrome *b*562 or *bh* and closer to the inner side of the membrane. The other pathway is myxothiazol sensitive. It goes through center o, near *b*566 or *bl*, closer to the outer side of the membrane.

Respiratory chain inhibitors like Antimycin A (Keilin and Hartree, 1955) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea DCMU (also called diuron) (Mukasa *et al.*, 1966; Inoue *et al.*, 1967; Hachimori *et al.*, 1968) have been known for several decades to interfere with the electron transfer chain between cytochromes *b* and c_1. HQNO (van Ark and Berden, 1977) as well as funiculosin (Nelson *et al.*, 1977) have also been identified as cytochrome *b* inhibitors. Diuron was found to reverse the cytochrome *b* red shift induced by HQNO, suggesting the same binding niche for both inhibitors (Convent and Briquet, 1978). Interestingly, DCMU appeared to interfere more specifically with the photosynthetic electron transfer chain by inhibiting the Hill reaction (Wessels and Van der Veen, 1956). It is now known to interfere with the interactions between plastoquinone and photosystem II (Trebst and Draber, 1986). Stigmatellin was likewise found to interfere in both the plastoquinone/photosystem II interactions (Oettmeier *et al.*, 1985) and the quinone/bc_1 complex interactions (Thierbach *et al.*, 1984). A decisive fact was that methoxyacrylate inhibitors such as myxothiazol, mucidin (identical to strobilurin—von Jagow *et al.*, 1986), and MOA stilbene, as well as chromone inhibitors (stigmatellin) inhibit center o whereas antimycin A, funiculosin, and HQNO are clear center i inhibitors (von Jagow and Link, 1986).

Inhibitor Resistance and the Cytochrome *b* Gene

The inability of the yeast *Saccharomyces cerevisiae* to grow on glycerol, a respiratory substrate, in the presence of appropriate concentrations of center i and center o inhibitors, offered the opportunity to select for resistance mutants with a view to identifying the inhibitory targets. Selection for resistance to inhibitors of both types was undertaken. The following resistance to mutations were mapped on the mitochondrial genome: mucidin-resistance mutations (Subik, 1975; Subik *et al.*, 1977; Colson and Slonimski, 1977, 1979; Takacsova *et al.*, 1981), antimycin-resistance mutations (Michaelis, 1976; Burger *et al.*, 1976; Pratje and Michaelis, 1977, Colson *et al.*, 1979), funiculosin-resistance mutations (Burger *et al.*, 1976; di Rago *et al.*, 1990a), diuron-resistance mutations (Colson *et al.*, 1977; Colson and Slonimski, 1977; 1979, Colson *et al.*, 1979), myxothiazol-resistance mutations (Thierbach and Michaelis, 1982; di Rago *et al.*, 1989), stigmatellin-resistance

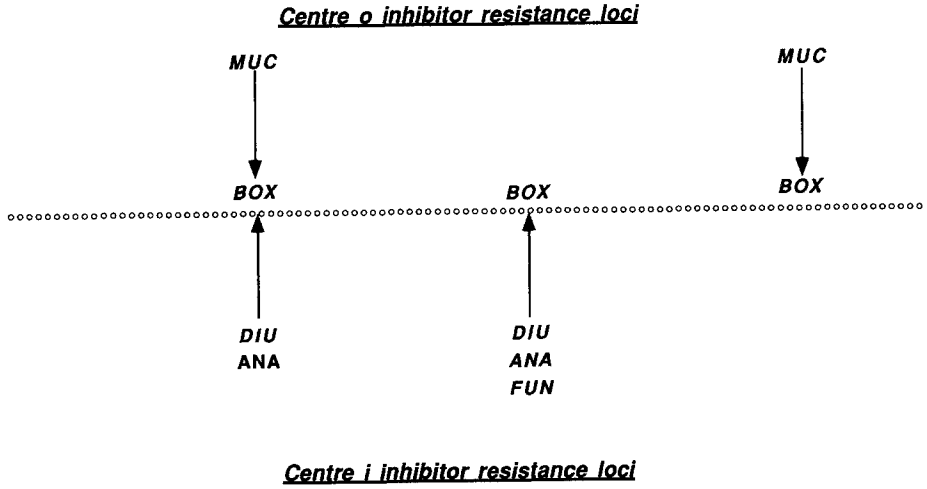

Fig. 1. Inhibitor resistance loci within the mitochondrial cytochrome *b* gene. Positions of center i and center o inhibitor resistance loci with respect to cytochrome *b* deficiency loci on the yeast mitochrondrial genetic map. MUC: mucidin-resistance locus; DIU: diuron-resistance locus; FUN: funiculosin-resistance locus; ANA: antimycin-resistance locus; MYX: myxothiazol-resistance locus; BOX: cytochrome *b* deficient locus.

mutations (di Rago *et al.*, 1989), and MOA-stilbene resistance mutations (Meunier, Di Rago, and Colson, unpublished results). The resistance mutations were located within a segment of the mitochondrial genome bearing the split mitochondrial cytochrome *b* gene. The concept of a split cytochrome *b* gene was formulated in 1978 (Slonimski *et al.*, 1978) and the split nature of the yeast cytochrome *b* gene was demonstrated two years later (Lazowska *et al.*, 1980; Nobrega and Tzagoloff, 1980a,b). The first inhibitor resistance mutations were located with respect to each other and to cytochrome *b*-deficient mutations (BOX mutations) without any knowledge of the split nature of the cytochrome *b* gene or even any concept of it (Colson and Slonimski, 1977, 1979; Colson *et al.*, 1979).

In 1977, three groups of mutated sites or loci were identified in the cytochrome *b* gene (Fig.1). The first group carried mutations conferring resistance to antimycin A, diuron, or mucidin. These sites were inseparable by genetic recombination. The second group carried mutations conferring resistance to antimycin, funiculosin, or diuron. The third carried mutations conferring mucidin-resistance. The three groups of loci were unlinked on the mitochondrial genomic map. Within each group, the resistance mutations were found to be genetically linked according to the mitochondrial recombination rules (see Dujon, 1981). All resistance mutants exhibited respiratory resistance in *in vitro* experiments performed on isolated mitochondrial membranes. Moreover, mucidin-resistance mutants were sensitive to antimycin as were antimycin-resistance mutants to mucidin, although the two types of mutations were genetically linked. One diuron-resistance mutant, however, presented some cross-resistance to antimycin A (Briquet and Goffeau, 1981). Also, all diuron-resistance mutants showed *in vitro* cross-resistance to HQNO (Briquet and Goffeau, 1981). HQNO, however, was not used as a center i inhibitor for selecting resistance mutants, because it failed to inhibit yeast growth on a respiratory substrate.

In 1980, knowledge of the split structure of the cytochrome *b* gene, (Lazowska *et al.*, 1980) made it possible to assign each inhibitor-resistance locus to its exon (Fig. 2): a diuron-resistance locus and an antimycin-resistance locus in exon 1 and again in exon 4; the funiculosin-resistance locus in exon 4; the two mucidin-resistance loci in exons 1 and 6.

Moreover, a third mucidin-resistance locus was attributed to exon 5 (Takacsova *et al.*, 1981). Thierbach and Michaelis (1982) identified two myxothiazol-resistance loci in exons 1 and 6.

In summary, four of the six exons of the split mitochondrial cytochrome *b* gene can carry inhibitor resistance mutations. Exon 4 carries center i inhibitor resistance mutations. Exons 5 and 6 carry center o inhibitor resistance mutations. Only exon 1 carries both center i and center o inhibitor resistance mutations.

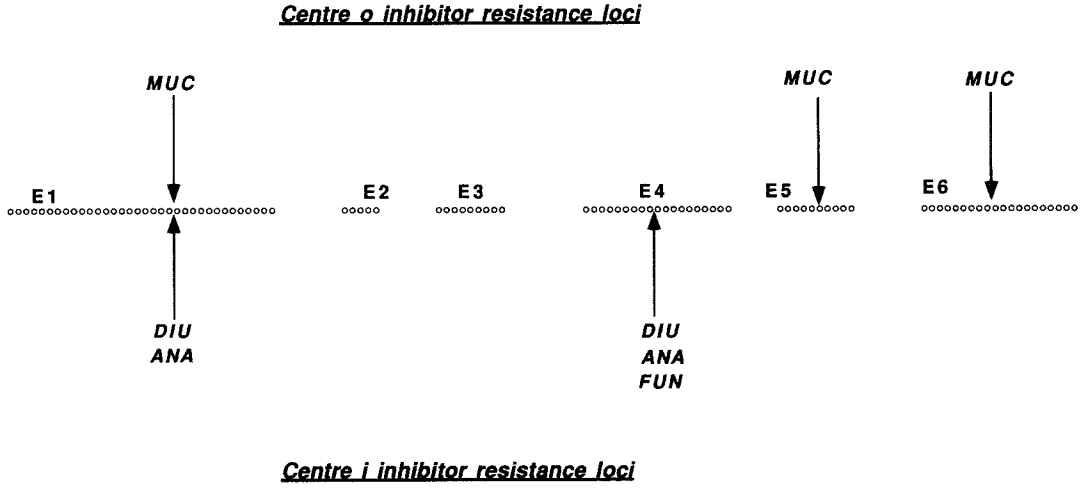

Fig. 2. Positions of center i and center o inhibitor-resistance loci with respect to the exons of the split cytochrome *b* gene; E1 to E6: exons 1 to 6.

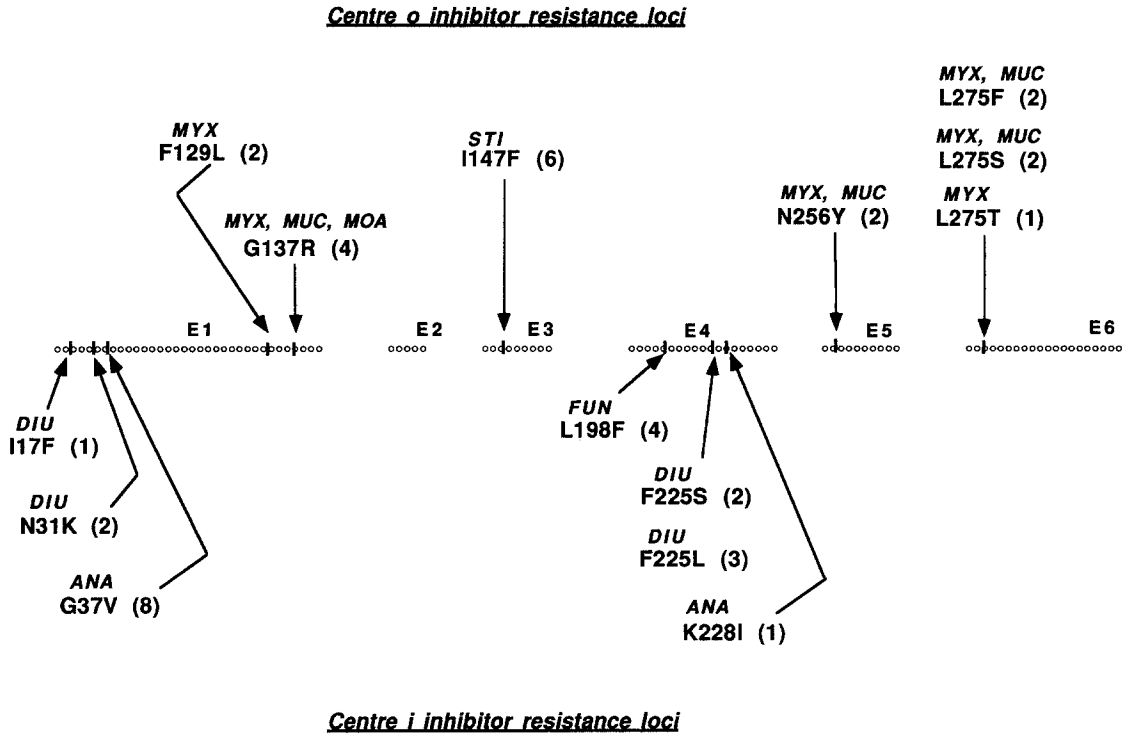

Fig. 3. Center i and center o inhibitor-resistance mutational positions and changes in the cytochrome *b* split gene. MYX: myxothiazol-resistance locus; STI: stigmatellin-resistance locus; MUC: mucidin-resistance locus; MOA: moa-stilbene-resistance locus; DIU: diuron-resistance locus; FUN: funiculosin-resistance locus; ANA: antimycin-resistance locus; E1 to E6: exons 1 to 6. Between parentheses, the number of independently identified mutational changes for each change. Bars indicate the mutations' positions in each exon.

Inhibitor Resistance and Sequence Analyses

It was necessary to identify the nucleotide substitutions responsible for all the resistance mutations in order to better understand their bearing on cytochrome *b* structure. A very efficient technique for analyzing the sequences of resistance mutants was primer elongation with labelled oligonucleotides hybridized to mitochondrial resistance-mutant messenger RNA (di Rago *et al.*, 1986; di Rago and Colson; 1988; di Rago *et al.*, 1989, 1990a). First, this technique avoided cloning each mutated cytochrome *b* gene. Secondly, it limited the sequence analyses to the exons, the large introns being spliced out of the messenger RNA. Sequences were obtained for 40 independent inhibitor-resistance mutations previously mapped to the exons of the split cytochrome *b* gene (di Rago *et al.*, 1986; di Rago and Colson, 1988; di Rago *et al.*, 1989, 1990; Meunier, Di Rago, and Colson, unpublished results). It is a pleasure for me to acknowledge the contributions to this study of three fellow geneticists who, at the dawning of the cytochrome *b* inhibitor resistance field, were willing to send me their original inhibitor resistance mutants and allowed me to analyze their sequences. Dr. Julius Subik kindly sent his mucidin-resistance mutations, and Drs. Georg Michaelis and Elke Pratje offered me their antimycin-resistance mutations.

The sequence analysis data significantly contributed to our understanding of the structure of the cytochrome *b* gene. They also provided a stimulus for further progress (Fig. 3). Most mutations were mononucleotide substitutions resulting in a single amino acid replacement. For most positions, more than one mutant was found to bear the same substitution. In some cases, dinucleotide substitutions were found, resulting in two adjacent or distant amino acid replacements (di Rago *et al.*, 1986, 1988, 1989). In all disubstitutions, one change was identical to a known monosubstitution which alone accounted for the resistance phenotype. The phenotypic effect of the second substitution remains

unknown. The combination of the two mutations may result in a minute difference in cytochrome *b* topology and function as compared to the monosubstitution.

The sequence data enabled us to somewhat refine the genetic mapping of the mutations. Center i (ANA: antimycin-resistance, DIU: diuron-resistance) and center o (MYX: myxothiazol-resistance; MUC: mucidin-resistance) inhibitor-resistance loci of exon 1 were found to be separate and located near opposite ends of exon 1. A third myxothiazol-resistance locus (MYX) was identified in exon 5. A stigmatellin resistance locus (STI) was located in exon 3.

The study of possible mutated codon positions revealed six small subregions of the cytochrome *b* gene which can carry resistance mutations. Forty independent mutations were identified and only 11 positions were found. All center i inhibitor resistance mutations are grouped in only two segments of the gene, each with three possible mutated positions. All center o inhibitor resistance mutations are grouped in only four segments of the gene, one with three possible mutated positions, each of the other three having only one mutated position. Interestingly, antimycin-, diuron-, and funiculosin-resistance positions are different though proximally located. The mutated-position data were quite informative. The most spectacular finding concerned resistance to methoxyacrylates such as myxothiazol, mucidin (same as strobilurin), and MOA stilbene. Mutants resistant to each of these center o inhibitors, which are structurally different but present similarities to the methoxyacrylate toxophore subgroup, were selected in different laboratories and derived from different parental strains. The same changes were found, however, involving identical amino acid substitutions at identical positions (Fig. 3). These results are consistent with those of a recent study aimed at characterizing the site of the inhibitory action of methoxyacrylates (Wiggins 1992a,b). Data on the activity of structural analogues of methoxyacrylate inhibitors confirmed the importance of the methoxyacrylate subgroup for inhibitory activity and suggested its precise fitting to its binding site (Wiggins, 1992a). Moreover, two tetrapeptides, GQMS (G at position 137, Fig. 3) and TVIT (I at position 147), were proposed as best candidates for binding to the ester and amide groups of the myxothiazol and mucidin toxophores (Wiggins, 1992b). Wiggins suggested how the myxothiazol and mucidin methoxyacrylate toxophores, although different, might be accommodated at the same binding site near the glycine at position 137, the only position found for methoxyacrylate resistance mutations whether selection had been for myxothiazol, mucidin, or MOA stilbene resistance. These results support the view that the positions identified in cytochrome *b* by sequence analysis of the resistance mutants belong to a region of the protein that is important for interactions between the inhibitors and cytochrome *b*.

Inhibitor Resistance Positions and Cytochrome *b* Folding Models

In the absence of *bc* complex crystals with a good resolution, structural data are poor. Structural predictions based on the deduced amino acid sequence of cytochrome *b* identify transmembrane alpha helices and the conserved histidine ligands of the heme groups. A structural model was proposed involving nine transmembrane alpha helices, with the same two helices carrying the ligands of the two hemes (Widger *et al.*, 1984; Saraste, 1984). Ohnishi *et al.*, (1989) confirmed the spatial organization of the active redox centers i and o, in the respective vicinities of heme *b*562 or *bh*, near the middle of the phospholipid bilayer, and heme *b*566 or *bl*, closer to the outer surface of the membrane.

The positions of the mutated codons conferring resistance to center i and center o inhibitors were superimposed on the corresponding amino acids in the nine-transmembrane-alpha-helices model (Fig. 4). Surprisingly, two center i inhibitor-resistance regions ended up on opposite sides of the membrane and so did two center o inhibitor resistance regions. On the basis of other calculations, a model with only eight transmembrane alpha helices was presented (Rao and Argos, 1986; Crofts *et al.*, 1987; Brasseur, 1988). The previous fourth transmembrane alpha helix, C–D in Fig. 4, having amphiphilic properties, was removed from the membrane (Fig. 5). In this second model, the two center i inhibitor-resistance regions are located on the proposed inner or negative side of the membrane and the center o inhibitor resistance regions on the outer or positive side of the membrane. These four subregions are the most conserved of the cytochrome *b* apoprotein; about 20 center i and center 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 (Howell and Gilbert, 1987, 1988;

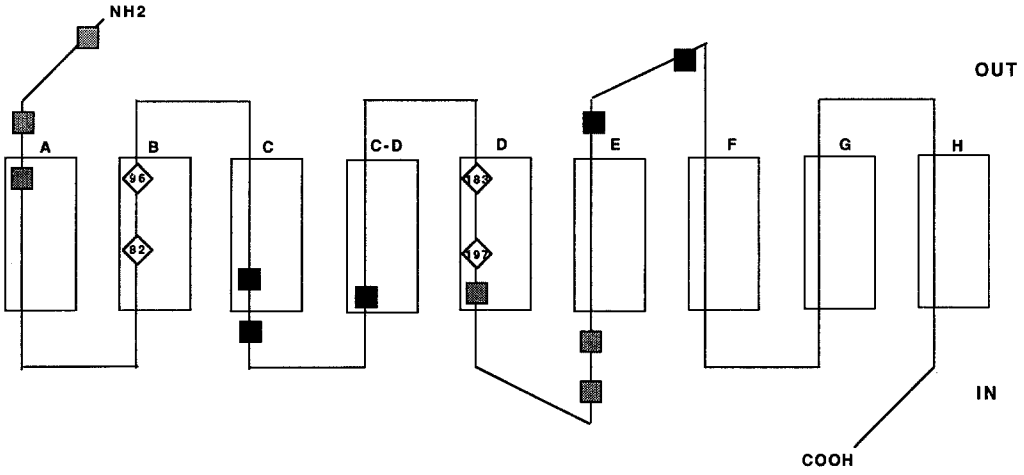

Fig. 4. Center i and center o inhibitor resistance positions in the nine-transmembrane-alpha-helices folding model of cytochrome *b*. A schematic representation of the model of Widger *et al.* (1984) and Saraste (1984). Dark squares are center o inhibitor-resistance positions and grey squares are center i inhibitor-resistance positions. Diamonds represent the histidine ligands of the hemes. Heme *bl* or *b*566 ligands are H82 and H183; heme *bh* or *b*562 ligands are H96 and H197. The transmembrane helices are designated by letters A–H. C–D represents the helix which is removed from the membrane in the eight-transmembrane-alpha-helices model (see Fig. 5) because of its amphiphilic properties. OUT indicates the outer side of the membrane; IN indicates the inner side of the membrane.

Weber and Wolf 1988; Daldal *et al.*, 1989; Coria *et al.*, 1989, Bennoun *et al.*, 1991). Prior to publishing our sequence analysis results (di Rago *et al.*, 1986; di Rago and Colson, 1988; Di Rago *et al.*, 1989), we communicated the positions of the various inhibitor resistance mutations to R. Brasseur, as experimental support to his model (Brasseur, 1988). A review of the combined mechanistic, genetic, and structural prediction approaches was presented by Colson *et al.* (1991). The eight-transmembrane-alpha-helices model provided a better link between the presumed structure and the known mechanism of the protonmotive Q cycle. The two center i inhibitor resistance regions are postulated as parts of center i and the

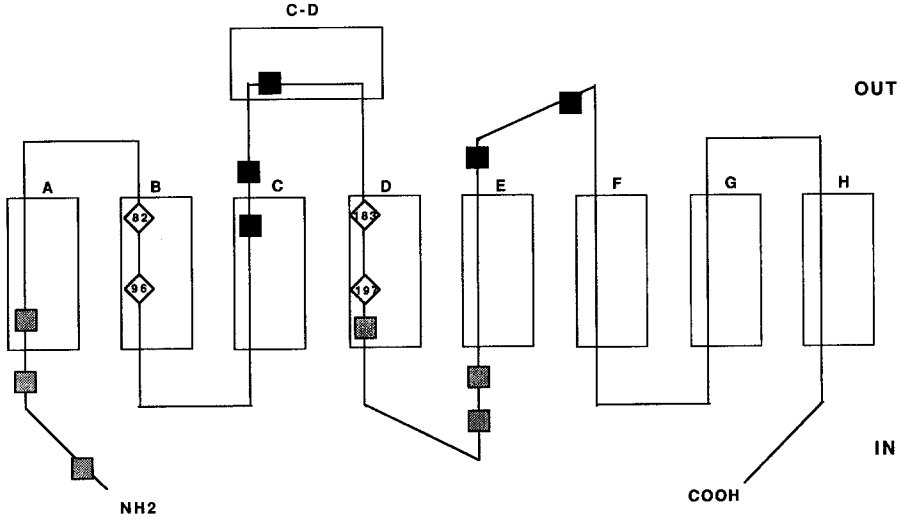

Fig. 5. Center i and center o inhibitor resistance positions in the eight-transmembrane-alpha-helices cytochrome *b* folding model. A schematic representation of the model proposed by Rao and Argos (1986), Crofts *et al.* (1987), and Brasseur (1988). Symbols are the same as in Fig. 4.

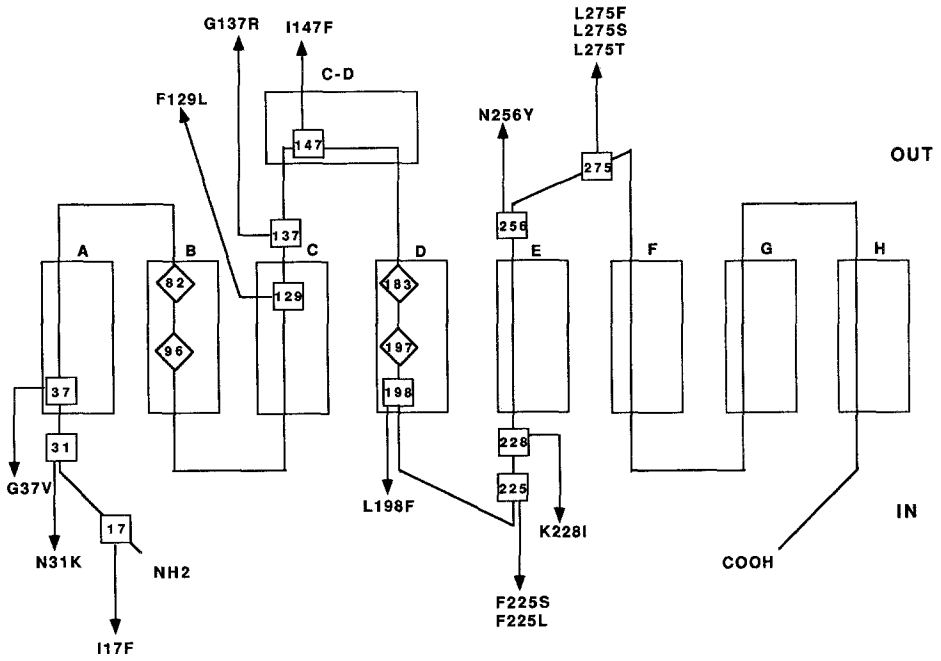

Fig. 6. Center i and center o inhibitor-resistance positions and amino acid changes in the yeast cytochrome *b* amino-acid sequence. Symbols are the same as in Fig. 4. Amino acids are represented by one-letter-code symbols.

two center o inhibitor-resistance regions as parts of center o.

Resistance Mutants and Structural Data

In the light of these structural predictions and of current knowledge of the bc_1 complex mechanism, inhibitory action sites, and bc_1 complex subunit composition, little is known about the structure and kinetics of the complex in yeast resistance mutants. Data derived from two recent studies, however, are worth stressing.

1. In one study, the circular dichroic spectra of two crude bc_1 complex preparations were compared. The preparations were derived from two center o inhibitor resistance mutants whose mutations each resulted in a substitution involving an aromatic residue (Tron *et al.*, 1991). One mutant was stigmatellin-resistant and carried an isoleucine-to-phenylalanine change at position 147, in amphiphilic helix C–D, on the outer side of the membrane. The other was a myxothiazol-resistant mutant with a phenylalanine-to-leucine substitution at position 129, in the middle of transmembrane alpha helix C (Fig. 6). The CD (circular dichroism) spectra obtained for the wild type and the myxothiazol-resistant mutant were similar, but the stigmatellin-resistant mutant exhibited a modified spectrum in the vicinity of heme *bl* (*b*566). This might be interpreted as probably meaning that residue 147 is in the close vicinity of heme *bl*, while residue 129 is not.

2. Two myxothiazol-resistance mutants, each carrying a single amino acid change (glycine to arginine at position 137 or asparagine to tyrosine at position 256), were characterised biochemically (Geier *et al.*, 1992). The substitution at position 256 was found to reduce the mutant's *in vivo* growth on glycerol, indicating a partial functional impairment. *In vitro*, the purified mutant bc_1 complex exhibited an altered quaternary structure, having lost the iron–sulfur protein, the second important catalytic subunit at center o. No similar effect was observed with the wild type complex or with the complex isolated from the mutant having the substitution at position 137. The loss is specific to position 256. It is accompanied by a 10,000-fold or higher decrease of inhibitor binding. Because the mutant presented a normal cytochrome *b* absorption spectrum and a normal red-shift spectrum, the authors suggested that the heme *b* domain is preserved. Thus, a single amino acid

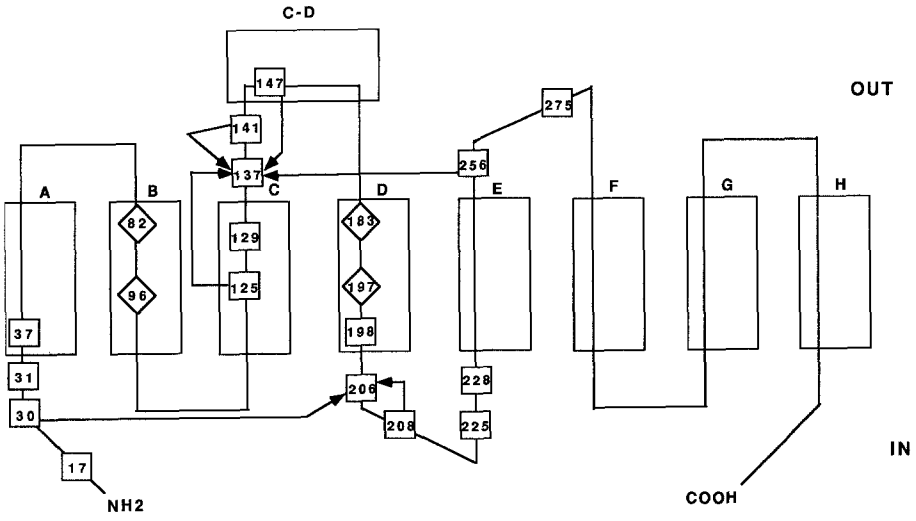

Fig. 7. Intragenic complementation between deficiency-causing and deficiency-reversing mutations in the center i and center o inhibitor resistance regions. Symbols are as in Fig. 4. The yeast numbering of the altered positions is shown. The arrows represent links between changed amino acids in the revertants, pointing from the position associated with the deficiency to the position associated with the deficiency-complementing substitution.

substitution in cytochrome *b* can dramatically affect the binding of the iron–sulfur protein without significantly altering heme *b* assembly.

Respiratory-Deficient Mutants and Intragenic Complementation in the Cytochrome *b* gene

Respiratory-deficient mutants are characterized by an inability to grow on glycerol. Revertants of cytochrome *b*-deficient mutants carrying a second mutation in the apocytochrome *b* gene have been sought and obtained; the positions and natures of the resulting residue substitutions have been determined (di Rago *et al.*, 1990b, 1990c; Coppée *et al.*, in preparation; Colson *et al.*, 1992). Interestingly, the distant reversions highlight pairs of amino acids which are at least functionally related and may be structurally in the same vicinity (Fig. 7). So far, these revertant studies link pairs of amino acids situated on the same side of the membrane. They provide an additional argument in favor of the spatial proximity of the two center i resistance regions, on one hand, and of the two center o resistance regions, on the other. Distant substitutions reversing the effects of position-137 replacements were found at (positions 147, 256) or near (positions 125, 141, 151) known center o inhibitor-resistance positions (Fig. 7) (di Rago *et al.*, 1990c). Also, substitutions reversing the effects of positions-133 and -131 replacements were found at positions 126 and 260, respectively. A thermosensitive revertant carrying its deficiency-causing substitution at position 133 and its deficiency-reversing replacement at position 126 was used to select new thermoresistant revertants (Tron *et al.*, 1991). Some secondary, position-133 revertants, while retaining the primary reversion at 126, presented an increased resistance to a center o inhibitor. Also, revertants with a deficiency-causing substitution at position 142 presented an increased resistance to myxothiazol, a center o inhibitor (Bruel *et al.*, 1992). Triple amino acid changes allowing the protein to function partially were also identified, the third substitution being at position 130 or 135 (Tron *et al.*, 1991). Likewise, distant reversions of position-206 deficiencies were found at positions 208 and 30, thus showing a relationship between the two center i inhibitor resistance regions (Coppée *et al.*, in preparation; Colson *et al.*, 1992).

At glycine 137 acid in the center o inhibitor-resistant region, a glycine-to-arginine substitution was found in mutants resistant to myxothiazol, mucidin, or MOA stilbene. These mutants grow poorly on glycerol but their growth is resistant to the above-mentioned inhibitors. They present only a slightly increased resistance to methoxyacrylate inhibitors *in vitro* (Geier *et al.*, 1992).

Two respiratory-deficient mutants were found to carry a glycine-to-glutamate or a glycine-to-valine substitution also at position 137 (Tron and Lemesle-Meunier, 1990). Interestingly, even though these changes led to a total inability to grow on glycerol, these deficient mutants retained some electron transfer activity, which one would have thought sufficient to support some growth. Why growth on glycerol was totally impaired is not understood. Moreover, the remaining electron transfer flow in these mutants presented an *in vitro* resistance to myxothiazol together with antimycin sensitivity.

The study of revertants of frame-shift mutations in the cytochrome *b* gene made it possible to isolate cytochrome *b* mutants carrying, adjacently, more than one amino acid change (Colson *et al.*, 1992). It appeared that cytochrome *b* can tolerate major structural changes in regions located outside the center i and center o inhibitor-resistance regions. Finally, revertants in positions 30, and 208 derived from a deficient mutant in position 206 (S206L) as well as revertants of a frame-shift mutation in 204, all positions located within the center i inhibitor-resistance regions (30, 204, 208), were obtained; the resulting changes caused a significant reduction of NADH oxidase activity. This suggests that bc_1 complex activity might become rate-limiting in such mutants and that these positions may be involved in the catalytic activity at center i (Coppée *et al.*, in preparation; Colson *et al.*, 1992; Brasseur *et al.*, 1992).

A number of deficient mutants were studied biochemically. In one case, a deficiency mutation resulting in a cysteine-to-tyrosine substitution at position 133 led to a significant diminution of heme b_l relative to heme b_h. In another, a deficiency mutation resulting in a serine-to-leucine substitution at position 206 produced a significant diminution of heme *bh* relative to heme *bl* (Meunier-Lemesle *et al.*, 1988). These data are consistent for the proximal locations of positions 133 and 206 to heme *bl* and heme *bh* ligands, respectively. Another key position in the center o inhibitor region is position 131, where a glycine-to-serine substitution has been found (Chevillotte-Brivet *et al.*, 1988).

The amino acids between positions 125 and 151 appear to play a key role in both center o inhibitor-resistance and in the catalytic activities at center o.

Future prospects

The use of random mutants as tools to improve our knowledge in cytochrome *b* structure and function will be pursued. The efficient generation and screening of random cytochrome *b* deficient mutants (unable to grow on respiratory substrate) with optically detectable cytochrome *b* will provide new series of mutants of potential interest for bioenergetic studies (Meunier *et al.*, in press, Brown *et al.*, 1992). These mutants will be made available for new series of revertants'-selection and characterization. Center i and center o inhibitors of high affinity and their analogues (Wiggins, 1992a; Tokutake *et al.*, in press; Miyoshi *et al.*, 1992) will be testable on the whole collection of mutants with optically detectable cytochrome *b* and retaining partial or complete activity; it will provide further insights into the topology of the two centers.

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