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CYTOCHROME *b* OF PROTOZOAN MITOCHONDRIA: RELATIONSHIPS BETWEEN FUNCTION AND STRUCTURE

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Abstract—1. The sensitivity of ubiquinol:cytochrome *c* reductase to its most powerful inhibitors has been characterized in mitochondria from three ciliate and two trypanosome protozoans and compared with that in mitochondria of animals and plants.

2. Mitochondria of ciliates, particularly those of *Tetrahymena pyriformis*, are resistant to antimycin.

3. Mitochondria of trypanosomes are quite resistant to stigmatellin, as they exhibit a 40-fold higher titer than that in ciliate or animals mitochondria.

4. Both ciliates and trypanosomes are highly resistant to myxothiazol.

5. Correlations have been drawn between the natural resistance of the protozoan mitochondria to antimycin, stigmatellin and myxothiazol and peculiar features in the structure of their apocytochrome *b*, on the basis of an accurate alignment of the sequences of this protein.

INTRODUCTION

Mitochondrial cytochrome *b* is the only subunit of the ubiquinol:cytochrome *c* reductase (bc_1 complex) that is encoded by the mitochondrial DNA (DeVries and Marres, 1987; Chomyn and Attardi, 1987). Cytochrome *b* contains two hemes in bis-histidine coordination (Degli Esposti, 1989) that are directly involved in the electron transfer from ubiquinol to the other cytochromes of the respiratory chain (DeVries and Marres, 1987; Trumpower, 1990).

Among the great many sequences of the cytochrome *b* gene that are now available, those from protozoan species exhibit the lowest degree of homology (DelaCruz *et al.*, 1984; Hauska *et al.*, 1988; Pritchard *et al.*, 1990a). This high sequence divergence is quite useful for enucleating the essential features in cytochrome *b*, because it is related to the extraordinary evolutionary distance between species of the Protista kingdom and any other species (Sogin *et al.*, 1989). We present herein a detailed study of the cytochrome *b* function and structure in protozoan mitochondria. This work is aimed, in particular, to provide structural explanations for the unusual responses of protozoan mitochondria to inhibitors that bind to cytochrome *b*, e.g. the low sensitivity to antimycin in paramecia (Kilpatrick and Erecinska, 1977; Doussiere *et al.*, 1979).

The large diversity in structure of protozoan cytochrome *b* makes this protein a potential target for drugs acting as quinone antagonists specific for the mitochondria of parasitic species (cf. Wan *et al.*, 1974). Clearly, only a detailed comparison of the cytochrome *b* sequences can reveal how peculiar structural features in the quinone binding sites of cytochrome *b* correlate with different responses to quinone antagonists (Degli Esposti *et al.*, 1990a, 1992). Towards this end we have elaborated a refined alignment of the protozoan sequences. The analysis of the aligned proteins has suggested correlations between structural features in cytochrome *b* and the relative sensitivity to the inhibitors that we have measured in mitochondria from ciliate and flagellate protozoans.

MATERIALS AND METHODS

Mitochondria were prepared from cells of the ciliate *Tetrahymena pyriformis* strain W as described by Kilpatrick and Erecinska (1977). Cultures of *Leishmania infantum* strain MCAN/IT/ISS29 were obtained essentially as described previously (Rioux *et al.*, 1970), whereas cultures of *Crithidia lucilae* were prepared as reported by Renger and Wolstenholme (1972). Mitochondria from trypanosomes cells were prepared essentially as described by Renger and Wolstenholme (1972). *In vivo* assays of the reduction of the infection by *L. infantum* in mice were carried out with the standard protocol described by Gradoni *et al.* (1989). Cultures of *Paramecium aurelia* and *Paramecium tetraaurelia* were purchased from Carolina Ltd and grown as specified in the purchaser kit. Mitochondrial fractions from the collected cells were prepared as described by Doussiere *et al.* (1979).

Mitochondria from fresh stocks of wheat-germ, obtained by a local mill, were prepared essentially as described by Pfeiffer *et al.* (1990). Mitochondria from mammalian tissues were prepared by standard procedures (see Degli Esposti *et al.*, 1992, and references therein). Optical spectra of the mitochondrial cytochromes were obtained as previously

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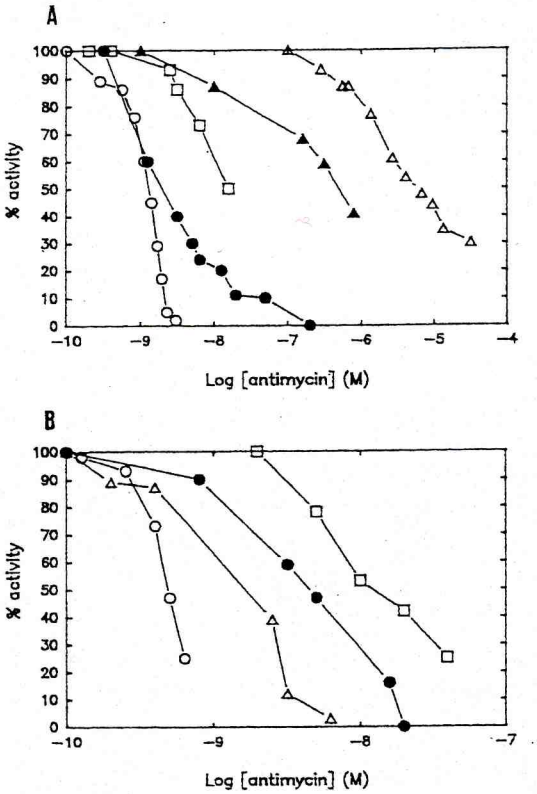

Fig. 1. Titration of antimycin in protozoan mitochondria. The conditions of assay are: ubiquinol-2 (14 μM), cytochrome *c* (10 μM) (Tron *et al.*, 1991; Degli Esposti *et al.*, 1990a) and the inhibitor is added to the concentrated mitochondrial solution, which is *ca* 0.4–2 μM in cytochrome *b*, unless otherwise stated. (A)—○——○ wheat-germ (1.2 nM bc_1 complex); ●——● *Leishmania infantum* (3 nM bc_1 complex); □——□, *Tetrahymena pyriformis* (1.6 nM bc_1 complex); △——△, *Tetrahymena pyriformis* (3 nM bc_1 complex) with the inhibitor added directly to the assay medium under continuous stirring; ▲——▲, *Tetrahymena pyriformis* (1.6 nM bc_1 complex) with the inhibitor added directly to the assay medium without continuous stirring. (B)—○——○, pig liver (0.9 nM bc_1 complex); △——△, *Paramecium aurelia* (0.75 nM bc_1 complex); ●——●, pig liver (0.9 nM bc_1 complex) with the inhibitor added directly to the assay medium; □——□, *Paramecium aurelia* (0.75 nM bc_1 complex) with the inhibitor added directly to the assay medium. Parallel experiments in mitochondria of *Paramecium tetraaurelia* showed identical titers to those of *Paramecium aurelia*.

described (Tron *et al.*, 1991). The activity of the ubiquinol:cytochrome *c* reductase of the mitochondrial preparations was assayed with nearly saturating concentrations of ubiquinol-2 (a generous gift from Eisai Co., Tokyo, Japan) or its alkyl analogs and horse heart cytochrome *c* as reported previously (Degli Esposti *et al.*, 1990a; Tron *et al.*, 1991). Antimycin and hydroxy-quinoline *N*-oxide (HQNO) were purchased from Sigma Chemical Company (St Louis, MO) myxothiazol from Boehringer (Lewis, U.K.) and the tridecyl analog of stigmatellin was a generous gift from ICI Agrochemicals (Bracknell, U.K.). Diuron was kindly donated by DuPont (Wilmington, DE U.S.A.). The inhibitors were dissolved and determined in ethanol (Von Jagow and Link, 1986) and usually incubated for at least 1 min with the concentrated mitochondrial solution.

Sequence analysis and comparison were performed with both standard methods like FASTP (Pearson and Lipman, 1988) or CLUSTAL (Higgings and Sharp, 1988) and the programs developed in house for evaluating the hydropathy (Degli Esposti *et al.*, 1990b) and the local homology (Degli Esposti, 1989; Crimi, 1991) in membrane cytochromes. A program was also created for translating DNA sequences into protein sequences using the yeast and animal code for mitochondrial genes (Chomyn and Attardi, 1987). The codon ATA was considered to specify for Ile in apicomplexan protozoans after extensive sequence comparisons. However, according to Ziaie and Suyama (1987), the single ATA codon in *Paramecium* cytochrome *b* was considered to specify for Met. Over 100 complete sequences of cytochrome *b* have been collected from the literature (Hauska *et al.*, 1988; Crimi, 1991) and those most different from each other have been aligned (Tron *et al.*, 1991; Crimi, 1991). From this master alignment we have compiled a consensus sequence normalized to the yeast cytochrome *b* that has been utilized as the reference in comparing all the sequences of protozoans.

RESULTS AND DISCUSSION

Functional properties of ubiquinol:cytochrome c reductase in mitochondria of ciliates and flagellates

There are two major groups of inhibitors of ubiquinol:cytochrome *c* reductase that specifically bind to cytochrome *b* (Von Jagow and Link, 1986; DiRago and Colson, 1988; DiRago *et al.*, 1989; Howell and Gilbert, 1988; Daldal *et al.*, 1989; Tron *et al.*, 1991; Trumpower, 1990). The group that is generally identified as center i inhibitors (DiRago and Colson, 1988) includes antimycin, HQNO, funiculosin and diuron and binds to the quinone site at the negative (matrix) side of the mitochondrial membrane. The other group [generally identified as center o inhibitors, (Tron *et al.*, 1991)] includes a variety of compounds such as myxothiazol and stigmatellin. A number of point mutations affecting the sensitivity (usually in the form of an increased titer) of some inhibitors of either group have been sequenced in the gene of cytochrome *b* of several species (see Trumpower, 1990, and Tron *et al.*, 1991, for an overview on this subject). These mutations induce amino acid substitutions in a few discrete regions of the protein that are now known to lie at the opposite sides of the mitochondrial membrane, where they define domains for each group of inhibitors (Daldal *et al.*, 1989; Trumpower, 1990).

The response to specific inhibitors of the cytochrome *c* reductase, therefore, can reveal structure–function relationships in mitochondrial cytochrome *b* (Degli Esposti *et al.*, 1990a, 1992). With this in mind we have investigated the sensitivity of the reductase to its inhibitors in mitochondria from protozoans for which sequences of cytochrome *b* are known. We have studied both *Tetrahymena pyriformis* and *Paramecium aurelia* as representative species of ciliates, since their mitochondrial genes are the best known for these protozoans (Ziaie and Suyama, 1987; Pritchard *et al.*, 1990a,b). The mitochondria of the two ciliates have similar, though not identical, spectral and redox properties of cytochrome *b* and cytochrome oxidase (results not shown, cf. Kilpatrick and Erecinska, 1977; Doussiere *et al.*, 1979). However, the two species seem rather distant in the evolution of the ciliate phylum (Sogin *et al.*, 1989; Douglas *et al.*, 1991).

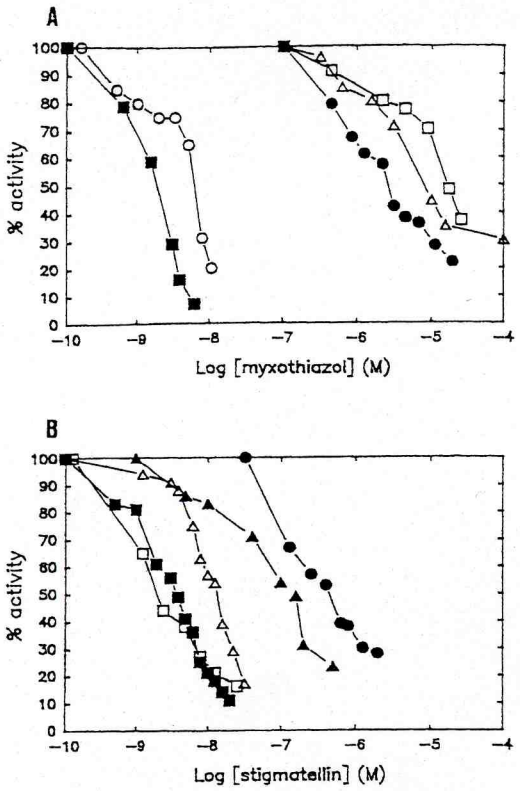

Fig. 2. Titration of myxothiazol and stigmatellin in various mitochondria. The conditions of the assay are as in Fig. 1. (A)—Myxothiazol titration. ■——■, pig liver (1.7 nM bc_1 complex); ○——○, wheat-germ (1.0 nM bc_1 complex); ●——● *Leishmania infantum* (3 nM bc_1 complex); △——△, *Tetrahymena pyriformis* (2 nM bc_1 complex); □——□, *Paramecium aurelia* (0.8 nM bc_1 complex). (B)—Titration of the tridecyl analog of stigmatellin. □——□, *Paramecium aurelia* (0.75 nM bc_1 complex); ■——■, pig liver (1.4 nM bc_1 complex); △——△, *Tetrahymena pyriformis* (3.3 nM bc_1 complex); ▲——▲, *Crithidia lucilae* (0.8 nM bc_1 complex); ●——●, *Leishmania infantum* (3 nM bc_1 complex).

Although several respiratory assays have been reported for the mitochondria of *T. pyriformis* (Turner *et al.*, 1971; Kilpatrick and Erecinska, 1977) and *P. tetraurelia* (Doussiere *et al.*, 1979), the ubiquinol:cytochrome *c* reductase activity of cyliate mitochondria has never been measured before. Such an activity can be completely blocked by specific inhibitors that bind to cytochrome *b*. In agreement with previous reports, the titer of antimycin is clearly higher in ciliates than in any other eukaryotic organisms, including flagellates (Fig. 1). This is especially the case for *T. pyriformis*, whose mitochondria show an I_{50} of *ca* 2×10^{-8} M under optimal conditions (Fig. 1A). The conditions of the assay heavily influence the quantitative estimation of the potency of antimycin in *Tetrahymena* mitochondria. In particular, if the inhibitor is added to the assay cuvette containing the mitochondrial fraction at nanomolar concentration in the reductase instead of incubating previously the inhibitor with the concentrated mitochondria, titers in the micromolar range are measured (Fig. 1A). This is due to two different phenomena: (i) the affinity of antimycin in *Tetrahymena* is unfavorable for the binding to such a diluted enzyme directly in the cuvette—with mammalian, plant and even paramecia mitochondria the titer increases only five-fold by adding antimycin to the diluted instead of the concentrated preparation (Fig. 1B); (ii) an inhibitor-insensitive reduction of cytochrome *c* is stimulated by the addition of antimycin in the cuvette, particularly with continuous stirring of the assay medium (Fig. 1A). The latter phenomenon is sensitive to superoxide dismutase and thus is likely to derive from the generation of oxygen radicals due to a diverted electron transfer from ubiquinol in the antimycin-treated bc_1 complex (Degli Esposti *et al.*, 1984). The antimycin titer in both *Paramecium* species that we have studied is much lower than that in *Tetrahymena* (Fig. 1). Clearly, previous respiratory and binding assays (Doussiere *et al.*, 1979) were not sufficiently specific for determining the antimycin affinity of these mitochondria, also because the inhibitor was added to the assay medium (cf. Fig. 1B and Doussiere *et al.*, 1979).

Although the respiratory chain of flagellates has been more widely studied than that from ciliates (e.g. Gutteridge *et al.*, 1979; Stoppani *et al.*, 1980), the activity of their ubiquinol:cytochrome *c* reductase has not been measured except for a purified reductase of *E. gracilis* (Mukai *et al.*, 1989) and of *L. tarentolae* (E. Berry, personal communication). Conventional respiratory assays with trypanosome mitochondria have established their functional similarity with those of higher plants in view of the presence of a hydroxy-salicylic-sensitive and cyanide-insensitive oxidase (Hryniewcka *et al.*, 1978; Fairlamb, 1982). Herein, we have characterized the reductase of two kinetoplast flagellates, *Leishmania infantum* and *Crithidia lucilae*, which are closely related to *Leishmania tarentolae* and *Crithidia fasciculata*, for which the sequence of cytochrome *b* is available (Feagin *et al.*, 1988). *Leishmania infantum* has been chosen also because of its impact on human health that demands a constant research of selective chemotherapic agents (Gradoni *et al.*, 1989). Moreover, preliminary assays *in vivo* have shown that diuron, a herbicidal compound which also binds to cytochrome *b* (DiRago and Colson, 1988; Von Jagow and Link, 1986), is capable of reducing the infection of this parasite in mice, with a 50% efficacy dose at 3–4 mg per kilogram of body weight (data not shown). This finding stimulated further a comparative investigation of the responses of mitochondria of trypanosomes to inhibitors of the cytochrome *c* reductase.

The ubiquinol:cytochrome *c* reductase in trypanosome mitochondria is often not completely blocked by bc_1 inhibitors, probably because of interferences from the alternative quinol oxidase. In agreement with data in other flagellates (e.g. Stoppani *et al.*, 1980), the ubiquinol:cytochrome *c* reductase of *L. infantum* and *C. lucilae* is very sensitive to antimycin, apparently as in plant or animal mitochondria (Fig. 1A and data not shown). However, mitochondria of both trypanosomes are resistant to the powerful inhibitor myxothiazol and, different to ciliate mitochondria, to stigmatellin too (Fig. 2 and Table 1). The resistance to myxothiazol is lower than that seen in ciliates (Fig. 2A) but, remarkably, the resistance towards stigmatellin is unique to try-

Table 1. Sensitivity to the most powerful inhibitors of the cytochrome *c* reductase in mitochondria from different species

Species	$[bc_1]$* (nM)	Reductase† activity (s^{-1})	Inhibitor I_{50}‡ (nM)		
			Antimycin	Myxothiazol	Stigmatellin
Yeast GM50-3C	5.6	238	2.9	3.7	4.0
Pig liver	1.4	220	0.7	1.4	3.5
Wheat-germ	2.6	130	1.3	7.8	—
Pea hypocotiles§	8.0	25	4.5	100	11.0
T. pyriformis	3.3	30	18.0	> 6000	10.0
P. aurelia	0.8	78	1.6	16,900	2.0
C. lucilae	1.4	50	0.8	1600	130.0
L. infantum	3.0	52	1.6	2300	380.0
R. rubrum‖	10.0	200	5.0	50	5.0

Conditions as in Fig. 1.

*The reductase concentration is estimated by either the content of cytochrome *b* or from extrapolation of the antimycin titration (Degli Esposti *et al.*, 1992). The yeast particles have been provided by T. Tron and D. Lemesle, CNRS Marseille.

†Average turnover of the reductase under the titrations experiments.

‡Concentration of the inhibitor that halves the reductase activity with ubiquinol-2 (or its nonyl, decyl and undecyl analog) and cytochrome *c* concentrations that are nearly saturating (Tron *et al.*, 1991).

§Percoll gradient pure mitochondria provided by Prof. D. Zannoni from our Biology Department. Condition of the assays as those of Berry *et al.* (1991).

‖Data from Guner *et al.* (1991) in isolated bc_1 complex.

panosomes so far. This latter resistance is confirmed in the purified reductase of *L. tarentolae* (E. Berry, personal communication). Interestingly, also chloroplast *bf* complex is sensitive to stigmatellin approximately as mitochondrial bc_1 complex, even if it is highly resistant to myxothiazol (Oettmeier *et al.*, 1987).

Both ciliate and flagellate mitochondria are quite resistant to other center i inhibitors like HQNO (results not shown). Conversely, *Leishmania* mitochondria are slightly more sensitive to diuron than ciliate mitochondria and those from plant or animal species (data not shown, cf. Berry *et al.*, 1991). Hence, they do not show a strong hypersensitivity to this compound that might explain its *in vivo* effect on infected mice. However, we note that addition of diuron stimulates an antimycin-insensitive reduction of cytochrome *c* exclusively in *L. infantum* mitochondria. This suggests that the compound indeed affects the redox pathway of ubiquinol in trypanosome mitochondria as to increase the production of oxygen radicals that are deleterious for the parasitic organism. Some antiparasitic drugs which are efficient in reducing trypanosomal infection do act in this way (Docampo and Stoppani, 1979).

Sequence analysis of protozoan cytochrome b

The unusual responses of ciliate and flagellate mitochondria to inhibitors of the bc_1 complex that have previously been discussed should originate from structural changes in the cytochrome *b* of these protozoans, by analogy with previous cases of natural resistance (Degli Esposti *et al.*, 1990a, 1992). To understand how the changes in inhibitors' titers could be correlated to specific amino acid variations in protozoan cytochrome *b*, it is necessary to compare and align accurately the amino acid sequences of all protozoans so far available. To our knowledge, there are eight complete sequences to cytochrome *b* from protozoans and they generally are from parasitic species owing to their biomedical importance. There are three trypanosomes; *Trypanosoma brucei brucei* (Benne *et al.*, 1983), *Leishmania tarentolae* (DelaCruz *et al.*, 1984) and *Crithidia fasciculata* (Feagin *et al.*, 1988). The RNA-editing of all these sequences has been clarified by Feagin *et al.* (1988). Recently, the sequences of three species of the *Plasmodium* genus have been obtained: *P. yoelii* (Vaidya *et al.*, 1989; 1990), *P. gallinaceum* (Aldritt *et al.*, 1989) and *P. falciparum* (J. Feagin and R. Wilson, personal communication). The sequence of another apicomplexan like plasmodia has also been reported, namely that of *Theileria annulata* (Megson *et al.*, 1991). The sequence of *Paramecium aurelia* (Pritchard *et al.*, 1990a,b) is the only one from ciliate protozoans that has been reported to date.

The original report of the cytochrome *b* sequence of *P. yoelii* (Vaidya *et al.*, 1989) and *P. gallinaceum* (Aldritt *et al.*, 1989) contained a number of sequencing errors that have been subsequently corrected (A. Vaidya, J. Feagin and D. Aldritt, personal communication, cf. Vaidya *et al.*, 1990). The recent sequencing (Feagin, 1992) of the same gene from the agent of human malaria *P. falciparum*, which is closely related to the two species above, has further clarified the amino acid assignment of the deduced DNA sequences We have been puzzled previously by the original uncorrect reports of the plasmodial sequences, particularly because the conserved histidines that ligate the hemes were missing. Consequently, we elaborated an alternative deduction of the reported DNA of *P. yoelii* that increased considerably the protein homology with other species (data not shown and Crimi, 1991). This deductive reinterpretation of the originally published DNA of Vaidya *et al.* (1989) differed only for 22 residues from the corrected sequence that has been subsequently reported and which differs in 130 residues from the original one (Vaidya *et al.*, 1990 cf. Vaidya *et al.*, 1989). This underscores the validity of deducing the most likely protein sequence of a poorly homologous cytochrome *b* on the basis of a detailed evaluation of the aligned sequences.

Fig. 3. Alignment of cytochrome *b* sequences. The amino acid sequence deduced from the mitochondrial gene of cytochrome *b* from protozoans, one plant, one alga and one purple bacterium are aligned to that of the yeast cytochrome *b*, to which the numeration is normalized (Crimi, 1991). The sequence of *T. aestivum*, *T. brucei* (Benne *et al.*, 1983) and *L. tarentolae* (DelaCruz *et al.*, 1984) are corrected for RNA editing (Gualberto *et al.*, 1989; Feagin *et al.*, 1988). The sequence of *P. aurelia* is taken from Pritchard *et al.* (1990b) and has been corrected for parsimony (see Table 3 and text). The sequence of *Rhodospirillum rubrum* (Majewski and Trebst, 1990), *Thelleria annulata* (Megson *et al.*, 1991), *Chlamydomonas reinhardtii* (Michaelis *et al.*, 1990), *Saccharomyces cerevisiae* (Nobrega and Tzagoloff, 1980) and *Plasmodium yoelii* (Vaidya *et al.*, 1990) are taken from the quoted references. The symbols * indicate the residues that appear to be conserved in all the protozoan sequences, including those (not shown) of *Plasmodium gallinaceum* (Aldritt *et al.*, 1989) and *Plasmodium falciparum* (Feagin, 1992). The limits of the transmembrane helices (underlined) have been evaluated by a detailed sequence and hydropathy analysis (Degli Esposti *et al.*, 1992).

Table 2. Per cent identity of cytochrome *b* sequences

Species		% Identity												
		SC	BT	SP	TA	OV	CR	PA	PY	PG	TH	LT	TB	RR
S. cerevisiae	(SC)	100												
B. taurus	(BT)	52	100											
S. pombe	(SP)	58	52	100										
T. aestivum	(TA)	50	53	50	100									
O villaricae	(OV)	51	53	51	95	100								
C. reinhardtii	(CR)	45	49	44	58	59	100							
P. aurelia	(PA)	19	21	25	24	24	23	100						
P. yoelii	(PY)	38	41	39	42	41	39	23	100					
P. gallinaceum	(PG)	35	40	40	39	40	41	24	67	100				
T. annulata	(TH)	34	37	37	36	36	34	21	39	38	100			
L. tarentolae	(LT)	23	25	24	25	26	23	21	30	27	25	100		
T. brucei	(TB)	23	26	25	25	27	24	21	30	27	25	86	100	
R. rubrum	(RR)	52	54	51	57	59	51	22	40	38	35	25	22	100
		SC	BT	SP	TA	OV	CR	PA	PY	PG	TH	LT	TB	RR

The sequence were aligned as shown in Fig. 3. The sequence of beef (*B. taurus*) was taken from Hauska *et al.* (1988), that of *Schizosaccharomyces pombe* from Lang *et al.* (1985) and that of *Oenothera villaricae*, corrected for RNA editing, from Schuster *et al.* (1990). The acronyms in the horizontal axis correspond to the species as indicated on the left of the table. Note that the sequence of *Plasmodium gallinaceum* as reported originally (Aldritt *et al.*, 1989) is incomplete and partially incorrect (J. Feagin and D. Aldritt, personal communication). The sequence of *Plasmodium falciparum* is 89% identical to that of *Plasmodium yoelii* (Feagin, 1992). It should be noted that the high per cent identity of the plasmodial sequences with that of beef is less significant than the high identity with the plants sequences because the latter derives from a larger sequence overlap with far less gaps and insertions than beef or other animals (Crimi, 1991).

With this in mind we have carefully aligned and compared the corrected sequences of protozoan cytochrome *b* with those of yeast, plants and algae mitochondria (Fig. 3). The alignment of the median and *C*-terminal part of the sequence had to be optimized manually owing to the large sequence variability. Several factors were considered for implementing the alignment of these regions: the hydrophobicity profile (Degli Esposti *et al.*, 1990b); the positions of the intron–exon junctions in the mosaic genes of fungi, yeast and algae (cf. Nobrega and Tzagoloff, 1980; Michaelis *et al.*, 1990); and the maximal homology with all other sequences (Bashford *et al.*, 1987). The result of this extensive analysis is shown in the alignment of Fig. 3, which includes the sequence of the purple bacterium *Rhodospirillum rubrum* (Majewski and Trebst, 1990), since this is the bacterial cytochrome *b* most similar to the mitochondrial ones (data not shown). The underlined sequences are predicted to form the putative transmembrane helices of the protein (see also Degli Esposti *et al.*, 1992).

The per cent identity of the sequences in Fig. 3 and some related sequences are shown in Table 2. Clearly, cytochrome *b* of plasmodia is most identical to that of higher plants. This is consistent with the established phylogenetic relationships between protozoan species and plants (Sogin *et al.*, 1989) and with the homology of extrachromosomal DNA in plasmodia with the DNA of plant chloroplasts that has been recently uncovered (Wilson *et al.*, 1991). The sequence of *P. yoelii* is also that most identical to the sequences of trypanosomes, the top score being 30% with *L. tarentolae* (Table 2). The degree of identity of the trypanosomal sequences with yeast and, in particular, plant mitochondria is now higher than previously measured (Table 2 cf. Hauska *et al.*, 1988) because of our improved alignment.

Considerations on the sequence of cytochrome b *of* Paramecium

The sequence of cytochrome *b* from the cyliate *P. aurelia* is extremely different from that of any other species (Table 2 and Pritchard *et al.*, 1990a). This disagrees with the phylogenetic relationships among cyliates and other eukaryotes that is inferred by sequence comparison of rRNA (Sogin *et al.*, 1989; Douglas *et al.*, 1991). We note, however, that there are several substitutions of otherwise invariant residues in *Paramecium* cytochrome *b* that can derive from a single base change in the corresponding codon. The previous re-evaluation of *P. yoelii* protein sequence (Crimi, 1991) and precedents of errors in sequencing of mitochondrial proteins (Jacobs *et al.*, 1990; Degli Esposti *et al.*, 1990a; Vaidya *et al.*, 1990) have led us to suspect that some of the unusual features in *Paramecium* cytochrome *b* may actually be erroneous assignments. Because our approach is essentially deductive, we must adopt a consistent criterion of structural parsimony that excludes those amino acid replacements that are most unusual and could derive from a minimal variation of the DNA sequence originally reported by Pritchard *et al.* (1990b). In this line, we have excluded all those replacements that were found to change an otherwise invariant residue by a single nucleotide variation of the corresponding codon, e.g. H instead of the invariant E272 and D instead of the invariant G75 (Table 3).

Table 3. Unusual residues substitution in *Paramecium aurelia* cytochrome *b*

Residue conserved*	Residue *Paramecium*†	Comments‡
G_{75}	D	Ignored for a single base change
R_{79}	F	Ignored as structurally implausible
S_{87}	D	Ignored for a single base change
Y_{132}	L	Maintained as seen also in a lizard
V_{146}	I	Maintained as seen in chloroplast b_6
P_{155}	M	Maintained because of two base changes
D_{229}	E	Ignored for a single base change
I_{269}	V	Maintained as seen also in a fish
E_{272}	H	Ignored for a single base change

*Residue conserved in all other species so far examined (cf. Degli Esposti *et al.*, 1992) according to yeast numbering (Fig. 3).
†Residue deduced from the DNA as reported by Pritchard *et al.* (1990a).
‡Our proposals dictated by structural parsimony (see Text).

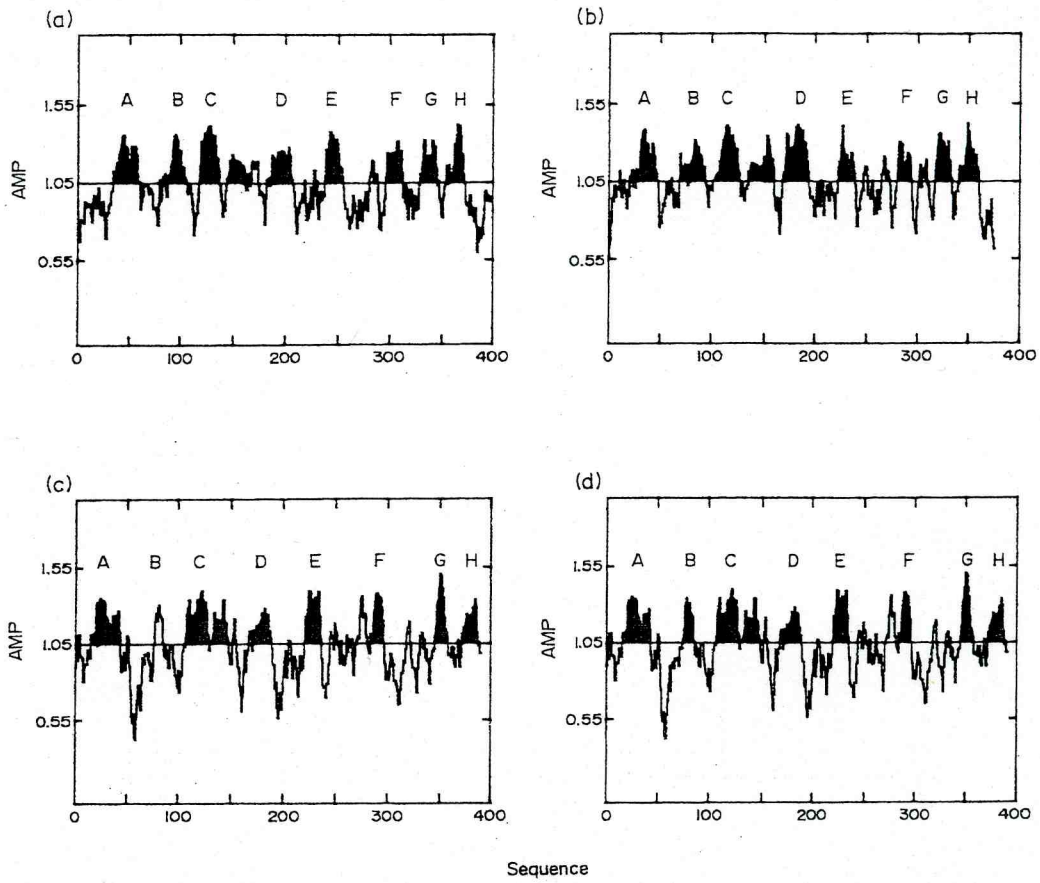

Fig. 4. Profile of hydropathy of some cytochrome *b* sequences. The relative hydropathy of the residues is according to the average membrane preference (AMP) scale and the baseline is lowered to 1.05 instead of 1.1 to avoid errors of negative predictions of transmembrane helices (Degli Esposti *et al.*, 1990b). The helices of the apocytochrome are indicated by capital letters as suggested by Crofts *et al.* (1990). The hydrophobic peaks colored in black are predicted to span the membrane. Note, however, that the black peak between helix C and D is a false positive prediction since this region is now known not to span the membrane (Degli Esposti, 1989). (A)—Wheat cytochrome *b* corrected for RNA editing (Gualberto *et al.*, 1989). (B)—*P. yoelii* cytochrome *b* as corrected by Vaidya *et al.* (1990). (C)—*P. aurelia* cytochrome *b* as reported by Pritchard *et al.* (1990b); note that helix B is not predicted to be transmembrane. (D)—*P. aurelia* sequence as modified by us (Table 3 and see text); note that now helix B is predicted to be transmembrane.

Even after the above tentative modifications by parsimony, the protein region spanning residues 70–88 (yeast numbering) in the reported sequence of *P. aurelia* is much too hydrophilic to form a transmembrane helix as in all other species (Fig. 4). This has not been noted previously (Pritchard *et al.*, 1990a) and its catastrophic consequence for the folding of cytochrome *b* suggested to us that some substantial errors or artifacts may be concentrated in this part of the translated *P. aurelia* gene. The simplest possibility that we considered is based on the alignment with the corrected DNA and protein sequence of *P. yoelii* (Vaidya *et al.*, 1990a) which provides clues for the following conservative modifications. It is first necessary to introduce a single gap in the alignment of the proteins at position 77 (yeast numbering, Fig. 3). Secondly, a −2 frameshift within the codon of the conserved H82 yields a reasonable conservation of the residues 83–87; and thirdly, a +2 frameshift at the codon of residue 87 restores the previous reading frame (Fig. 3). With such modifications the protein region is now sufficiently hydrophobic to span the membrane (Fig. 4). Finally, we note that it is implausible that the invariant Arg 79 is substituted by Phe only in this species (Pritchard *et al.*, 1990a). By following the criteria used in aligning the globin sequences (Bashford *et al.*, 1987), such a substitution can be tentatively ignored in view of the opposite chemical nature of the exchanged amino acids.

Alternative possibilities could also be considered for increasing the protein homology of *Paramecium* cytochrome *b* in the extrinsic loop between helix A and B. We note, for instance, that the stretch LAFSSV corresponding to residue 40–46 of the DNA-deduced sequence of *Paramecium* (Pritchard *et al.*, 1990a) is very homologous to the stretch LAFSSV which is conserved in most yeast and animal species at position 60–66 (Hauska *et al.*, 1988; Degli Esposti *et al.*, 1992, cf. Fig. 3). If these two stretches were aligned together, the subsequent sequence of the extrinsic loop A to B in *Paramecium* could be

alternatively translated A67RTVRDERGVIIRSLH-82 by postulating some frameshifts up to the beginning of helix B as discussed before. Such a hypothetical amino acid translation would much increase the protein homology of the region (cf. Fig. 3) and also restore its usual hydrophobic character which is apparently lost in the sequence deduced originally (Fig. 4). However, this would imply a series of modifications of the genomic DNA of *Paramecium*, and/or the presence of small introns in the cytochrome *b* gene. These alterations of DNA translation are difficult to postulate solely on the basis of protein homology and thus, for the moment, we discuss them just as extremely tentative hypotheses for future experiments.

In spite of the modifications of the original sequence that we propose in helix B, *P. aurelia* cytochrome *b* is still the most unusual and generally shows a degree of identity below 23% (Table 2). The highest homology is seen with the cytochrome *b* of *Plasmodium* and, among all other eukaryotes, with that of *S. pombe* and higher plants (Table 2). Such a pattern is now more consistent with phylogenetic data (Douglas *et al.*, 1991) than before. It is interesting to note that most insertions of the *P. aurelia* sequence occur near exon–intron junctions in the *cob* gene of yeasts (Fig. 3).

Structure–function correlations in protozoan cytochrome b

Having analyzed the protein sequences of cytochrome *b*, we now consider how the unusual responses of protozoan mitochondria to inhibitors of cytochrome *c* reductase (Table 1) can be correlated to structural features in this protein. The alignment in Fig. 3 and the knowledge of resistant mutants in yeast and other species (Trumpower, 1990; Tron *et al.*, 1991) indicate some correlations between natural resistance and unusual replacements in the sequence of cytochrome *b*. The most straightforward of such correlations regards the resistance to stigmatellin in trypanosomes (Table 1 and Fig. 2B), since the sequence of cytochrome *b* from these organisms shows the same replacement Ile 147 to Phe as the yeast stigmatellin mutant *Sti1*-5 (DiRago *et al.*, 1989; Tron *et al.*, 1991, cf. Fig. 3). The titer of this inhibitor is about 40-fold higher in trypanosomes than in cyliate or mammalian mitochondria (Table 1), being thus comparable to the 20-fold resistance in the yeast mutant *Sti1*-5 (Tron *et al.*, 1991). This suggests that the above replacement in the flagellate cytochrome *b* is the principal reason for the insensitivity to stigmatellin.

We note that trypanosomal cytochrome *b* shows unusual replacements also at two positions whose mutation induces resistance to myxothiazol and mucidin (but not stigmatellin, DiRago *et al.*, 1989), namely Gly 137 which is changed to Thr, and Asn 256 which is changed to Ser (Fig. 3). The former substitution is shared by the sequence of *Paramecium*, which also changes Asn 256 with Phe (Fig. 3). However, it is likely that the very strong resistance of ciliate mitochondria towards myxothiazol (Fig. 2 and Table 1) is also contributed by the unique substitution of Gly 143 with Thr (Fig. 3 and Daldal *et al.*, 1989; Degli Esposti *et al.*, 1990a). Position 143 is sterically critical for the turnover of ubiquinol oxidation as deduced by saturation mutagenesis in bacterial cytochrome *b* (Atta-Asafo-Adjei and Daldal, 1991). Clearly, the center o of *Paramecium* cytochrome *b* must have protein compensations for the steric hindrance due to the substitution of Gly with the bulkier Thr, since the turnover rate of the reductase in paramecia is comparable to that of other species including trypanosomes (Table 1 and data not shown). In this line, by using the information gathered from secondary site revertants in yeast cytochrome *b* (DiRago *et al.*, 1990), we propose that the following changes—that are uniquely seen in *Paramecium* cytochrome *b*—may naturally compensate for the increased hindrance at position 143: the substitution of the very conserved Tyr 132 with the smaller Leu, the change of the aromatic at position 141 with Glu and the substitution of the bulky Ile 148 with the smaller Ala (Fig. 3).

Cytochrome *b* in ciliates is most unusual for its resistance to antimycin (Fig. 1). *Tetrahymena* mitochondria are one order of magnitude more resistant to antimycin than *Paramecium* mitochondria (Fig. 1 cf. Turner *et al.*, 1971) and generally also than yeast resistant mutants (M. Degli Esposti and T. Tron, unpublished results). Unfortunately, the sequence of *T. pyriformis* cytochrome *b* is not available yet, and thus it is impossible to infer molecular reasons for this natural resistance. In the case of *P. aurelia* the unique substitution of Gly 37 with Phe (Fig. 3) might be the major reason for the antimycin resistance of this species. Indeed, although positions 228 and 232—whose mutation induces resistance to antimycin as for position 37 (DiRago and Colson, 1988; Howell, 1989)—are unusually substituted in *Paramecium* cytochrome *b*, such positions are similarly substituted also in the cytochrome *b* of trypanosomes (Fig. 3), which are apparently as sensitive as other species to antimycin (Fig. 1 and Table 1). We note, additionally, the substitution of Trp 30 with Ser that is uniquely seen in *Paramecium* (Fig. 3 and data not shown). Position 30 lies nearly on top of position 37 in a helical wheel representation of transmembrane helix A (Tron *et al.*, 1991), thereby suggesting that there is a compensatory exchange of an aromatic residue between these positions in center i of *Paramecium* cytochrome *b*. This may reduce the intrinsic effect of the change at position 37 in the affinity for antimycin in this cyliate.

CONCLUSIONS

On the basis of the analysis of a few sequences and of the nature of the mutated residues in resistant mutants, predictions of natural resistance towards antimycin in trypanosome cytochrome *b* have been advanced (DiRago and Colson, 1988; Howell and Gilbert, 1988). Similar predictions were subsequently advanced for *Paramecium* cytochrome *b* (Crimi, 1991). These predictions have been tested here by the characterization of the response of the ubiquinol:cytochrome *c* reductase to its inhibitors in mitochondria of trypanosomes and ciliates. Ciliate mitochondria, but apparently not trypanosome mitochondria, are resistant to antimycin (Fig. 1). Such a resistance is, however, mild in the case of *Paramecium*

species (Fig. 1B), thereby implying that the structure of the transmembrane helices A and E in the cytochrome *b* of these protozoans is similar to that of other species. In fact, these helices contain the loci of resistance for antimycin in several organisms (DiRago and Colson, 1988; Howell, 1989; Trumpower, 1990). However, the sequence of *Paramecium* cytochrome *b* as originally deduced from the DNA sequence (Pritchard *et al.*, 1990a) may not correspond to the most likely protein structure of helix B and probably also of the extrinsic loop connecting helix A and B (Table 3). Since helix B contains a resistance locus for stigmatellin (Daldal *et al.*, 1989), the remarkable change of its structure that was deduced previously (Pritchard *et al.*, 1990a, cf. Fig. 4) would imply a reduced binding for this specific inhibitor. Instead, stigmatellin is very powerful in inhibiting the reductase of *Paramecium* (Fig. 2B), thereby indicating that the structure of helix B must be more conserved than originally deduced. Thus, functional data support conservative translations of the protein sequence of *Paramecium* cytochrome *b*, and our proposals in this line (Fig. 3) are the simplest that are compatible with the reported DNA (Table 3) and the membrane folding (Fig. 4). We plan to determine the protein sequence of the *N*-terminus part of apocytochrome *b* of ciliates by sequencing directly the isolated subunit, in order to establish the real structure of this mitochondrial protein and thus test the present deductions dictated by parsimony.

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