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The cytochrome *b* of the sea urchin *Paracentrotus lividus* is naturally resistant to myxothiazol and mucidin

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The ubiquinol:cytochrome *c* reductase activity of *Paracentrotus lividus* mitochondria is relatively insensitive to the specific inhibitors myxothiazol and mucidin. The I_{50} of myxothiazol and mucidin are three and two orders of magnitude higher, respectively, in *P. lividus* than in bovine heart mitochondria. The natural resistance of the *P. lividus* reductase to these inhibitors can be correlated with a single amino replacement, an alanine for a glycine at position 143, in the sequence of cytochrome *b*. This position is located in a conserved region of the molecule, believed to be important in the oxidation of ubiquinol by the reductase.

Cytochrome *b*; Cytochrome bc_1 complex; Myxothiazol; Inhibitor resistance; (Mitochondria)

1. INTRODUCTION

Dihaem cytochrome *b* of the ubiquinol:cytochrome *c* reductase (cytochrome bc_1 complex) is now among the best characterized membrane proteins [1–3]. The knowledge of this cytochrome has expanded greatly due to the availability of two classes of inhibitors that specifically interact with one or other of the two redox centres of the protein, centre 'i' and centre 'o' according to the Q-cycle scheme [3–5].

Structure–function correlations have been made possible by the determination of the sequence of several mutants of cytochrome *b* that are resistant to either centre 'i' or centre 'o' inhibitors [3,6–12]. These mutants generally possess a single amino acid replacement that strongly decreases the inhibitors' potency either in vivo or in vitro [6–8,10–12]. It is widely recognized that the positions conferring inhibitor resistance in cytochrome *b* are either close to one of its redox centres or constitute parts of its ubiquinol/ubiquinone binding sites [3,8,10,12]. In particular, most of the mutations conferring resistance to centre 'o' inhibitors map within a highly conserved region of cytochrome *b* that is located between the third and the fourth transmembrane helices in the current models [2,3,7,8,10,12].

Amino acid replacements that are similar to those found in resistant mutants can be deduced also from the comparison of the various sequences of cytochrome *b* which are now available [7–12]. This suggests that natural resistance towards inhibitors that bind to cytochrome *b* may be present in some species. Here we report that the ubiquinol:cytochrome *c* reductase in mitochondria of the sea urchin *Paracentrotus lividus* is naturally resistant to the centre 'o' inhibitors myxothiazol and mucidin.

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2. MATERIALS AND METHODS

Bovine (*Bos taurus*) heart mitochondria and *P. lividus* egg mitochondria were prepared according to [13] and [14], respectively. The cytochrome *b* content of the mitochondria was measured by the reduced minus oxidized difference spectrum at 560–575 nm using an extinction coefficient of 25 $mM^{-1}\ cm^{-1}$. Ubiquinols were prepared as in [15]. Ubiquinone-2 was a generous gift of Eisai Co., Tokyo, Japan and 2,3-dimethoxy-5-methyl-6-nonyl benzoquinone was donated by Professor G. Von Jagow, University of Frankfurt, FRG. Myxothiazol was purchased from Boehringer and mucidin was kindly provided by Dr J. Subik, University of Bratislava, Czechoslovakia. The concentration of the inhibitors in ethanol was measured as in [5]. Ubiquinol:cytochrome *c* reductase was assayed as described in [16].

3. RESULTS

The highly conserved region spanning residues 128–150 (using yeast as the reference [2,3,8]) in the sequence of cytochrome *b* is believed to form an important portion of the binding pocket of centre 'o' inhibitors [3,8,10,12]. This region contains 8 of the approximately 30 invariant residues present in the mitochondrial and bacterial sequences examined (Fig. 1 and [17]). As shown in Fig. 1, the sequence of cytochrome *b* from *P. lividus* mitochondria [18] has the single amino acid replacement of a highly conserved glycine by an alanine at position 143. The same

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SPECIES	AMINO ACID SEQUENCE
	130 140 150
Saccharomyces cerevisiae	AFLGYCCVYGQMSHWGATVITNL
Bos taurus	AFMGYVLPWGQMSFWGATVITNL
Rhodobacter capsulatus	AFMGYVLPWGQMSFWGATVITGL
Paracentrotus lividus	AFWGYVLVWGQMSFWAATVITNL
CONSENSUS	AFMGYVLPWGQMSFWGATVITNL
(mitochondria and bacteria)	

Fig. 1. Alignment of some phylogenetically-representative sequences of cytochrome *b* of ubiquinol:cytochrome *c* reductase. The 4 upper sequences are aligned to that of *S. cerevisiae* [2,3] and are representative of yeasts, mammals, bacteria and sea urchins. In the consensus of bacterial and mitochondrial sequences (taken from those listed in [2,3,8,10,12,17–19]) shown below, the apparently invariant residues are underlined, the residues that are conserved in all but one or two species are indicated by dots and the positions at which resistance to myxothiazol and mucidin occurs [8,10,12] are marked by asterisks. This protein region is believed to contain the C-terminal part of the third transmembrane helix (up to ca. position 135) and an amphipathic loop that was previously predicted to span the membrane [2,3,7–10]. The sequence of *P. lividus*, the numeration of which is identical to that of yeast in the region shown [17,18], differs slightly from that previously published [18]. Further cloning and DNA sequencing revealed that positions 138 and 148 are Q and T rather than R and A, respectively. The assignment of A at position 143 (indicated by the arrow) was confirmed by many independent sequencing data.

substitution is observed only in another sea urchin cytochrome *b* [19] and is remarkable for it is identical, even in the base exchange (G-C at the second position of the codon [18]), to that found in mouse and bacterial mutants showing resistance to the centre 'o' inhibitors myxothiazol and mucidin [10,12].

This observation strongly suggests that *P. lividus* mitochondria may have an ubiquinol:cytochrome *c* reductase activity less sensitive to myxothiazol and mucidin than, for instance, mammalian mitochondria. Indeed, this is the case, as shown in Fig. 2. The inhibitor titre of myxothiazol is three orders of magnitude higher in *P. lividus* than in bovine heart mitochondria, the I_{50} being 4.0×10^{-6} M instead of 5.6×10^{-9} M (Fig. 2A). A large increase in the inhibitor titre is also seen for mucidin, since its I_{50} is 2.8×10^{-6} M in *P. lividus* mitochondria whereas it is 3.2×10^{-8} M in bovine heart mitochondria (Fig. 2B). The latter value is almost identical to the K_i of 30 nM measured under similar conditions with the reductase complex purified from bovine heart [20].

4. DISCUSSION

Herein we report the first example of a mitochondrial ubiquinol:cytochrome *c* reductase that is naturally resistant to myxothiazol and mucidin, two powerful centre 'o' inhibitors [5]. The increased titres of the inhibitors of *P. lividus* as compared with bovine heart are similar to those measured in resistant mutants of mouse with respect to their wild-type [10]. Interestingly, myxothiazol is somewhat less efficient than mucidin in *P. lividus* mitochondria (Fig. 2). By contrast, in all other species myxothiazol binds one order of magnitude more tightly than mucidin [5,8,12,20].

It is highly probable that the decrease in potency of myxothiazol and mucidin in the *P. lividus* reductase

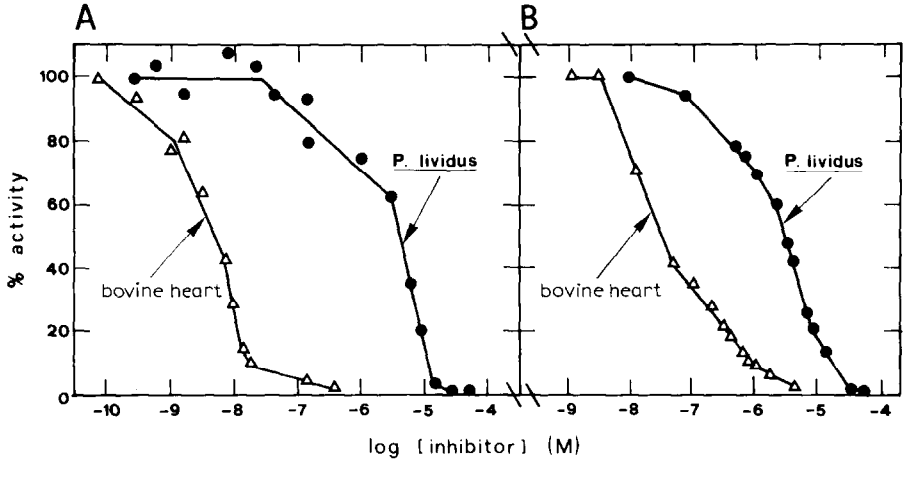

Fig. 2. Comparative inhibition of the ubiquinol:cytochrome *c* reductase by myxothiazol and mucidin in bovine heart and *P. lividus* mitochondria. (A) Myxothiazol titration of ubiquinol-2 (10 μM):cytochrome *c* (20 μM) reductase, assayed with mitochondria containing 4 and 7 nM cytochrome *b* of bovine heart and *P. lividus*, respectively. The uninhibited turnover of the reductase was 384 s^{-1} in bovine and 118 s^{-1} in *P. lividus*. (B) Mucidin titration of 2,3-dimethoxy-5-methyl-6-nonyl benzoquinol (5 μM):cytochrome *c* reductase assayed as in A, but with different preparations of both mitochondria. The uninhibited rate was 230 s^{-1} in bovine heart and 75 s^{-1} in *P. lividus*. Note that the reductase activity in *P. lividus* is as sensitive to antimycin as that in bovine mitochondria under the above conditions.

(Fig. 2) is primarily derived from amino acid substitutions in the sequence of its cytochrome *b* (see also [1,3,5–12,20] for supporting evidence). The only significant residue change, with respect to species that are normally very sensitive to these inhibitors (e.g. yeast), is the replacement of the otherwise highly conserved glycine by an alanine at position 143 (Fig. 1, cf. [1,3,8,12,18]). This can be considered as the major molecular reason for the resistance to these inhibitors in *P. lividus* cytochrome *b*. The introduction of a methyl group in the glycine to alanine substitution presumably causes steric hindrance, preventing full recognition of myxothiazol and mucidin.

Myxothiazol differs from mucidin in the structure and volume of its hydrophobic tail, since both inhibitors possess a similar methoxyacrylate group [5]. The loss of steric recognition of such compounds that function as ubiquinol antagonists [5,20], therefore, suggests that position 143 of cytochrome *b* could be involved in the specific binding of the polyisoprenoid tail of ubiquinol at centre 'o'. Preliminary experiments of steady-state activity (M. Degli Esposti, unpublished results) support this idea, since in *P. lividus* mitochondria the relative affinity for ubiquinol homologues and analogues appears to be different from that in other mitochondria. Further studies are under way to clarify this point.

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