

Structure/function relationships in mitochondrial cytochrome *b* revealed by the kinetic and circular dichroic properties of two yeast inhibitor-resistant mutants

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The kinetic and circular dichroic properties of two yeast mutants that are resistant towards specific inhibitors of the mitochondrial cytochrome *bc*₁ complex have been characterized. Both of these mutants have an altered cytochrome *b* gene in which aromatic residues are exchanged with non-polar residues in a highly conserved region of the protein.

The mutant resistant to myxothiazol and mucidin that contains the substitution Phe129 → Leu is not greatly affected either in its ubiquinol:cytochrome *c* reductase or in the spectral properties of cytochrome *b*. On the other hand, the mutant resistant to stigmatellin that contains the substitution Ile147 → Phe shows a large decrease of the catalytic efficiency for ubiquinol and of the maximal turnover of its reductase activity. This stigmatellin mutant also shows an altered circular-dichroic spectrum of the low-potential haem of cytochrome *b*.

This study provides biochemical and biophysical information for identifying a region in mitochondrial cytochrome *b* that may fulfill a crucial role in the binding of ubiquinol to the *bc*₁ complex. The results are discussed also in terms of the structural model of cytochrome *b* having a core of four transmembrane helices.

The mitochondrial *bc*₁ complex catalyzes the electron transfer from ubiquinol to cytochrome *c*. In yeast, this enzyme is composed of at least nine subunits, three of which have a redox function: cytochrome *b*, cytochrome *c*₁ and the Rieske iron-sulphur protein [1]. Cytochrome *b*, the only subunit that is encoded by the mitochondrial DNA [1], is an integral protein which carries two haems, *b*_H-562, the high-potential form (*b*_H) and *b*_L-566, the low-potential form (*b*_L). These haems are located towards the inner and outer side of the mitochondrial membrane, respectively [2–6], and form a transmembrane electron circuit between the two redox sites of ubiquinone within the *bc*₁ complex, Q_i and Q_o, according to the Q-cycle scheme for mitochondria [1, 4, 7] (or Q_c and Q_m, respectively, in the equivalent schemes for bacteria [6, 8]).

Inhibitors like antimycin, diuron and funiculosin act at the Q_i (Q_c) site as they block the oxidation of cytochrome *b* by ubiquinone and affect the *b*_H haem, whereas inhibitors like myxothiazol, mucidin and stigmatellin affect the Q_o (Q_m) site since they block the reduction of the Rieske protein and alter the optical properties of the *b*_L haem [8–12]. Stigmatellin also affects the EPR and redox properties of the Rieske cluster [13].

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Abbreviations. *b*_H, high-potential cytochrome *b* or *b*-562; *b*_L, low-potential cytochrome *b* or *b*-566; *I*₅₀, concentration of inhibitor which reduces by one-half the reductase activity; *k*_{min}, *V*_{max}/*K*_m or second-order rate constant of the reaction between ubiquinol and the reductase; Q₂, ubiquinone-2; Q₂H₂, ubiquinol-2.

Enzyme. Ubiquinol:cytochrome *c* reductase (EC 1.10.2.2).

The availability of several potent inhibitors of the *bc*₁ complex has favoured the selection of many mutants that are resistant *in vivo* to any of these compounds. These mutants generally show a single amino acid substitution in the gene for cytochrome *b* [14–17], which indicates that both Q_i and Q_o inhibitors bind to this subunit of the complex [1, 4, 6]. The location of the genetic lesions responsible for resistance towards inhibitors of the two different classes has suggested a revision of the former nine-helices model for the structure of cytochrome *b* [1, 6, 15–19]. In the current model, only eight helices are considered to transverse the membrane and the former helix IV is believed to form an amphipathic helix which possibly runs parallel to the membrane plane [1, 6, 18].

Several mutations leading to the resistance towards Q_o inhibitors map around or within the region corresponding to the former helix IV. In yeast such mutations are at position 129, 137 and 147 [16], in mouse at positions 143 and 148 [19] and in *Rhodobacter capsulatus* at positions 125, 129, 137, 143 and 148 [20] (using the alignment to the yeast sequence [16]). Natural resistance towards myxothiazol and mucidin in sea urchin mitochondria is also related to the substitution of the highly conserved Gly143 with Ala [21] as in mouse and *Rb. capsulatus* mutants [19, 20]. It is increasingly clear, therefore, that the region spanning residues 129–148 is directly involved in the binding of Q_o inhibitors and, presumably, also of ubiquinol as these compounds have structural properties in common with quinols [6, 16, 19–22].

In order to characterize the structural and functional changes that are associated with the specific amino acid replacements in mutants resistant to inhibitors of the *bc*₁ complex, we report here results on the enzymatic and CD proper-

ties of two yeast mutants, one resistant to myxothiazol (Phe129→Leu) and the other resistant to stigmatellin (Ile147→Phe). We have chosen to study these mutants, in which a phenylalanine is either replaced or inserted, as the CD spectra of oxidized cytochrome *b* are essentially due to the dipole coupling of each haem with surrounding aromatic residues, especially phenylalanines [23]. The results reveal novel structure/function relationships in mitochondrial cytochrome *b* since the stigmatellin mutant, contrary to the myxothiazol mutant, shows a remarkable decrease in the catalytic efficiency of the ubiquinol:cytochrome *c* reductase in parallel with a modification of the CD signal specific for the b_L haem.

MATERIALS AND METHODS

Strains and growth conditions

Parental strains: KM91, 777-3A (α *ade op1*) × KL 14-4A (a *his1 trp2*); GM50-3C (a *his1 trp2*); KL 14-4A a *his1 trp2*). The myxothiazol-resistant mutant is GM50-3C/103 (*myx1-103*) [24], whereas the stigmatellin mutant is KL 14-4A/*sti1-5* [16].

The cells were grown aerobically in YPGal medium containing 1% yeast extract, 2% bactopectone, 2% galactose, 0.1% glucose, 0.005% alanine and 0.12% ammonium sulphate, pH 6.5, in a 10-l fermentor [15, 16].

Mitochondria and bc_1 complex preparation

Mitochondrial particles were prepared by the method described in [25] modified by Meunier-Lemesle et al. [26]. Crude bc_1 complex was prepared from each strain as a soluble succinate:cytochrome *c* reductase by a simplified procedure derived from the method of Siedow et al. [27] (G. Palmer, personal communication).

The total content of cytochrome *b* of either mitochondrial particles or crude bc_1 complex was measured from the dithionite-reduced minus ferricyanide-oxidized spectrum at 561–575 nm by using an absorption coefficient of $28 \text{ mM}^{-1} \text{ cm}^{-1}$ [27]. The content of b_H and b_L was estimated from their optical α band reduced by appropriate redox mediators [8, 9, 23] and also from the intensity of the negative band at 424 nm with respect to the positive band at 411 nm in the CD spectrum of the oxidized preparation (the former band is specific for b_L [23]). In the crude bc_1 complex of the various strains, the ratio of the two *b* haems was very close to 1 as previously found in baker's yeast [23, 27]. The concentration of bc_1 complex was assumed to be equal to one-half of that determined for cytochrome *b*.

Reductase assay

The ubiquinol:cytochrome *c* reductase activity of the mitochondrial fractions was measured at 25°C as previously described [28], generally with quasi-saturating concentrations of horse-heart cytochrome *c* (type VI Sigma). The crude bc_1 complex was diluted to about 2–5 μM cytochrome *b* in 25 mM Tris/Cl pH 8 containing 20% glycerol (the same buffer as that used in the CD measurements [23]).

CD measurements

The CD measurements were carried out at room temperature in a Jasco J-500 spectropolarimeter under the conditions

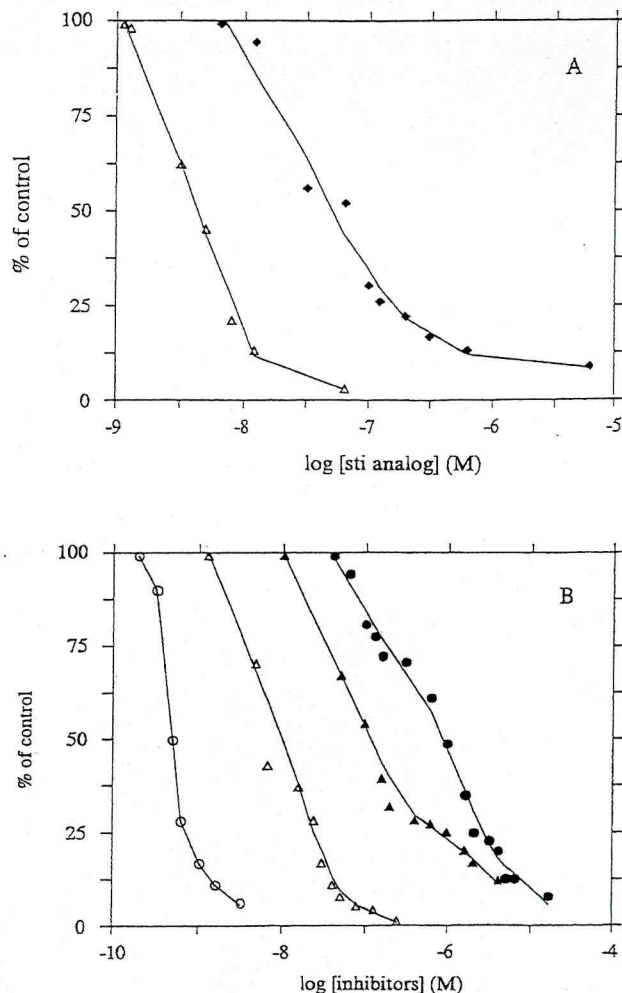


Fig. 1. Inhibition of ubiquinol:cytochrome *c* reductase by myxothiazol, mucidin and stigmatellin in the crude bc_1 complex of three yeast strains. The reductase activity was measured as described previously [21] with 15 μM cytochrome *c* and 4 μM 2,3-dimethoxy-5-methyl-6-nonylbenzoquinol. (A) Inhibition by the tridecyl analog of stigmatellin (sti analog): (Δ) wild-type KM91, 12.8 nM cytochrome *b*; (\blacklozenge) *sti1-5* mutant, 10 nM cytochrome *b*. (B) Inhibition by myxothiazol (\circ , \bullet) and by mucidin (Δ , \blacktriangle); (\circ , Δ) wild-type KM91 with 3.2 nM cytochrome *b*; (\bullet , \blacktriangle) *myx1-103* mutant with 4.1 nM cytochrome *b*. The titer of the inhibitors is very similar in all wild-type strains to that in baker's yeast (results not shown, see also [9]).

described previously [23]. Optical spectra of the same CD samples were recorded in a Jasco Uvidec-610 spectrophotometer.

Reagents

Ubiquinols were prepared and assayed as in [28, 29]. Q_2 was a generous gift from Eisai Co. (Tokyo, Japan) and nonylbenzoquinone was donated by Prof. G. Von Jagow (University of Frankfurt, FRG). The stigmatellin analog was provided by Dr T. Wiggins (ICI Agrochemicals, UK), whereas stigmatellin was purchased from Fluka. Myxothiazol was purchased from Boehringer and mucidin was kindly provided by Dr J. Subik (University of Bratislava, Czechoslovakia). The concentration of inhibitors was determined in ethanol using the absorption coefficients reported in [22].

Table 1. I_{50} values of the inhibition of ubiquinol-cytochrome *c* reductase activities of crude bc_1 complex from various yeast strains

Conditions used are described in the legend of Fig. 1. I_{50} represents the concentration of drug which decreases the rate of nonylbenzoquinol:cytochrome *c* reductase by 50% [21]

Strains	I_{50} for		
	stigmatellin	myxothiazol	mucidin
	nM		
Wild type	4	0.7	8
<i>Myx1</i> –103	3	743	115
<i>Sti1</i> –5	61	—	—

RESULTS

Inhibition of the reductase activity by myxothiazol, mucidin and stigmatellin

The sequencing of the split gene of cytochrome *b* has established that phenylalanine at position 129 is replaced by a leucine in the *myx1*–103 mutant and that isoleucine at position 147 is replaced a phenylalanine in the *sti1*–5 mutant [16]. To perform a detailed characterization of these mutants, we have titrated the activity of their crude bc_1 complex with three Q_o inhibitors (Fig. 1). We have chiefly used the stigmatellin analog having a tridecyl side chain, a compound which, although less potent, exhibits effects similar to those of stigmatellin in the bc_1 complex [30].

The *sti1*–5 mutant shows an order-of-magnitude increase in the I_{50} for this analog (Fig. 1A) as for stigmatellin (results not shown). When expressed as the ratio to the bc_1 complex, the stigmatellin mutant displays an about 20-fold increase in the titer for the stigmatellin analog (Table 1). There is no cross resistance to myxothiazol in the *sti1*–5 mutant (results not shown).

Myxothiazol and mucidin possess a methoxyacrylate group that is different from the chromone ring of stigmatellin and resembles part of the substituted ring of ubiquinol [12, 13, 22, 30]. Owing to their different effects on the Rieske cluster [30, 31], methoxyacrylate inhibitors such as myxothiazol seem to bind to a pocket in the mitochondrial bc_1 complex that is not identical to that of stigmatellin [22, 30, 31]. Consistent with this, the *myx1*–103 mutant shows a three orders-of-magnitude increase in the I_{50} of myxothiazol in the ubiquinol:cytochrome *c* reductase activity, but no cross resistance towards the stigmatellin analog (Table 1). In *Rb. capsulatus*, however, the identical substitution Phe → Leu at the position equivalent to 129 in yeast induces resistance to myxothiazol and also, though small, to stigmatellin [20, 32].

The results obtained here provide the first accurate evaluation of the titer for Q_o inhibitors in yeast resistant mutants and can be directly compared with previous results obtained in mouse mutants [19], with those reported in the natural resistant *Paracentrotus lividus* [20] and with the more recent data in *Rb. capsulatus* mutants [32]. Table 2 collects all such data and their comparison suggests the following considerations.

First, it appears that the yeast mutant having the substitution Ile147 → Phe shows the highest level of resistance towards stigmatellin, but much lower than the resistance towards myxothiazol exhibited by some myxothiazol mutants. Secondly, the level of myxothiazol resistance in the mutants (either yeast or *Rb. capsulatus*) carrying the mutation at posi-

tion 129 is lower than that of mutants or species where Gly143 is substituted by Ala [19–21]. Thirdly, resistance to mucidin is 10-fold higher in *P. lividus* (position 143) than in the yeast mutant *myx1*–103 (position 129, see als [32]).

The last two observations suggest that the mutation Phe129 → Leu affects more specifically the binding of myxothiazol, whereas the mutation Gly143 → Ala impairs the binding of the methoxyacrylate group that is common to both myxothiazol and mucidin.

Ubiquinol:cytochrome c reductase in the inhibitor-resistant mutants and their wild types

We have characterized the properties of the ubiquinol:cytochrome *c* reductase of the *sti1*–5 and *myx1*–103 resistant mutants and compared it to that of their respective wild-type strains for investigating the functional effect of the mutations. Alterations in the catalytic properties of the bc_1 complex can be deduced from the reductase assays by considering that its steady-state mechanism conforms to a ping-pong/two-site scheme in which ubiquinol and cytochrome *c* interact with independent sites [28, 33, 34]. Although the interaction of exogenous ubiquinols involves both the Q_i and the Q_o site, the latter site seems to be more rate-determining, especially in yeast [34, 35].

Classically, the functional effect of mutations involving a single amino acid is evaluated by the experimental value of V_{max}/K_m (herein abbreviated as k_{min}), which corresponds to the apparent second-order rate constant for the reaction of enzyme and substrate to form product [36]. The independent reaction of ubiquinol to its site (chiefly formed by cytochrome *b* [3, 6, 35]) and the ping-pong nature of the mechanism of the reductase render k_{min} particularly valuable for appreciating variations in the catalytic efficiency of the bc_1 complex for ubiquinol. This parameter, which is equivalent to the inverse of the slope in the reciprocal plots of the rates as a function of quinol concentration, is in fact independent of the concentration and reactivity of the other substrate, cytochrome *c* [33, 34].

An additional advantage given by the ping-pong/two-site mechanism is the possibility of measuring the dissociation constant of the product ubiquinone from its competitive inhibition versus ubiquinol [33, 34]. Indeed, the K_i of ubiquinone-2 derived from these steady-state measurements is in very good agreement with the K_d for the same quinone measured by static binding [34, 37]. Information on other properties of the interaction of ubiquinol at its site could be derived from pre-steady-state experiments [35], which, due to the large amount of enzyme required, have not been possible in this study.

The double-reciprocal plots of the Q_2H_2 and nonylbenzoquinol titrations performed in the bc_1 complex extracted from the mutants *myx1*–103 and *sti1*–5 and one related wild type are shown in Fig. 2. Although *myx1*–103 exhibits a decrease in V_{max} (which is, however, within the range of variability seen in various wild-type strains, results not shown), the slope of the plots is not substantially changed from that of the wild type. This suggests that the catalytic efficiency of the bc_1 complex for ubiquinol is not much altered in this mutant. Moreover, only a slight increase of the dissociation constant for ubiquinone is seen in *myx1*–103 (Table 3).

In the case of the *sti1*–5 enzyme, however, the reciprocal plots with both quinols show a one order-of-magnitude decrease of the value of k_{min} relative to any wild type (Fig. 2 and

Table 2. Overview of the various mutants resistant to Q_0 inhibitors

The data are taken from [16, 19–21, 40, 50, 51]. The number refer to the positions in the sequence of yeast cytochrome *b*, to which the sequences of the other species have been carefully aligned [16, 21, 42]. The relative ratio of resistance expresses the ratio between the I_{50} of the resistant mutant and that of its wild-type strain, obtained *in vitro*. In the case of the natural resistant *P. lividus* [21] and beef, this ratio has been calculated using the I_{50} found here in the wild-type strain of yeast (Table 1) after normalization to the same content of cytochrome *b*. Yes and no indicate the phenotypic resistance towards an inhibitor found *in vivo* and/or *in vitro* (this work and [16, 20]). The dash signifies that data are not available

Species	Position and substitution	Relative ratio of resistance to		
		myxothiazol	mucidin	stigmatellin
<i>Rb. capsulatus</i>	Met125 → Leu	20	no	8
<i>S. cerevisiae</i>	Phe129 → Leu	1000	14	no
<i>Rb. capsulatus</i>	Phe129 → Leu	530	no	5
<i>Rb. sphaeroides</i>	Phe129 → Ser	28	—	—
<i>Rb. capsulatus</i>	Phe129 → Ser	530	yes	no
<i>C. reinhardtii</i>	Phe129 → Leu	yes	yes	—
<i>S. cerevisiae</i>	Gly137 → Glu	20	—	—
<i>S. cerevisiae</i>	Gly137 → Arg	yes	yes	no
<i>Rb. capsulatus</i>	Gly137 → Ser	37	yes	2
<i>M. musculus</i>	Gly143 → Ala	2110–19380	yes	1–4
<i>P. lividus</i>	Gly143 → Ala	1990	147	—
<i>Rb. capsulatus</i>	Gly143 → Ala	yes	yes	—
<i>S. cerevisiae</i>	Ile147 → Phe	no	no	17
<i>M. musculus</i>	Thr148 → Ala	no	—	6
<i>Rb. capsulatus</i>	Thr148 → Met	3	no	6
<i>S. cerevisiae</i>	Asn256 → Tyr	yes	yes	no
<i>S. cerevisiae</i>	Leu275 → Ser, Phe, Thr	yes	yes	no
<i>B. taurus</i>	Leu275 → Phe	2	3	—
<i>Rb. capsulatus</i>	Val292 → Ala	5	no	7
<i>M. musculus</i>	Leu295 → Phe	4	no	5

Table 3). We exclude that such a dramatic change is due to an enhanced lability to the extraction conditions of this mutated enzyme, as the same decrease of V_{max}/K_m is seen in the mitochondrial particles of *sti1-5* with respect to those of its parent strain KL 14-4A (the actual values with Q_2H_2 being $6.4 \times 10^6 M^{-1} s^{-1}$ for *sti1-5* and $6.0 \times 10^7 M^{-1} s^{-1}$ for the wild type). Therefore, it is clear that the substitution Ile147 → Phe in cytochrome *b* of the *sti1-5* mutant produces a large decrease of the catalytic efficiency for ubiquinol. On the other hand, the K_i of Q_2 is not changed in this mutant (Table 3), thereby indicating that the affinity for the quinone product is not modified.

Circular-dichroic properties of the crude bc_1 complex from mutants and wild-type strains

The resolved properties of cytochrome *b* within the bc_1 complex from yeast and other species have been characterized [23]. The bisignate Cotton effect in the Soret region is essentially derived from the dipole coupling of each of the two haems of cytochrome *b* with surrounding aromatic residues plus, especially in the reduced state, an exciton signal due to haem–haem coupling [23]. This latter signal does not greatly contribute to the spectral features of the oxidized state, in which the negative band at about 424 nm is specifically attributed to the interaction of the b_L haem with its protein environment [23]. This band is so characteristic of the b_L haem that it can be recognized even in crude preparations of the complex [23].

With this in mind, we have studied here the CD spectra of the resistant mutants *myx1-103* and *sti1-5* as they both

involve a modification of aromatic residues that are postulated to be near the b_L haem in the current models for the structure of cytochrome *b* [6, 18, 19, 20, 23, 32]. Any significant alteration of the CD signal specific to the b_L haem in these mutants, therefore, could be correlated to the structural position of the mutated residues and also provide information on the topology of b_L within the protein.

The CD spectra of the oxidized crude bc_1 complex from the two resistant mutants and one related wild type are similar in their overall intensity (Fig. 3A). However, the spectrum of the *sti1-5* complex is completely shifted towards negative $\Delta\epsilon$ and shows a wider negative band with respect to the spectrum of the wild type or to that of the purified enzyme from baker's yeast (cf. [23]). These alterations suggest that the replacement of Ile147 with Phe in the stigmatellin-resistant mutant leads to a different electronic environment of the b_L haem. Modifications in the haem–haem coupling are unlikely to be the cause of the observed CD changes as the peak-to-through intensity is identical in the *sti1-5* mutant and in the wild type (Fig. 3A, cf. [23]).

The oxidized CD spectrum of the crude bc_1 complex from the *myx1-103* mutant similar to that of the wild type (Fig. 3A). Thus, the replacement of Phe129 with Leu in the myxothiazol-resistant mutant induces a weaker effect than the introduction of Phe at position 147 on the environment of the b_L haem.

In agreement with previous conclusions that the couplings of the haems with aromatic residues are considerably overlapped by the haem–haem coupling in reduced cytochrome *b* [23], there is no substantial difference in the reduced CD spectra of all strains (Fig. 3B). We have checked the optical

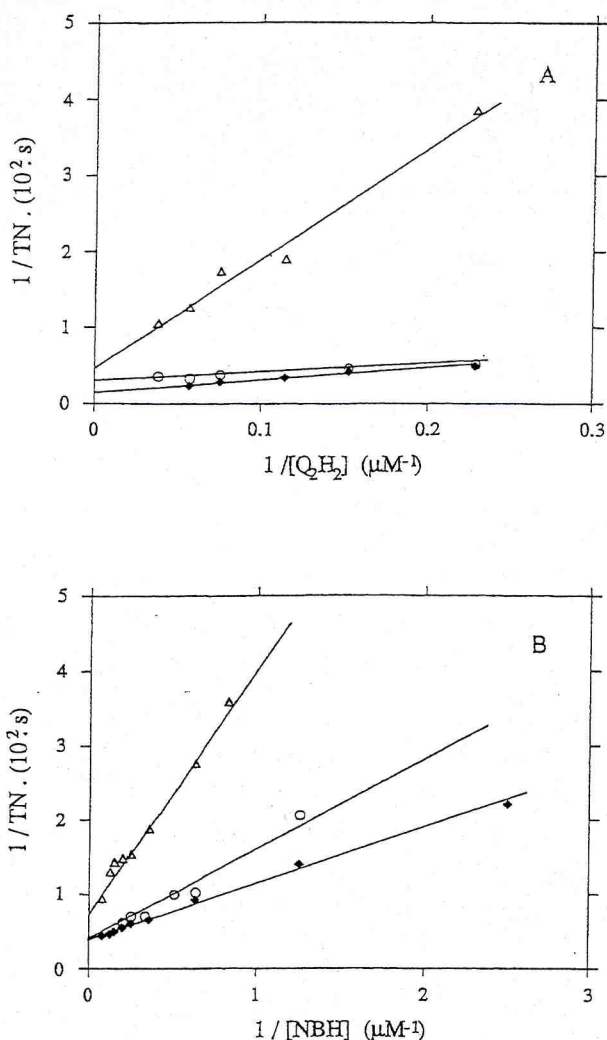


Fig. 2. Ubiquinol:cytochrome *c* reductase of the crude bc_1 complex from various yeast strains. The reductase activity was measured with a fixed concentration of 15 μM cytochrome *c* under the conditions described previously [28]. The enzymatic rates are expressed in turnover numbers (TN) normalized to the concentration of the bc_1 complex as in [21, 28]. (A) Double-reciprocal plot of the rates as a function of the Q_2H_2 concentration: (\blacklozenge) wild type KM91; (\circ) *myx1-103*; (\triangle) *sti1-5*. (B) Double-reciprocal plot of the rates as a function of the concentration of nonylbenzoquinol (NBH): (\blacklozenge) wild-type KM91; (\circ) *myx1-103*; (\triangle) *sti1-5*

spectra of the mutants and found no significant alteration of the reduced α and Soret bands that are characteristic of each *b* haem, at least within the resolution of our instruments (results not shown).

The present CD data are interpreted to mean that, in the native cytochrome *b*, Ile147 is presumably close to the b_L haem, whereas Phe129 may be located beyond the limit of 1.5 nm at which aromatic-haem coupling can be discerned [23]. However, the latter conclusion is not so straightforward, since the loss of the eventual dipole coupling of Phe129 with the b_L haem might be partially compensated by some adjustments in the tertiary structure resulting in stronger interactions of this haem with other aromatic residues.

DISCUSSION

Binding sites of Q_o inhibitors and ubiquinol in cytochrome *b*

The mutation Ile147 \rightarrow Phe of *sti1-5* is located in a region of cytochrome *b* which is topologically not equivalent, though close in the sequence, to the mutation Phe129 \rightarrow Leu. The former position belongs to an amphipatic loop or helix (corresponding to the former helix IV [6, 18]), whereas the latter position lies in a hydrophobic region which is consistently predicted to form the C-terminus part of transmembrane helix III or C [6, 18–21]. This different location of the mutation within the structure of cytochrome *b* can be correlated with: (a) their different effects on the binding of methoxyacrylate inhibitors and stigmatellin (Tables 1 and 2); and (b) their different alteration of the catalytic efficiency of the bc_1 complex for ubiquinol (Table 3).

We note first that, among the stigmatellin-resistant mutants, the mutation Ile147 \rightarrow Phe (yeast strain *sti1-5* [16]) induces the highest level of resistance to this inhibitor (Table 2). So, this mutation can be considered to have altered the binding pocket of the chromone ring which is the active part of stigmatellin [22]. As the replacement Ile147 \rightarrow Phe dramatically decreases the catalytic efficiency of the bc_1 complex for ubiquinol (Fig. 2 and Table 3), it is conceivable that this position is somehow critical also for the binding of the benzoquinol ring of ubiquinol. Note, in fact, that the ring group of stigmatellin strongly resembles the ubiquinol ring [22]. Furthermore, the b_L haem may lie within 1.5 nm of the ring-binding site for ubiquinol or stigmatellin, as deduced from the type and intensity of the CD change in the oxidized spectrum of the *sti1-5* mutant (Fig. 3A).

Table 3. Catalytic properties of the ubiquinol:cytochrome *c* reductase of the crude bc_1 complex in various yeast strains

The assay conditions are those described in the legend of Figs 1 and 2. The concentration of cytochrome *c* was always 15 μM , which ensured quasi-saturation of the enzyme as its K_m is 2–3 μM in all strains (results not shown). V_{\max} values are expressed in turnover numbers, i.e. mol cytochrome *c* reduced \times (mol bc_1 complex) $^{-1}$ s $^{-1}$. Note that the values of k_{\min} for ubiquinol-2 (i.e. V_{\max}/K_m) in other wild-type strains are very similar to those of KM91. NBH = 2,3-dimethoxy-5-methyl-6-nonylbenzoquinol

Kinetic parameter and unit	Ubiquinol	Value in yeast strain		
		KM91 (wt)	<i>myx1-103</i>	<i>sti1-5</i>
V_{\max} (s $^{-1}$)	Q_2H_2	556	333	208
K_m (μM)	Q_2H_2	8.1	3.3	23.9
k_{\min} (M^{-1} s $^{-1}$)	Q_2H_2	6.9×10^7	1.0×10^8	8.7×10^6
K_i (μM)	Q_2	21	25	20
V_{\max} (s $^{-1}$)	NBH	250	228	139
K_m (μM)	NBH	1.9	2.3	4.9
k_{\min} (M^{-1} s $^{-1}$)	NBH	1.4×10^8	1.0×10^8	2.8×10^7

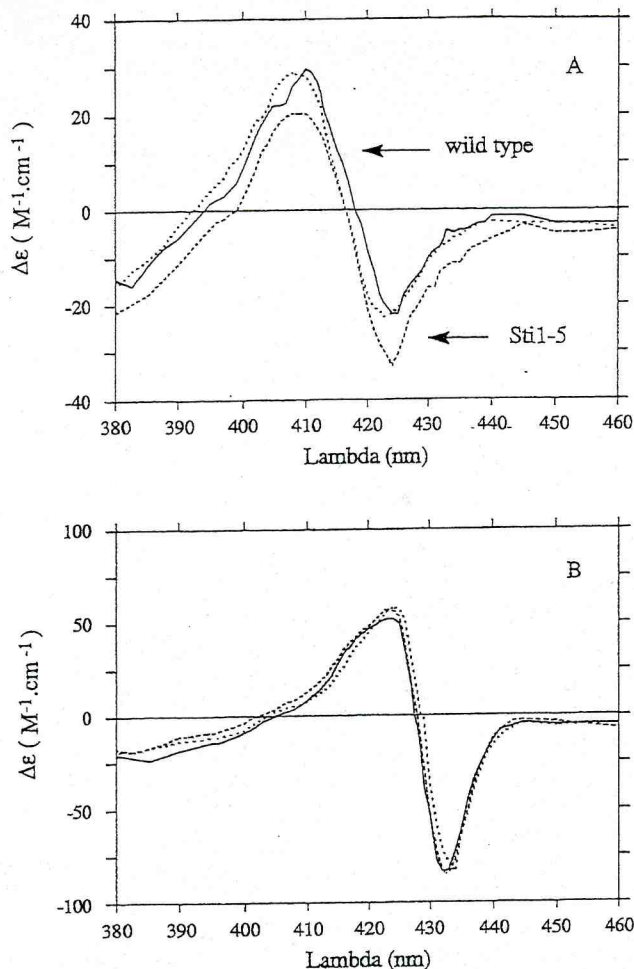


Fig. 3. Circular dichroic spectra of crude bc_1 complex of three yeast strains. The preparations from the wild-type KM91 and the resistant mutants $myx1-103$ and $sti1-5$ contained 3.45, 3.73 and 3.93 μM cytochrome b , respectively, dissolved in 25 mM Tris/Cl pH 8 containing 20% glycerol and 0.1% recrystallized sodium cholate [23]. (—) Wild type; (.....) $myx1-103$ mutant; (---) $sti1-5$ mutant. (A) Spectra of the oxidized enzymes. (B) Spectra of the dithionite-reduced enzymes. The optical spectra in the Soret region were indistinguishable in the three preparations. It is interesting to note that the overall shift towards the negative $\Delta\epsilon$ of the oxidized spectra of the $sti1-5$ mutant may be caused by the gain of a single Cotton effect due to the coupling of Phe147 with the b_L haem. Indeed, a similar shift is seen in the CD spectrum of iso-cytochrome c with respect to its mutants in which Phe82, that is known from the crystals to lie about 0.5 nm from the haem, is replaced by Ile or Leu [49]

By contrast, the mutations conferring resistance to stigmatellin in *Rb. capsulatus*, which occur at positions equivalent to the residues 125, 129, 148 and 292 in yeast cytochrome b , do not induce substantial changes in the reactivity of the bc_1 complex with ubiquinol as judged by their flash-induced transient kinetics [32]. This, together with the low level of resistance towards stigmatellin (Table 2, see also [19, 20, 32]), suggests that these positions are not largely involved in the binding of the ring of either the inhibitor or the quinol substrate. As the stigmatellin-sensitive positions 125, 129, 292 [20] and 295 [19] are located within the transmembrane helices C and F [16–20], it is possible that they are involved rather in the binding of the hydrophobic tail of stigmatellin. The same helices C and F may also contribute to the binding of the hydrophobic tail of myxothiazol, since a

weak resistance towards this inhibitor is seen in the above positions conferring stigmatellin tolerance in mouse and *Rb. capsulatus* (Table 2). By inference, it might be that helices C and F are partially involved in the interaction with the isoprenoid tail of ubiquinol as well.

The mutation Gly143 \rightarrow Ala induces the strongest resistance towards the methoxyacrylate inhibitors myxothiazol and mucidin (Table 2 and [16, 19–21]). Presumably, as such a position is located in an extrinsic loop of cytochrome b [6, 16–20], it is primarily involved in the binding of the methoxyacrylate group, the active and most hydrophilic part of the inhibitors. This moiety resembles part of the dimethoxy-substituted ring of ubiquinol [22] and thus can occupy part of the binding pocket for ubiquinol in cytochrome b . Indeed, the mutation Gly \rightarrow Asp in *Rb. capsulatus* at the position equivalent to Gly143 of yeast [20] produces a deficient mutant (R126) which has a non-functional Q_z (or Q_o) site [32, 38], thereby suggesting a critical role of this position in the binding of ubiquinol [20, 32].

The binding of myxothiazol also involves position 129, which lies in transmembrane helix C and seems to be much less important for the binding of mucidin than position 143 (Table 2). Hence, by considering that these two inhibitors essentially differ in the structure of their hydrophobic tail [22], it is likely that position 129 is more essential for the recognition of their hydrophobic tail than for their methoxyacrylate group, as discussed before.

Apparently, several results obtained in the mitochondrial bc_1 complex do not sustain the concept that myxothiazol binds exactly at the same site as that of ubiquinol (for instance, see [22, 37, 39]). However, in *Rb. capsulatus* one myxothiazol-resistant mutant (showing the same substitution as that of yeast $myx1-103$ [20]) is heavily affected in the rate constants of reactions that are characteristic of ubiquinol oxidation at the Q_z or Q_o site [32]. These observations are interpreted to mean that the replacement Phe144 \rightarrow Leu (at a position equivalent to position 129 in yeast) induces a weakening in the binding of both ubiquinone and ubiquinol at the Q_z (or Q_o) site in the bc_1 complex [32]. The transient kinetics measurements in [32] are not directly comparable to the present steady-state data, but it seems unlikely that alterations such as seen in this *Rb. capsulatus* mutant would not affect clearly either the catalytic efficiency or the dissociation constant for ubiquinone in the yeast $myx1-103$ mutant that carries the same residue substitution. On the other hand, neither of these parameters is much affected in $myx1-103$ with respect to the wild-type strains (Fig. 2 and Table 3).

Nevertheless, this discrepancy does not necessarily imply a controversy, for several points indicate that the same Phe \rightarrow Leu substitution in transmembrane helix C induces different phenotypic effects in the bacterial and in the mitochondrial bc_1 complex. First, the myxothiazol-resistant mutant in *Rb. capsulatus* exhibits a cross-resistance towards stigmatellin that is not seen in the yeast $myx1-103$ mutant (Tables 1 and 2, see also [16, 20, 32]). Secondly, another myxothiazol-resistant mutant of *Rb. capsulatus* shows the substitution Phe144 \rightarrow Ser but its kinetic properties are little affected [32] and its phenotype looks quite similar to that of the yeast $myx1-103$ mutant (Table 2, cf. [32]). Thirdly, in the alga *C. reinhardtii* the Phe129 \rightarrow Leu substitution confers resistance towards myxothiazol without altering significantly the mitochondrial respiration [40].

Furthermore, the sequence of both cytochrome b and the Rieske protein in bacteria have long insertions located at the Q_o side of the membrane [6, 20, 41, 42] which are absent in

these subunits of mitochondria [41]. It is possible that such extra protein regions are involved in contacts that are specific to the bacterial bc_1 complex with the part of transmembrane helix C containing position 129 (Phe144 in *Rb. capsulatus* [20]). One might speculate that this explains the observed effect of the Phe144 → Leu mutation on the bacterial Rieske cluster [32]. Certainly, in the light of the different functional effects in *Rhodobacter* and yeast or algal cytochrome *b*, the proposal that an aromatic in position 129 of cytochrome *b* is essential for the function the Q_z (Q_o) site [32] can not be extended to the mitochondrial bc_1 complex.

Further information for the structure of cytochrome *b*

The present work not only suggests possible structure/function correlations between the inhibitors and the ubiquinol binding site in the bc_1 complex, but also gives additional information for implementing the modeling of the structure of cytochrome *b*. In particular, the finding that position 147 is within the dipole interaction region (i.e. < 1.5 nm [23]) of the b_L haem (Fig. 3) agrees with previous evidence indicating that this haem is not far from the water-lipids interphase [5, 8], since this position belongs to an amphipathic region that is now considered to protrude at the positive side of the membrane [6, 16–21, 42]. On the other hand, the slight effect on the CD spectra induced by the loss of a phenylalanine at position 129 *myx1*–103 mutant, Fig. 3) is helpful for locating the side of transmembrane helix C that may be most distant from the b_L haem.

In the current model of cytochrome *b*, the position of helices B and D are constrained since each of them possesses two invariant histidines that form the axial ligands of the two haems [3, 6, 18, 41, 42]. In our opinion, the following arguments, when considered together with the above discussion, strengthen the concept that transmembrane helices A–D form the central core of cytochrome *b*.

a) By using the sequence alignment of 23 species selected from all those available [42], it is seen that helices A, B, C and D have a periodical distribution of the conserved residues resembling that previously seen in bacterial reaction centres [42–44]. The majority of the most conserved and of the invariant residues are located on the same side of the helices; in helices B and D they surround the ligand histidines (i.e. they are haem-sided, Fig. 4).

b) Among the few invariant residues, there are four glycines, Gly33 and Gly47 in helix A and Gly117 and Gly131 in helix C, each pair spaced by 13 residues, which is the same distance separating the pair of invariant histidines in helices B and D [41, 42]. These glycines, which have the smallest side chain of all the amino acids, may be just positioned symmetrically in the tertiary structure to give some 'spatial comfort' to the two haems of cytochrome *b* (i.e. they form part of the haem pocket). Consequently, they may be critical for the packing and assembly of the protein and even of the whole bc_1 complex. This idea is supported by the partial loss in the assembly of the complex when Gly131 is replaced by Ser in a respiratory deficient mutant of *S. cerevisiae* [45].

c) There is a clear similarity in the sequence motifs that characterize the probable ends of helices A and C and those of helices B and D (M. Degli Esposti, unpublished results; see also [41, 42, 44]). This, together with the equally spaced doublets of histidines in helices B and D and of glycines in helices A and C, is reminiscent of a duplicated motif of two helices, as it is generally seen in proteins organized in antiparallel bundles of four helices [43, 46–48].

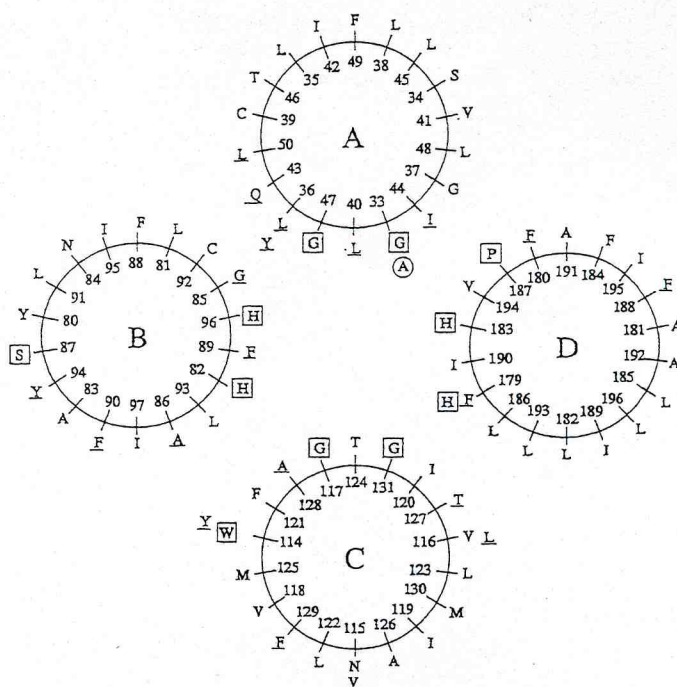


Fig. 4. Helical wheel representation of the transmembrane helices A, B, C and D in the haem-binding domain of cytochrome *b*. The numbers refer to the sequence of cytochrome *b* in yeast (*S. cerevisiae*), to which 22 other sequences, selected among the 100 or so available, were aligned [42]. For helix D only the consensus of 21 sequences minus those of cytochrome b_6 has been used to avoid the insertion of a threonine after the invariant His182 that is present in the b_6 sequence [41]. Invariant residues are squared, conserved residues in cytochrome *b* but not in b_6 are circled and residues conserved in more than 75% of all the sequences are underlined. Note that the orientation of helix C has been chosen so as to locate Phe 129 at a side not facing the haem pocket, which is supposed to lie at the centre of the helical bundle [44–48], on the basis of the CD spectra of the Phe129 → Leu mutant (Fig. 3A). The protein is viewed along the membrane plane from the positive side

The core formed by the transmembrane helices A to D (Fig. 4). May also constitute the essential catalytic unit of the quinol reaction sites in the bc_1 complex [47, 48], as the present characterization of the Ile147 → Phe mutation outlines.

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REFERENCES

1. De Vries, S. & Marres, C. A. (1987) *Biochim. Biophys. Acta* **895**, 205–239.
2. Berry, E. A. & Trumpower, B. L. (1985) in *Coenzyme Q* (Lenaz, G. ed.) pp. 365–389, John Wiley, Chichester.
3. Trumpower, B. L. (1990) *Microbiol. Rev.* **54**, 101–129.
4. Trumpower, B. L. (1990) *J. Biol. Chem.* **265**, 11409–11412.
5. Ohnishi, T., Shagger, H., Meinhardt, S. W., Lobrutto, R., Link, T. A. & Von Jagow, G. (1989) *J. Biol. Chem.* **264**, 735–744.

6. Crofts, A., Robinson, H., Andrews, K., Van Doren, S. & Berry, E. (1987) in *Cytochrome systems* (Papa, S., Chance, B. & Ernster, L. eds) pp. 617–624, Plenum Press, New York.
7. Mitchell, P. (1976) *J. Theor. Biol.* 62, 327–367.
8. Robertson, D. E. & Dutton, P. L. (1988) *Biochim. Biophys. Acta* 935, 273–291.
9. Tsai, A. L., Kauten, R. & Palmer, G. (1985) *Biochim. Biophys. Acta* 806, 418–426.
10. Convent, B., Briquet, M. & Goffeau, A. (1978) *Eur. J. Biochem.* 92, 137–145.
11. Thierbach, G. & Reichenbach, H. (1981) *Biochim. Biophys. Acta* 638, 282–289.
12. Thierbach, G., Kunze, B., Reichenbach, H. & Hofle, G. (1984) *Biochim. Biophys. Acta* 765, 227–235.
13. Von Jagow, G. & Ohnishi, T. (1985) *FEBS Lett.* 185, 311–315.
14. Di Rago, J. P., Perea, J. & Colson, A. M. (1986) *FEBS Lett.* 208, 202–210.
15. Di Rago, J. P. & Colson, A. M. (1988) *J. Biol. Chem.* 263, 12564–12570.
16. Di Rago, J. P., Coppeé, J. Y. & Colson, A. M. (1989) *J. Biol. Chem.* 264, 14253–14548.
17. Di Rago, J. P., Perea, J. & Colson, A. M. (1990) *FEBS Lett.* 263, 93–98.
18. Bresseur, R. (1988) *J. Biol. Chem.* 263, 12571–12575.
19. Howell, N. & Gilbert, K. (1988) *J. Mol. Biol.* 203, 607–618.
20. Daldal, F., Tokito, M. K., Davidson, E. & Faham, M. (1989) *EMBO J.* 8, 3951–3961.
21. Degli Esposti, M., Ghelli, A., Butler, G., Roberti, M., Mustich, A. & Cantatore, P. (1990) *FEBS Lett.* 263, 95–98.
22. Von Jagow, G. & Link, T. A. (1986) *Methods. Enzymol.* 126, 253–271.
23. Degli Esposti, M., Palmer, G. & Lenaz, G. (1989) *Eur. J. Biochem.* 181, 27–36.
24. Thierbach, G. & Michaelis, G. (1982) *Mol. Gen. Genet.* 186, 501–506.
25. Guerin, B., Labbe, P. & Somlo, M. (1979) *Methods Enzymol.* 55, 149–159.
26. Meunier-Lemesle, D., Chevillotte-Brivet, P. & Pajot, P. (1980) *Eur. J. Biochem.* 111, 151–159.
27. Siedow, J. N., Power, S., De la Rosa, F. F. & Palmer, G. (1978) *J. Biol. Chem.* 253, 2392–2399.
28. Degli Esposti, M., Avitabile, E., Barilli, M., Schiavo, G., Montecucco, C. & Lenaz, G. (1986) *Comp. Biochem. Physiol.* 85B, 543–552.
29. Rieske, J. S. (1967) *Methods Enzymol.* 10, 239–245.
30. Ohnishi, T., Brandt, U. & Von Jagow, G. (1988) *Eur. J. Biochem.* 176, 385–389.
31. Von Jagow, G., Ljungdahl, P. O., Graf, P., Ohnishi, T. & Trumpower, B. L. (1984) *J. Biol. Chem.* 259, 6318–6326.
32. Robertson, D. E., Daldal, F. & Dutton, P. L. (1990) *Biochemistry* 29, 11249–11260.
33. Speck, S. H. & Margolias, E. (1984) *J. Biol. Chem.* 259, 1064–1072.
34. Degli Esposti, M. & Lenaz, G. (1991) *Arch. Biochem. Biophys.* in the press.
35. Kauten, R., Tsai, A. L. & Palmer, G. (1987) *J. Biol. Chem.* 262, 8658–8667.
36. Bone, R., Silen, J. L. & Agard, D. A. (1989) *Nature* 339, 191–195.
37. Samworth, C. M., Degli Esposti, M. & Lenaz, G. (1988) *Eur. J. Biochem.* 171, 81–86.
38. Robertson, D. E., Davidson, E., Prince, R. C., Van der Berg, W. H., Marrs, B. L. & Dutton, P. L. (1986) *J. Biol. Chem.* 261, 584–591.
39. Brandt, U., Shägger, H. & Von Jagow, G. (1988) *Eur. J. Biochem.* 173, 499–506.
40. Bennoun, P., Delosme, M. & Kuck, U. (1991) *Genetics* 127, 335–363.
41. Hauska, G., Nitschke, W. & Herrmann, R. G. (1988) *J. Bioenerg. Biomembr.* 20, 211–227.
42. Degli Esposti, M. & Ghelli, A. (1990) in *Sixth EBEC Short Reports*, p. 16, Elsevier, Amsterdam.
43. Rees, D. C., Komya, H., Yeates, T. O., Allen, J. P. & Feher, G. (1989) *Annu. Rev. Biochem.* 58, 607–633.
44. Crofts, A., Wang, Z., Chen, Y., Mahalingham, S., Yun, C. H. & Gennis, R. B. (1990) in *Highlights in ubiquinone research* (Lenaz, G. et al., eds) pp. 98–103, Taylor & Francis, London.
45. Chevillotte-Brivet, P. & Di Rago, J. P. (1989) *FEBS Lett* 255, 5–9.
46. Link, T. A., Shägger, H. & Von Jagow, G. (1986) *FEBS Lett.* 204, 9–15.
47. Degli Esposti, M., Ghelli, A., Luchetti, R., Crimi, M. & Lenaz, G. (1989) *Ital. J. Biochem.* 38, 1–22.
48. Degli Esposti, M. (1989) *Biochim. Biophys. Acta* 977, 249–265.
49. Rafferty, S. P., Pearce, L. L., Barker, P. D., Guillemette, J. G., Kay, C. M., Smith, M. & Mauk, A. G. (1990) *Biochemistry* 29, 9365–9369.
50. Tron, T. & Lemesle-Mevnier, D. (1990) *Curr. Genet.* 18, 413–419.
51. Yum, C.-H., Beci, R., Crofts, A. R., Kaplam, S. & Gennis, R. B. (1990) *Eur. J. Biochem.* 194, 399–411.