Cytochrome b of Fish Mitochondria Is Strongly Resistant to Funiculosin, a Powerful Inhibitor of Respiration

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We report here some unusual properties of ubiquinol: cytochrome c reductase of eel and other fish mitochondria. The turnover rate of the reductase is clearly higher than in mammalian mitochondria and the binding constant for ubiquinone seems to be larger than in other vertebrates. Additionally, the reductase activity of fish mitochondria is resistant to some powerful inhibitors that bind to cytochrome b, in particular to funiculosin. After sequencing most of the gene of eel cytochrome b and comparing the deduced amino acid sequence with that of other fish and animals, we hypothesize that the decreased binding of funiculosin could be due to a few amino acid replacements in the third and fourth transmembrane helix of the protein. In particular, the presence of methionine instead of alanine at position 125 seems to be largely responsible for the strong resistance to funiculosin and also to the partial resistance to myxothiazol in all fish mitochondria. Correlations between some residue substitutions in cytochrome b and the different effects of funiculosin in different species are also considered.
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Mitochondrial cytochrome b, the central subunit of ubiquinol:cytochrome c reductase (the bc_1 complex), is one of the best known membrane proteins to date (1–3). A major contribution to this knowledge derives from studies on mutants resistant toward some of the several inhibitors of the reductase (1–6). According to the Q-cycle scheme for the bc_1 complex (7), there are two quinone-reacting centers in cytochrome b, center i at the negative side of the membrane and center o at the positive side of the membrane (1–8). Inhibitors, which often have a quinone-like structure, are recognized to bind to one or another of these centers essentially from their effects on the redox reactions and spectroscopic properties of the b hemes (3, 7–10).

A detailed picture of structure–function relationships at center o of cytochrome b is now emerging from the characterization of bacterial (11) and yeast (12) mutants resistant to inhibitors such as myxothiazol, as well as from the analysis of revertants of deficient mutants of yeast (13, 14). Conversely, the understanding of the essential features of cytochrome b at center i is limited, for a number of reasons. For instance, mutants resistant to center i inhibitors cannot be selected in bacteria (11). Moreover, there is little biochemical characterization of the resistant mutants selected in yeasts, excluding the early work in (15).

To obtain more information on the structure–function relationships of cytochrome b we have previously compared the properties of ubiquinol:cytochrome c reductase in different animals (16–18). Here we extend this theme to include the many gene sequences of cytochrome b from fish species which are now available from studies of molecular systematics (19–22). We found some clear differences with respect to the properties commonly seen in mammalian mitochondria, the most striking of which regards the strong resistance of all fish mitochondria to funiculosin, a powerful center i inhibitor. This natural resistance may be correlated to a few amino acid substitutions in the sequence of fish cytochrome b.

MATERIALS AND METHODS

Mitochondria and bc_1 complex were isolated from beef heart as described in (18). Mitochondria and submitochondrial particles from beef,

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pig, chicken, fish, and amphibian livers were prepared essentially according to the method described in (23, 24). Adult eels (*Anguilla anguilla*), reared in the Comacchio lagoon, were provided by the Istituto di Malattie Infettive, Faculty of Veterinary Medicine in Bologna (Dr. G. Poglayen). African toad (*Xenopus laevis*), common toad (*Bufo bufo*) and *Tilapia mossambica* hybrid *nilotica/mariae*—a cichlid fish taken from aquacultures in Piacenza—were kindly provided by M. Bigazzi from our Department of Biology. White sturgeon (*Acipenser transmontanus*) reared in aquaculture was kindly given by Agroittica Lombarda, Viadana. Whiting (*Merluccius merluccius*) was purchased from fishermen at Cervia on the Adriatic sea. The american eel (*Anguilla rostrata*) used for DNA sequencing was collected at Stony Brook, New York, and the *Cichlasoma* fishes in Nicaragua, by A. Meyer. Mitochondrial particles from various yeast strains were provided by the laboratory of Dr. D. Lemesle, LCB-CNRS, Marseille.

Ubiquinol-2:cytochrome c reductase was assayed as reported previously (12, 17, 18, 25). The mitochondrial preparations were diluted to less than 1 μM cytochrome b [determined at 561(2)–575 nm after dithionite reduction using an extinction coefficient of 25 mM^{-1} cm^{-1} (18)] in 250 mM sucrose, 30 mM Tris-Cl, EDTA 1 mM, pH 7.2. In the two-substrate titrations of the reductase activity, the submitochondrial particles were treated with 0.5% Tween-20 as in (25) to disrupt the lipid membrane. The concentration of the bc_1 complex was routinely determined from the total antimycin binding sites extrapolated in the inhibitor titrations of the activity (9). Inhibitors were determined in ethanol (8, 9). Funiculosin, a generous gift by Dr. P. Bollinger, Sandoz, Basel, was dissolved in ethanol containing 2–3 mM KOH for complete solubilization and incubated with the preparations for at least 2-min (9).

Sequencing of the gene for mitochondrial cytochrome b of eel and cichlid fish was performed by A. Meyer after amplification with the polymerase chain reaction as described previously (19–22). Sequencing of the cytochrome b gene of *T. mossambica* was carried out as in (22) in collaboration with P. Cantatore, Department of Molecular Biology, University of Bari. The eel sequence is deposited in GenBank, accession number M85080.

RESULTS

Unusual Properties of the Ubiquinol Cytochrome c Reductase in Eel Mitochondria

During the characterization of the respiratory chain of eel liver mitochondria (24) we realized that their ubiquinol:cytochrome c reductase displays unusual features. Of the various redox enzymes of the respiratory chain, cytochrome c reductase alone exhibits turnover rates much higher than those seen in any mammalian, avian, or amphibian mitochondria (results not shown). In order to understand whether this higher activity of the reductase derives from significant variations of its kinetic parameters in fish mitochondria, we analyzed the two-substrate kinetics of the enzyme with ubiquinol-2. This assay was carried out *in situ* in submitochondrial particles since the fish reductase is inactivated by detergent extraction (18). These particles are about threefold enriched in the content of the inner membrane enzymes with respect to mitochondria (24) and should retain, as in the case of beef submitochondrial particles, the same kinetic properties as the isolated bc_1 complex (26). Indeed, the graphical pattern of the titrations is identical in mitochondrial particles and the isolated enzyme [results not shown, see also (18, 25, 26)]. As shown in Table I, the ubiquinol-2:cytochrome c reductase of eel submitochondrial particles has a maximal turnover that is approximately threefold higher

TABLE I

Kinetic Parameters of the Ubiquinol-2:Cytochrome c Reductase in Eel and Pig Liver Mitochondrial Particles[a]

Parameter and unit	Eel liver	Pig liver
TN_{max} (s^{-1})	1220	357
K_m ubiquinol-2 (μM)	11.0	2.9
K_{min} ubiquinol-2 (M^{-1}s^{-1})	1.1×10^8	1.2×10^8
K_i ubiquinone-2 (μM)	15.1	5.8
K_m cytochrome c^{3+} (μM)	11.6	3.0
K_i cytochrome c^{2+} (μM)	6.0	7.9

[a] Calculated as described by Degli Esposti and Lenaz (25).

than that of pig [or beef, cf. (26)] submitochondrial particles, even if the catalytic efficiency for the quinol substrate [$k_{min} = TN_{max}/K_m$ ubiquinol (25)] is very similar in all species. This is due to the much larger value for the k_m for ubiquinol in eel, a difference that is paralleled by the larger value for the inhibition constant, which is closely related to the binding constant (25), for the oxidized ubiquinone. Similar enzymatic behavior is seen in other fish mitochondria, e.g., whiting (data not shown). Consequently, it is inferred that the kinetic interaction of ubiquinol and ubiquinone with the fish reductase is altered with respect to other vertebrates, in particular mammals.

Fish Mitochondria Are Resistant to Funiculosin

One peculiar characteristic of the cytochrome c reductase of fish mitochondria is its strong resistance to funiculosin, a powerful center i inhibitor. For instance, the titer of funiculosin is 50-fold higher in eel than in mammalian mitochondria (Fig. 1), where the I_{50} is in the nanomolar region. Conversely, the inhibitory potency of antimycin, the classical center i inhibitor (7–9), is identical in fish and mammalian mitochondria, consistent with the high toxicity of this antibiotic for all fish (9). Other, less powerful center i inhibitors like hydroxyquinolines and diuron also show similar titers in fish and mammalian mitochondria (results not shown).

It is likely that the lower sensitivity to funiculosin derives from a reduced binding in the fish bc_1 complex, as results similar to those in Fig. 1 were obtained in crude preparations of the enzyme and with different concentrations of the substrates. Funiculosin is the only inhibitor of the reductase that dramatically increases the midpoint redox potential of the b_H heme (10, 27, 28); it also shifts both the EPR signal and the optical spectrum of this heme (10, 27, 28). All such effects imply that the binding site of the inhibitor is in close proximity to cytochrome b_H and therefore, as this metal group is deeply embedded in the membrane sector of the reductase (29), funiculosin binds within the transmembrane helices of cytochrome b. It then follows that a decreased potency of funiculosin,
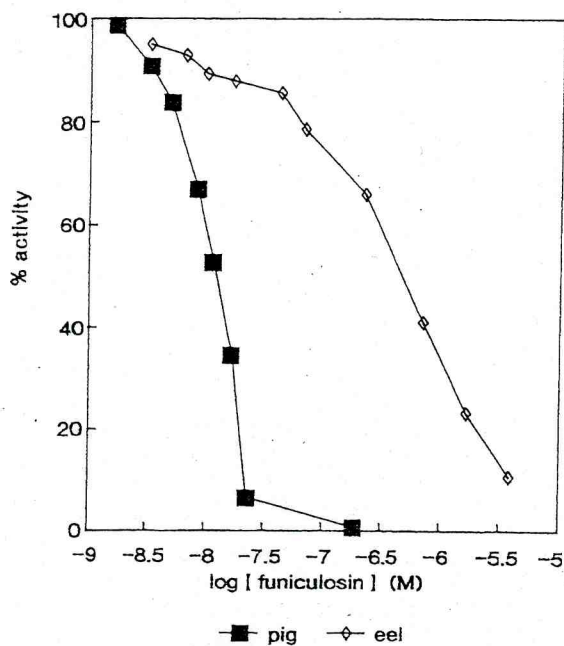
FIG. 1. Titration of the ubiquinol-2 (14 μM):cytochrome c (15 μM) reductase in pig and eel liver mitochondria. The final concentration of bc_1 complex is 2.4 nM in pig and 2.9 nM in eel.

as in eel mitochondria, reflects a decreased affinity of the eel cytochrome b for funiculosin and, consequently, it is expected that the above effects of the inhibitor would be diminished in this species. Indeed, Fig. 2 clearly shows that neither the level of reduction nor the spectral features of the b_H heme are affected by funiculosin in eel mitochondria conversely to mammalian mitochondria [see also (27, 28)]. However, similar results were obtained in other fish mitochondria.

Comparison of the Sequences of Cytochrome b from Fish

By analogy with other systems (2–6, 17, 30), it is highly probably that variations in the primary structure of cytochrome b are responsible for the natural resistance toward funiculosin in fish mitochondria. Therefore, we sequenced the gene of eel cytochrome b taking advantage of the procedures developed by studies of molecular systematics (19, 21, 22). Figure 3 presents the alignment of the deduced amino acid sequence of eel, other fish, two mammals, and one amphibian against that of yeast [cf. (2, 12)]. There is little variation between the eel sequence and that of other fish (20, 21, 31), but none of the positions known to confer resistance to inhibitors of the bc_1 complex are altered with respect to other animal species (Fig. 3).

The sequences of fish cytochrome b show a few unique amino acid substitutions between the N-terminus and the sixth transmembrane helix, where all the mutations giving resistance to center **i** inhibitors map (2–5, 30). We considered several points in order to deduce which of such

substitutions may be responsible for the decreased binding of funiculosin to fish cytochrome b. First, funiculosin is an atypical center **i** inhibitor, because it also alters properties of the metal groups of center **o**, such as the optical spectrum and of the b_L heme [Fig. 2C, see also (10, 28, 32)]. Second, the mutated residues giving resistance to center **i** inhibitors generally have a larger volume than the native residues. For instance, Gly37 → Val induces resistance to both antimycin and funiculosin (4, 5, 15).

Guided by the above considerations and by a detailed analysis of the sequences of cytochrome b (12, 16, 33), we concluded that the replacements Ala125 → Met and Ala193 → Thr (corresponding to positions 126 and 194 of the yeast sequence, Fig. 3) might be responsible for the natural resistance of fish mitochondria to funiculosin. To test our conclusion, we have investigated the inhibitory titer of funiculosin in several fish groups and compared it to that in amphibians [the cytochrome b of which is most closely related to that of fish (22) but has Ala at position 126 and Ser at position 194, Fig. 3] and to that in chicken [the cytochrome b of which has Thr at position
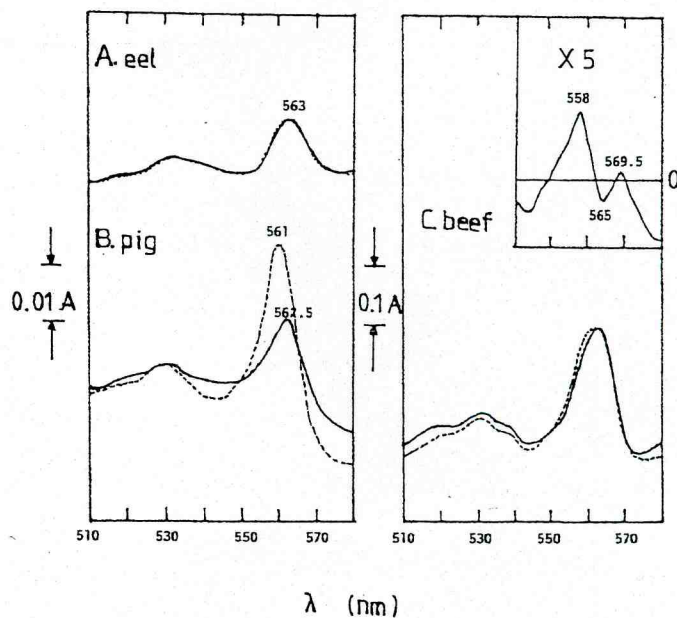
FIG. 2. Effect of funiculosin on the optical spectra of cytochrome b. (A) Eel liver submitochondrial particles. The spectra are the difference of the ubiquinol-reduced minus the ascorbate-reduced particles, where only the b_H heme is partially reduced (25). Solid spectrum, without funiculosin; dashed spectrum, plus 100 μM funiculosin. (B) Pig liver submitochondrial particles under conditions as in A. Note the spectral blue shift of the maximum after the addition of funiculosin. (C) Beef heart mitochondrial particles. The spectra are the difference between the dithionite-reduced and the ascorbate-reduced sample, i.e., both b hemes are fully reduced. The insert shows the five-fold-enlarged difference in absorption of the α band, which is induced by the addition of funiculosin to the fully reduced hemes. The peak at 558 nm and the trough at 565 nm are indicative of the large blue shift of the b_H heme [cf. (28)], whereas the small peak at 569.5 nm is indicative of the red shift of the b_L heme, whose spectrum is also broadened at ca. 558 nm [cf. (28)].

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              1          10        20        30        40        50
Beef       MT-NIRKSHPLMKIVNNAFIDLPAPSNISSWWNFGSLLGICLILQILTGLFLAMHY
Pig        MT-NIRKSHPLMKIINNAFIDLPAPSNISSWWNFGSLLGICLILQILTGLFLAMHY
Toad       MAPNIRKSHPLIKIINNSFIDLPTPSNISSLWNFGSLLGVCLIAQIITGLFLAMHY
Cod        MA-SLRKTHPILKIANSALVDLPAPSNISVWWNFGSLLGLCLITQLLTGLFLAMHY
Eel        MA-NLRKTHPLLKIANDALVDLPTPSNISAWWNFGSLLGLCISQILTGLFLAMHY
Cichlasoma MA-NLRKTHPLLKIANDALVDLPAPSNISVWWNFGSLLGLCLAAQILTGLFLAMHY
Sturgeon   MA-NIRKTHPLLKIINGAFIDLPTPSNISVWWNFGSLLGLCLITQILTGLFLAMHY
Yeast      MA--FRKSNVYLSLVNSYIIDSPQPSSINYWWNMGSLLGLCLVIQIVTGIFMAMHY
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              60        70        80        90        100       110
Beef       TSDTTTAFSSVTHICRDVNYGWIIRYMHANGASMFFICLYMHVGRGLYYGSYTFLET--
Pig        TSDTTTAFSSVTHICRDVNYGWVIRYLHANGASMFFICLFIHVGRGLYYGSYMFLET--
Toad       TADTSMAFSSVAHICRDVNYGLLIRNLHANGLSFFFICIYLHIGRGLYYGSFLYKET--
Cod        TSDIETAFSSVVHICRDVNYGWLIRNMHANGASFFFICLYMHIARGLYYGSYLFVET--
Eel        TSDISTAFSSVAHICRDVNYGWLIRNLHANGASFFFICLYLHIARGLYYGSYLYKET--
Cichlasoma TSDIATAFSSVAHICRDVNYGWLIRNMHANGASFFFICIYLHIGRGLYYGSYLYKET--
Sturgeon   TADISTAFSSVAHICRDVNYGWLIRNIHANGASFFFICLYLHVARGMYYGSYLQKET--
Yeast      SSNIELAFSSVEHIMRDVHNGYILRYLHANGASFFFMVMFMHMAKGLYYGSYRSPRVTL
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                                                    B
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              120       130       140       150       160       170
Beef       WNIGVILLLTVMATAFMGYVLPWGQMSFWGATVITNLLSAIPYIGTNLVEWIWGGFSVD
Pig        WNIGVLLLFTVMATAFMGYVLPWGQMSFWGATVITNLLSAIPYIGTDLVEWIWGGFSVK
Toad       WNIGVILLFLVMATAFVGYVLPWGQMSFWGATVITNLLSAIPYIGNVLVQWSLGGFSVD
Cod        WNIGVVLFLLVMMTSFVGYVLPWGQMSFWGATVITNLMSTVPYVGDALVQWIWGGFSVD
Eel        WNIGVVLILLVMMTAFVGYVLPWGQMSFWGATVITNLLSAVPYVGNSLVQWIWGGYSVD
Cichlasoma WNVGVVLLLLTMMTAFVGYVLPWGQMSFWGATVITNLLSAIPYIGNSLVQWLWGGFSVV
Sturgeon   WNIGVILLLLTMMTAFVGYVLPWGQMSFWGATVITNLLSAFPDIGDTLVQWIWGGFSVD
Yeast      WNVGVIIFILTIATAFLGYCCVYGQMSHWGATVITNLFSAIPFVGNDIVSWLWGGFSVS
                           #   #   #    #     #    ##
                       C
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              180       190       200       210       220
Beef       KATLTRFFAFHFILPFIIMAIAMVHLLFLHETGSNNPTGISSDVDKIPFHPYYTIK
Pig        KATLTRFFAFHFILPFIITALAAVHLMFLHETGSNNPTGISSDMDKIPFHPYYTIK
Toad       NATLTRFFAFHFLLPFILAGASILHLLFLHETGSTNPTGLNSDPDKVPFHPYFSYK
Cod        NATLTRFFAFHFLFPFVVAAFTMLHLLFLHETGSNNPTGINSNADKIPFHPYFTYK
Eel        NATLTRFFAFHFLFPFVVAALTMLHLLFLHETGSNNPVGLNSDADKIPFHPYFSYK
Cichlasoma NATLTRLFAFHFLLPFIIAAMTMIHLIFLHETGSTNPAGLNSDTDKILFHPYFLYK
Sturgeon   NATLTRFFAFHFLLPFVIAGASMIHLLFLHQTGSNNPTGLNSDADKVTFHPYFSYK
Yeast      NPTIQRFFALHYLVPFIIAAMVIMHLMALHIHGSSNPLGITGNLDRIPMHSYFIFK
                                           *
                       D                                 ** *
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              230       240       250       260       270
Beef       DILGALLLILALMLLVLFAPDLLGDPDNYTPANPLNTPPHIKPEWYF...
Pig        DILGALFMMLILLILVLFSPDLLGDPDNYTPANPLNTPPHIKPEWYF...
Toad       DLLGFLIMLTALTLLAMFSPNLLGDPDNFTPANPLITPPHIKPEWYF...
Cod        DLLGFAVMLLGLTALALFAPNLLGDPDNFTPANPIVTPPHVKPEWYF...
Eel        DLLGFIIML---------------------------------------
Cichlasoma DLLGFAILL---------------------------------------
Sturgeon   DLFGFTLMLVGLTSVALFSPNLLGDPDNFTPANPLVTPPHIKPEWYF...
Yeast      DLVTVFLFMLILALFVFYSPNTLGHPDNYIPGNPLVTPASIVPEWYL...
              ***                          #              #
                       E
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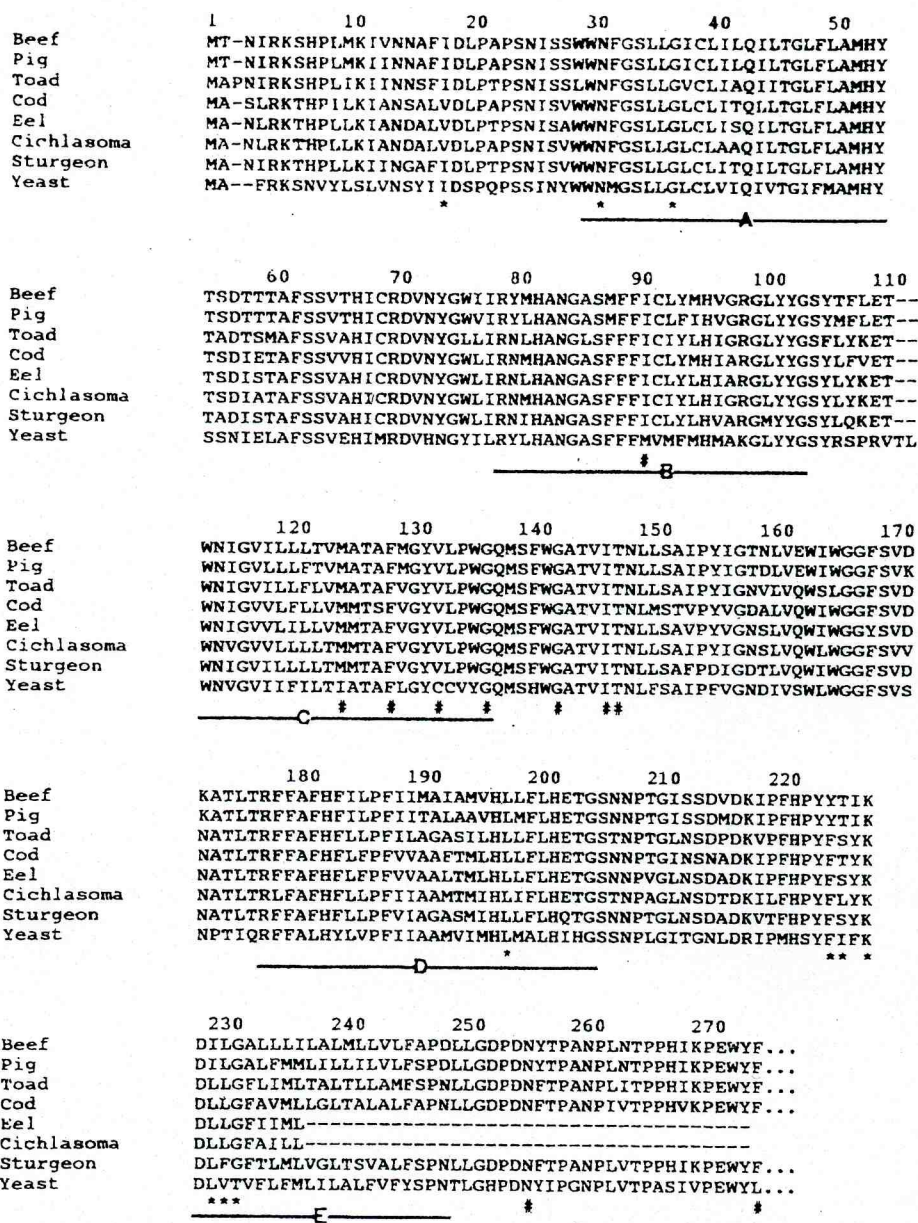
FIG. 3. Alignment of the amino acid sequences deduced from the mtDNA of cytochrome b from some mammals, amphibians, and fishes to that of yeast cytochrome b. The eel sequence and the C-terminal part of the consensus sequence of two closely related cichlid fishes, *Cichlasoma citrinellum* and *spilorum*, have been obtained by A. Meyer in this work, whereas the cod sequence has been kindly given by Dr. S. Johansen, Dept. of Cell Biology, University of Tromsö. The first four residues of the *Cichlasoma* sequence are considered to be identical to those in the closely related *Hemichromis* (21). Other sequences are taken from (1, 21, 31), but the sequence of *Xenopus laevis* has been corrected for some very likely errors, e.g., R70 and not F. * Positions conferring resistance to center i inhibitors (3–5, 30, 39); # positions inducing resistance to center o inhibitors [see (12) for an overview]. The numeration follows that of yeast cytochrome b for consistency with previous alignments (2, 4, 12, 16, 30). Note that this numeration differs slightly from that of animal cytochrome b, so that positions 126 and 194 in yeast correspond to residues 125 and 193, respectively, in the mammalian and fish sequences. The limits of the transmembrane helices [underlined, named as in (6, 12)] have been evaluated by a detailed sequence and hydropathy analysis (12, 33) of the over 240 sequences available to us. The sequence of *Tilapia* cytochrome b is identical to that of *Cichlesoma* in the 60–110 region except for the substitution of Val65 with Ile (unpublished results).

194 (34) like in ray-finned fish]. Moreover, in all these species we have measured the titer of myxothiazol, a powerful center o inhibitor whose binding is affected by mutations at positions close to 126 (11, 12, 14, 35).

As summarized in Table II, funiculosin is over one order of magnitude less powerful in fish than in either amphibian or mammalian mitochondria. However, sturgeon mitochondria are significantly less resistant to funiculosin

TABLE II

Inhibition Titer of Funiculosin and Myxothiazol on the
Ubiquinol-2:Cytochrome c Reductase in Liver
Mitochondria from Various Animal Species

Species	Cytochrome b residue		I_{50} (mol/mol of bc₁ complex)	
	at 125	at 193[a]	Funiculosin	Myxothiazol
Beef	Ala	Ala	3	1.3
Pig	Ala	Ala	4	1.1
Chicken	Ala	Thr	30	1.0
Toad[b]	Ala	Ser	6[c]	1.1
Sturgeon	Met	Ser	65[c]	3.8
Eel	Met	Thr	170	2.0
Whiting	Met[d]	Thr[d]	111	2.4
Tilapia	Met[e]	Thr[e]	226	3.0

Note. Conditions as described in the legend to Fig. 1.

[a] Corresponding to positions 126 and 194 in yeast (Fig. 3).

[b] Mean values in African and common toad.

[c] The time dependence of inhibition is longer than in other species.

[d] Considered to be the same as in the closely related cod (Fig. 3).

[e] Considered to be the same as in all the other species of the Cichlid family (Fig. 3), as also demonstrated by the very strong homology of part of the *Tilapia* sequence with all the other cichlid sequences.

than any ray-finned fish mitochondria (Table II and results not shown). Conversely, all fish including sturgeon are also partially resistant to myxothiazol with respect to either amphibians or mammals (Table II). Chicken, however, shows a resistance to funiculosin but no resistance to myxothiazol (Table II).

Volume Patterns of Cytochrome b in Fish and Other Vertebrates

The hydrophobicity (or hydropathy) and volume of the residues in the interior of proteins are the principal factors that influence the packing and consequently the catalytic properties of enzymes (36). These principles should be even more important for a transmembrane catalytic protein such as cytochrome b, as it reacts with a very hydrophobic and quite voluminous substrate, ubiquinone. Quinone-antagonist inhibitors like funiculosin are likely to bind within the ubiquinone binding pockets of cytochrome b like quinone-antagonist herbicides bind to the Q_B site of the photosynthetic reaction center (6, 25, 37). For these reasons we have thought it to be of interest to compare the profile of relative hydrophobicity and volume of the cytochrome b sequence in species where funiculosin has a largely different binding as judged by its different titers. By utilizing the programs developed previously for the hydropathy of integral proteins (38), we have therefore combined the profile of the relative volume with that of the relative hydropathy of the residues for cytochrome b of representative fish, amphibian, and mammalian species (Fig. 4). Only the region of the sequence spanning helices
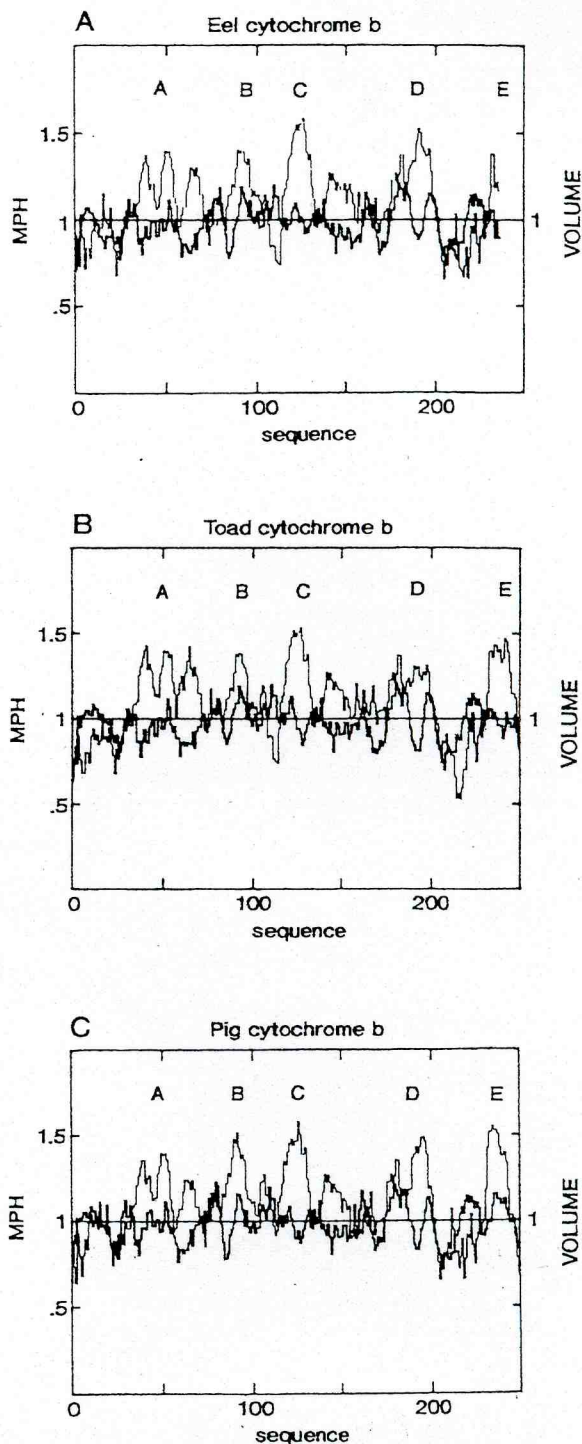

FIG. 4. Profile of the relative volume and hydropathy of some cytochrome b sequences. The relative hydropathy of the residues is according to the MPH scale (38), whereas the relative volume of the residues (bolder profile) is calculated by using the known value for Thr of 1, since the average volume of membrane cytochromes is close to that of this residue (33, 44). The profiles are averaged with a window of seven residues for both parameters. (A) Eel cytochrome b (cf. Fig. 3). (B) Toad cytochrome b (cf. Fig. 3). (C) Pig cytochrome b (20). The hydrophobic peaks corresponding to the known transmembrane helices of the protein are identified by capital letters according to (6).

A to E has been considered as this region contains all the positions that affect funiculosin binding (5, 15, 30).

Our attention was focused on local minima of volume that are conserved in size and depth in most species and can be located within the transmembrane helices of cytochrome b (cf. Fig. 3) near either the middle or the negative side of the membrane. The most conserved of such minima is seen in helix A around residues 35–37 (yeast numbering), a region that forms part of the ubiquinone binding niche at center i as it contains mutations conferring resistance to inhibitors of this center (3–5, 39). A prominent conserved minimum is centered around residue 205 (Fig. 4); position 198 of the yeast funiculosin-resistant mutant (30) is at the border of this region. The other two local minima can be positioned near the middle of the membrane in helices C and D and are present in most species. The minimum in helix C is centered around residue 126 and is clearly reduced in fish (Fig. 4A) and plant (data not shown) owing to the substitution of the small Ala with bulkier residues at this position. Position 125 and 129, which are critical for center o inhibitors like myxothiazol (11, 12, 40), also lie in the same region. The minimum within helix D is quite conserved in mammalian and amphibian cytochrome b but it is diminished in fish cytochrome b (Fig. 4), with the only exception being sturgeon (data not shown). In the latter species, the depth of this local minimum is identical to that in toad (Fig. 4B) owing to the presence of the same AGAS motif at positions 191 to 194 in the sequence (cf. Fig. 3).

There seems to be a consistent correlation between the dimensions in the volume minima in helices C and D and the relative sensitivity to funiculosin in the various species investigated (Table II cf. Fig. 4). When either of these minima of local protein volume is reduced, the titer of funiculosin is clearly higher than when these minima have their average depth, e.g., eel vs. toad. It is to be noted that the volume profiles of Fig. 4 are sensitive enough to detect the steric change of a single methyl group, e.g., a Ser to Thr substitution.

DISCUSSION

We have combined complementary approaches of bioenergetics and molecular biology to understand the reasons of the strong resistance of the bc_1 complex to funiculosin in fish mitochondria (Table II). This resistance very likely derives from some residue replacements in cytochrome b, where funiculosin binds (8, 10, 27, 28, 30). The replacement of the highly conserved Ala126 (yeast numbering, Fig. 3) with Met, unique to fish cytochrome b [Fig. 3 cf. (1, 19–22, 33) and A. Meyer, unpublished data], clearly increases the local protein volume (Fig. 4) and is probably responsible for the resistance to funiculosin. Indeed, yeast mutants that replace Ala126 with the bulkier Thr in their cytochrome b (14) show a significant increase in the titer of both funiculosin and myxothiazol

(Tron, T., Lemesle, D., Bruel, C., Coppée, J. P., Colson, A. M., Ghelli, A. and Degli Esposti, M., manuscript in preparation). To support further the idea that position 126 is involved in funiculosin binding, the bc_1 complex from potato is strongly resistant to funiculosin (40), and the cytochrome b of all plants shows the replacement of Ala126 with the bulkier Val (1).

We believe that also position 194 (yeast numbering) is involved in the binding of funiculosin to mitochondrial cytochrome b and that an increase of the residue volume at both this position and position 126 reduces the affinity for this inhibitor. This idea is sustained by the following points: (i) chicken cytochrome b is clearly resistant to funiculosin (Table II) and this can be correlated with a decrease in the minimum of volume within helix D due to the substitution of Ala194 with the bulkier Thr [34]; (ii) all ray-finned fish show a significantly higher titer for funiculosin than sturgeon (Table II), the cytochrome b of which shows a deep volume minimum in helix D, essentially due to the exchange of Thr194 with the smaller Ser (Fig. 3); (iii) in some mammals like rabbit in which cytochrome b has the bulky Val at position 194 (20, 41), the ubiquinol:cytochrome c reductase is highly resistant to funiculosin (ca. 80-fold with respect to other mammals, M. Degli Esposti, A. Ghelli and M. Crimi, unpublished results). Additionally, yeast cytochrome b also has Val at position 194 (cf. Fig. 3), and the titer of funiculosin in both the purified bc_1 complex and mitochondrial particles is at least 5-fold higher in yeast than in beef [M. Degli Esposti, T. Tron and A. Ghelli, unpublished results, see also (9, 10, 32)].

In a helical representation of helix D, position 194 lies within the quadrant that contains both His197, one ligand of the b_H heme (3–6), and Leu198, whose mutation to Phe (again an increase of the local protein volume) confers resistance to funiculosin (12, 30, 42). Thus, it is reasonable to conceieve that position 194 forms part of the binding pocket for funiculosin at center i. From the above discussion position 126 is also critical for funiculosin binding. However, this does not necessarily imply that both positions 126 and 194 form part of the same quinone and inhibitor binding site at center i. In fact, the transmembrane regions where these residues lie may define parts of two distinct sites for ubiquinone antagonists in cytochrome b that phenomenologically could correspond to both center i and o, given that position 126 is close to mutation sites inducing resistance to center o inhibitors (11, 12, 30, 42). Alternatively, it may be considered that the regions containing residues 126 and 194 are connected by long-range interactions within the membrane sector of the protein, so that binding of funiculosin at center i would transmit alterations at center o through conformational changes (32, 43).

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