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Review

Mitochondrial cytochrome *b*: evolution and structure of the protein

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Contents

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Summary

Cytochrome *b* is the central redox catalytic subunit of the quinol: cytochrome *c* or plastocyanin oxidoreductases. It is involved in the binding of the quinone substrate and it is responsible for the transmembrane electron transfer by which redox energy is converted into a protonmotive force. Cytochrome *b* also contains the sites to which various inhibitors and quinone antagonists bind and, consequently, inhibit the oxidoreductase.

Ten partial primary sequences of cytochrome *b* are presented here and they are compared with sequence data from over 800 species for a detailed analysis of the natural variation in the protein. This sequence information has been used to predict some aspects of the structure of the protein, in particular the folding of the transmembrane helices and the location of the quinone- and heme-binding pockets.

We have observed that inhibitor sensitivity varies greatly among species. The comparison of inhibition titrations in combination with the analysis of the primary structures has enabled us to identify amino acid residues in cytochrome *b* that may be involved in the binding of the inhibitors and, by extrapolation, quinone/quinol.

The information on the quinone-binding sites obtained in this way is expected to be both complementary and supplementary to that which will be obtained in the future by mutagenesis and X-ray crystallography.

I. Introduction

Cytochrome *b* is the central catalytic subunit of ubiquinol : cytochrome *c* reductase (or bc_1 complex, EC 1.10.2.2), an enzyme that is present in the respiratory chain of mitochondria [1–6], and in the respiratory chain or cyclic photo-redox chain of many bacteria [3,7–9]; it is functionally homologous to the plastoquinol : acceptor reductase (or *bf* complex) of chloroplasts that is involved in both cyclic and non-cyclic light-driven electron transfer [3,10–12]. With the exception of protozoans lacking mitochondria (e.g., *Trychomonas*), all eukaryotic organisms require this general class of redox enzyme, and consequently cytochrome *b*, for energy conservation [3,9,12].

Cytochrome *b* is the transmembrane protein involved in the vectorial oxidation of ubiquinol or plastoquinol and in the electrogenic portion of the catalytic pathway [3,5–16]. The Q-cycle mechanism, originally proposed by P. Mitchell [13], is now widely accepted to be a good description of the redox reactions of the bc_1 complex [5,7–9,14,15] and it predicts that cytochrome *b* forms a ubiquinone-reacting center at each side of the membrane. Present research on cytochrome *b* is focused upon how its structure is related to function [5–12].

Our knowledge of mitochondrial cytochrome *b* is expanding very rapidly, in particular through the analysis of protein sequences predicted from the DNA sequences [5,6,9–12]. The importance of aquiring a vast number of protein sequences is that it enables us to observe just how the protein has evolved while maintaining its function. New sequences of cytochrome *b* are presented here to further document the natural variation of the protein. For uncovering additional structure–function relationships, we report a screening of the inhibitor responses of the bc_1 complex from several different species. Since bc_1 inhibitors bind directly to native cytochrome *b* [5,6,9,17–19], significant changes in their affinity may arise from variations in the primary structure of the protein [6,19]. A systematic analysis of the sequences available and the integration of this analysis with a survey of the properties of inhibitor resistant mutants is presented. In this way, we provide a framework for proposing and testing correlations between inhibitor responses and natural variations in the sequence of cytochrome *b*. Some conclusions have been drawn regarding the possible folding of the protein and the connections of its transmembrane helices. In view of the flood of sequence information provided recently, comments on the relation between sequence variation in cytochrome *b* and evolution are also presented in this work.

II. Nomenclature of cytochrome *b*

Before discussing the data, it is necessary to clarify some issues of nomenclature that concern cytochrome *b*.

II-A. The b-hemes

It is well established that cytochrome *b* contains two distinct hemes with different spectroscopic and redox properties [2,3,7,8,18,20]. Unfortunately, various overlapping designations of such hemes have accumulated in the literature. Designation according to the maximum of the reduced alpha band (the recommended nomenclature by IUB) is questionable, since the maximum of the *b*-562 heme (in beef heart mitochondria [20,21]) varies between 558 and 563 nm in other species [3,9,21]. It is particulary inappropriate for the chloroplast cytochrome b_6, in which the electronic absorption spectra of two hemes are not easily distinguishable [3,10]. Among the properties that are suggested for distinguishing the hemes in diheme cytochromes (cf. IUB nomenclature for redox proteins, G. Palmer and J. Redijk (1992) Eur. J. Biochem. 200, 599–611), we believe that the relative difference in the midpoint redox potential is sufficiently general to be applicable

for the cytochrome *b* in all quinol:acceptor reductases [3,7,8,18,21]. Hence, we shall refer here to *b*-562 as the high potential heme, identified as b_H, and to *b*-566 (558) as the low potential heme, identified as b_L.

TABLE I

Species whose cytochrome b is sequenced [a]

Species (common name) and [reference]

Metazoans

Mammals and marsupials

Complete sequences:
Homo sapiens (man) [164]; *Bos taurus* (beef) [165]; *Capra hircus* (goat), *Ovis aries* (sheep), *Antilocapra americana* (pronghorn), *Giraffa camelopardalis* (giraffe), *Dama dama* (fallow deer), *Odocoileus hemionus* (black-tailed deer), *Tragulus napu* (Malay chervotain), *Camelus dromedarius* (dromedary camel), *Sus scrofa* (domestic pig), *Tayassu tajacu* (collared peccary), *Equus grevyi* (zebra), *Diceros bicornis* (black rhino), *Loxodonta africana* (African elephant), *Stenella longirostris* (dolphin1), *Stenelea attenuata* (dolphin2) [32]; *Mus musculus* (mouse) [166]; *Rattus rattus* (rat1) [167]; *Rattus norvegicus* (rat2) [168]; *Oryncholatus cuniculus* (rabbit) (F. Mignotte, unpublished cf. [169]); *Balaenoptera physalus* (fin whale) [170]; *Phoca vitulina* (harbor seal) [171].
Partial sequences:
Equus asinus (donkey), *Equus caballus* (horse), *Oryncholatus cuniculus* (rabbit *), *Felis catus domesticus* (domestic cat) this work; 2 *Hylobate* monkeys (J.C. Garza and Woodruff, D.S., unpublished, cf. L02766); *Akodon aerosus* and other 11 akodontine rodents [86]; 4 kangaroo rats and *Thomomys townsendi* [29]; *Cephalorhyncus commersonii* (small dolphin) [172]; *Canis canis* (dog) and other 4 canids [173]; *Canis aureus* (golden jackal) and other 9 canids (A. Meyer & R. Wayne, unpublished); *Ursus maritimus* (polar bear), *Ursus arctos* (brown bear), *Ursus americanus* (black bear) [87]; *Thylacinus* (marsupial wolf) and other 6 marsupials [85]; mandrill, drill, giraffe (R.H. Crozier, unpublished); elk and 5 *Odocoileus* deers (S.M. Carr and G.A. Hughes, unpublished, cf. M9484); *Ornithorhynchus* (platypus), *Tachiglossus, Zaglossus* and ca. 10 marsupials (M. Waskman, unpublished); 15 African bovids and reindeer (P. Arctander, unpublished); sheep and European muflon [174]; *Thylacinus cynocephalus,* 14 dasyuroid marsupials and bandicoot [252]; blue whale [253]; 2 squirrels (P.J. Wettstein, unpublished, M97277–79).

Birds

Complete and almost complete sequences:
Gallus gallus (chicken) [63]; *Coturnix coturnix* (quail) [197]; *Colaptes rupicoea* (andean flicker), *Scytalopus magellanicus* (andean tapaculo), *Asthenes dorbignyi* (canastero), *Ampelion stresemanni* (cotinga), *Pitta sordida* (pitta), *Pomatostomus temporalis* (babbler), *Pomatostomus isidori* (rufous babbler), *Amblyornis macgregoriae* (bowerbird), *Epimachus albertisii* (sicklebill), *Ptiloprora plumbea* .(honeyeater), *Gymmorhina tibicen* (magpie), *Catharus guttatus* (hermit thrush), *Parus inornatus* (plain titmouse) [33]; 17 *Phylloscopus* species (warblers), *Cettia fortipes* (Cetti's warbler), *Regulus satrapa* (gold crest), *Sylvia melanocephalus* (blackcap) [91]; 9 deep-node birds, 7 pipits (P. Arctander, unpublished), *Meleagris galiopavo* (turkey) and other 7 gallinaceous birds [255].
Partial sequences:
Emberyza shoeniculus (reed bunting) this work; 3 babblers [29,62]; *Corcorax melanorhamphos* (crow) [29]; ca. 10 birds of paradise and warblers (J. Cracraft, S.V. Edwards, unpublished); 7 *Laniarius* species (shrikes) [175]; 6 cowbirds, 25 blackbirds, *Sphyrapicus varius, Aulacorhynchus derbianes, Capito niger* (S.M. Lanyon, unpublished, cf. [176]); 11 bowerbirds (R.H. Crozier, unpublished); ca. 70 passeriforms suboscines (P. Arctander, unpublished); 2 blue tits and great tit [177]; 12 parrots and rock dove [246].

Reptiles

Partial sequences:
Uta sp. (lizard) this work; *Lepidophyma smithii* (Lepi lizard) and other 5 xantusiid lizards, *Ameiva auberi* (teiid lizard) [60]; ca. 10 *Lacerta* lizards (C. Moritz, unpublished); ca. 10 *Anolis* lizards (C. Schneider, unpublished).

Amphibians

Complete sequences:
Xenopus laevis (African toad) [55].
Partial sequences:
5 *Ambystoma* sp. (axolotl) and *Plethodon yonahlossee* [61]; ca. 10 *Ensatina* salamanders (T. Jackman, unpublished); 23 toads (A. Graybial, unpublished); *Rana catesbeiana* (bullfrog) (Y. Yoneyama, unpublished, D00198).

Fishes

Complete sequences:
Gadhus morhua (atlantic cod) (C. Johansen, unpublished cf. [178]); *Acipenser transmontanus* (white sturgeon) [179]; *Cyprinus carpio* (carp) (F.L. Huang, unpublished, X61010); *Carcharhinus plumbeus* (shark1), *Carcharodon carcharias* (white shark), *Sphyrna tiburo* (bonnet-head shark) and other 9 sharks [180]; *Lythrurus roseipinnis* (T.R. Schmidt and J.R. Gold, unpublished, X66456); *Crossostoma lacustre* (Taiwan loach) [249]; *Thunnus thynnus* (mediterranean tuna), *Sarda sarda* (sard), *Sgomber sgombrus* (mackerel), *Boops boops* (bogue) and *Trachurus trachurus* (horse mackerel) (P. Cantatore and M. Roberti, unpublished).

II-B. The transmembrane helices

During the past 10–12 years, as the primary sequences of numerous mitochondrial cytochrome *b* have

TABLE I (continued)

Partial sequences:
Astronotus ocellatus (cichlid), *Tilapia mossambica* (tilapia), *Hemichromis bimaculatus* (cichlid), *Salmo trutta marmorata* (trout) this work; *Dicentrarchus labrax* (bass) (P. Cantatore, M. Crimi and T. Patarnello, unpublished, cf. [181]); 4 European trouts [182]; *Sparus auratus* (sea bream), *Mugil cefalus* (grey mullet) and other 4 mugilides (T. Patarnello, unpublished); *Lycodicthys dearborni, Austrolycichtys brachycefalum* (eel pouts) (L. Bargelloni and T. Patarnello, unpublished); *Lepidosiren paradoxa* (lungfish1), *Protopterus* sp. (lungfish2), *Latimeria chalumnae* (coelacanth) [31]; *Salmo trutta* (trout2), *Promoxis nigromaculatus* (crappie), *Gomphosis varius* (bird wrasse), *Ceophagus steindachneri* (cichlid), *Polipterus* (bichir), *Megalops atlanticus* (tarpon), *Atractosteus spatula* (alligator gar), *Lepisosteus oculatus* (spotted gar), *Amia calva* (bowfin), *Pantodon buchholzi* (butterflyfish), *Scaphirhynchus platorynchus* (sturgeon) [64]; *Julidochromis regani* and other 6 *Cichlasoma* sp. (cichlids) [29,90]; *Astatoreochromis alluaudi, Buccochromis atritaeniatus, Pseudotropheops tropheops* [30] and ca. 150 other cichlids, 4 cyprinodonts, ca. 60 poeciliids, 8 goodeids, 8 characins, 9 sticklebacks, *Gambusia* sp. (A. Meyer, unpublished cf. [88,89]), 32 scombroid fishes (B. Block, unpublished); ca. 10 pomacentrids (B. Birmingham, unpublished); *Anguilla rostrata* (eel) [90]; 8 salmonids [183], *Salmo salar* (salmon) and brown trout [184]; 4 *Thunnus* sp. [185], swordfish, sailfish, blue marlin [186]; 3 Baikalian sculpins [187]; 3 prickly sharks (G. Bernardi and D.A. Powers, unpublished, M91183–5).

Echinoderms

Complete sequences:
Strongylocentrotus purpuratus (sea urchin1) [45], *Paracentrotus lividus* (sea urchin2) [46,188]; *Arbacia lixula* (sea urchin3) (F. DeGiorgi, unpublished); *Pisaster ochraceus* (sea star) [189], *Asterina pectinifera* (starfish) [190].

Arthropods

Complete sequences:
Drosophila yakuba (fly1) [191]; *Drosophila melanogaster* (fly2) [248]; *Anopheles quadrimaculatus* (mosquito) [192]; *Artemia franciscana* (shrimp1) (R. Marco and R. Garesse, unpublished); *Daphnia* (shrimp2) [193], lobster (D. Stanton, unpublished), *Apis mellifera* (honeybee) [194]; *Tetraponera rufoniger* (ant) [254].
Partial sequences:
Euphasia superba (krill) this work; *Pandalus borealis* (Greenland shrimp) (H. Lund, unpublished); 2 *Artemia* shrimps (J.R. Valverde, unpublished, X67264); 9 ants, 4 bees, 3 wasps (R.H. Crozier, unpublished).

Helminths

Complete sequences:
Ascaris suum (round worm), *Caenorhabditis elegans* (free-living worm) [54].
Partial sequences:
Parascaris equorum (M. Degli Esposti, unpublished); *Fasciola hepatica* (liver fluke), *Melenogyne jovanica* (F. DeGiorgi, unpublished, cf. [195]).

Other invertebrates

Complete and partial sequences:
Mytilus edulis (blue mussel) [196]; *Metridium senile* (D.R. Wolstenholme, unpublished cf. [195]); ca. 10 clams (E. Boulding unpublished); *Lombricus terrestris* (earthworm) (M. Degli Esposti, unpublished); 2 Antarctic clams (T. Patarnello, unpublished).

Non metazoans [b]

Yeasts and fungi

Saccharomyces cerevisiae [37]; *Saccharomyces douglasii* [41]; *Schizosaccharomyces pombe [57]; Kluyveromyces lactis* [198]; *Candida glabrata* (G.D. Clark-Walker, unpublished); *Aspergillus nidulans* [199]; *Neurospora crassa* [200]; *Podospora anserina* [201]; *Strobilurus tenacellus*, 2 *Mycaema* sp. (G. Von Jagow, unpublished), *Pneumocystis carinii* (partial [202]); *Allomyces macrogynus, Spizellomyces puctuatus, Rhizopus stolonifer, Rhizophlyctis rosea* (B.F. Lang, unpublished[c]).

Protozoans

Trypanosoma brucei (flagellate) [48,49]; *Leishmania tarentolae* (flagellate) [49,203]; *Crithidia fasciculata* (flagellate) [49,204]; *Leishmania infantum* (flagellate) (partial, L. Gradoni and M. Degli Esposti, unpublished); *Plasmodium gallinaceum* (apicomplexan) [205]; *Plasmodium yoelii* (apicomplexan) [47]; *Plasmodium falciparum* (apicomplexan) [163]; *Theileria annulata* (apicomplexan) [160]; *Toxoplasma gondii* (apicomplexan, partial [162]); *Paramecium aurelia* (cyliate) [56]; *Tetrahymena pyriformis* (cyliate), *Acanthamoeba castellanii* (ameboid, M.W. Gray, unpublished[c]); *Phytophthora infestans* (B.F. Lang, unpublished[c]); *Physarum polycephalum* (slime mould) (D. Miller, unpublished cf. [50]).

Algae (mitochondria)

Chlamydomonas reinhardtii [124,206]; *Chlamydomonas smithii* [206]; *Chlamydomonas moewusii* [207]; *Chlorella* [208]; *Ochromonas danica, Prototheca wickerhamii, Porphyra* sp., *Plocamiocolax pulvinata, Gracilariopsis lemaneiformis* (G. Burger and B.F. Lang, unpublished[c]).

Plants (mitochondria)

Triticum aestivum (wheat) [51,209]; *Zea mays* (maize) [210]; *Oryza sativa* (rice) [211]; *Oenothera villaricae (berteriana)* (evening primrose) [52,53]; *Solanum tuberosus* (potato) [212]; *Vicia faba* (broad bean) [213]; *Helianthus annuus* (sunflower) (R. Gallerani, unpublished and R.H. Koehler, unpublished); *Arabidopsis thaliana* (A. Brennicke, unpublished [53]); *Marchantia polymorpha* (liverwort) [214].

TABLE I (continued)

Purple bacteria

Rhodospirillum rubrum [43]; *Rhodopseudomonas viridis* [2151; *Bradyrhizobium japonicum* [216]; *Rhodobacter capsulatus* [217]; *Rhodobacter capsulatus* strain Ga [96]; *Rhodobacter sphaeroides* [44]; *Paracoccus denitrificans* [218]; *Thiosphaera pantotropa* (T. DeBoer, unpublished).

Other bacteria

Chlorobium limicola (G. Hauska, unpublished); *Heliobacillus chlorus* (V. Vermaas, unpublished); *Bacillus* PS3 (b_6-like) [82]; *Sulfolobus acidocaldarius* (SoxC subunit binding heme *a* in a quinol oxidase) [70].

Cytochrome b_6 of the cytochrome b_6f complex

Plants and algae (chloroplasts)

Spinacia oleracea (spinach) [23,219]; *Nicotiana tabacum* (tobacco) [220]; *Zea mays* (maize) [221]; *Pisum sativum* (pea) [222]; *Triticum aestivum* (wheat) [223]; *Hordeum vulgaris* (barley) [224]; *Oryza sativa* (rice) [225,251]; *Marchantia polymorpha* (liverwort) [226]; *Chlorella protothecoides* (green alga) [227]; *Chlamydomonas reinhardtii* (green alga) [228].

Cyanophyta

Nostoc PCC7906 [229]; *Agmenellum quadruplicatum* [12].

Subunit IV of cytochrome b_6f complex

Plants and algae (chloroplasts)

Spinacia oleracea (spinach) [23,219]; *Nicotiana tabacum* (tobacco) [220]; *Zea mays* (maize) [221]; *Pisum sativum* (pea) [230]; *Triticum aestivum* (wheat) [223]; *Hordeum vulgaris* (barley) [224]; *Oryza sativa* (rice) [251]; *Cuscuta reflexa* [250]; *Marchantia polymorpha* (liverwort) [226]; *Chlorella protothecoides* (green alga) [227]; *Chlamydomonas reinhardtii* (green alga) [2281; *Scenedesmus obliquus* [231]; *Chlamydomonas eugametos* (green alga) [232].

Cyanophyta

Nostoc PCC7906 [228]; *Agmenellum quadruplicatum* [12]; *Synechocystis* sp. PCC6803 [233].

[a] Partial sequences of cytochrome *b* have been obtained herein after extraction of mitochondrial DNA (from either mitochondria or frozen tissues) and PCR amplification with the primers and the experimental conditions described previously [29–32,89,90]. Our PCR sequence of rabbit (*), which was obtained in collaboration with Prof. P. Cantatore (University of Bari), is identical to the sequence of the cloned gene obtained by F. Mignotte (personal communication). Sequences that are under way or unpublished are referred to the principal scientists who are working on them. In some cases of unpublished sequences the EMBL-Genbank accession number is reported. Our DNA sequences of donkey, reed bunting and krill are deposited in the EMBL bank. Note that for some species, e.g., axolotl [61], babbler [62], cod [178] and cichlids [30,88], sequences from several individuals are reported. The list is updated to february 1993 and includes a survey of the releases of Genbank and EMBL databanks that was performed by Dr. M. Attimonelli, University of Bari, Italy.

[b] All complete sequences except when otherwise stated.

[c] Species being sequenced within the Canadian Organelle Genome Sequencing Project (G. Burger and B.F. Lang, personal communication, cf. [247]).

been determined, a number of overlapping designations of the predicted structural elements of the protein have accumulated. This is particularly the case for the putative transmembrane helices, which were initially designated with roman numerals [22,23]. Later, either letters [8,18,24,25] or arabic numbers [9,19,26] have been used concomitantly with the roman numerals [10–12,21,27]. Herein, we shall conform to the nomenclature proposed by Crofts [24,28] in which the likely transmembrane helices are defined by capital letters and the extramembrane loops by the lower case letters of the helices connected by them.

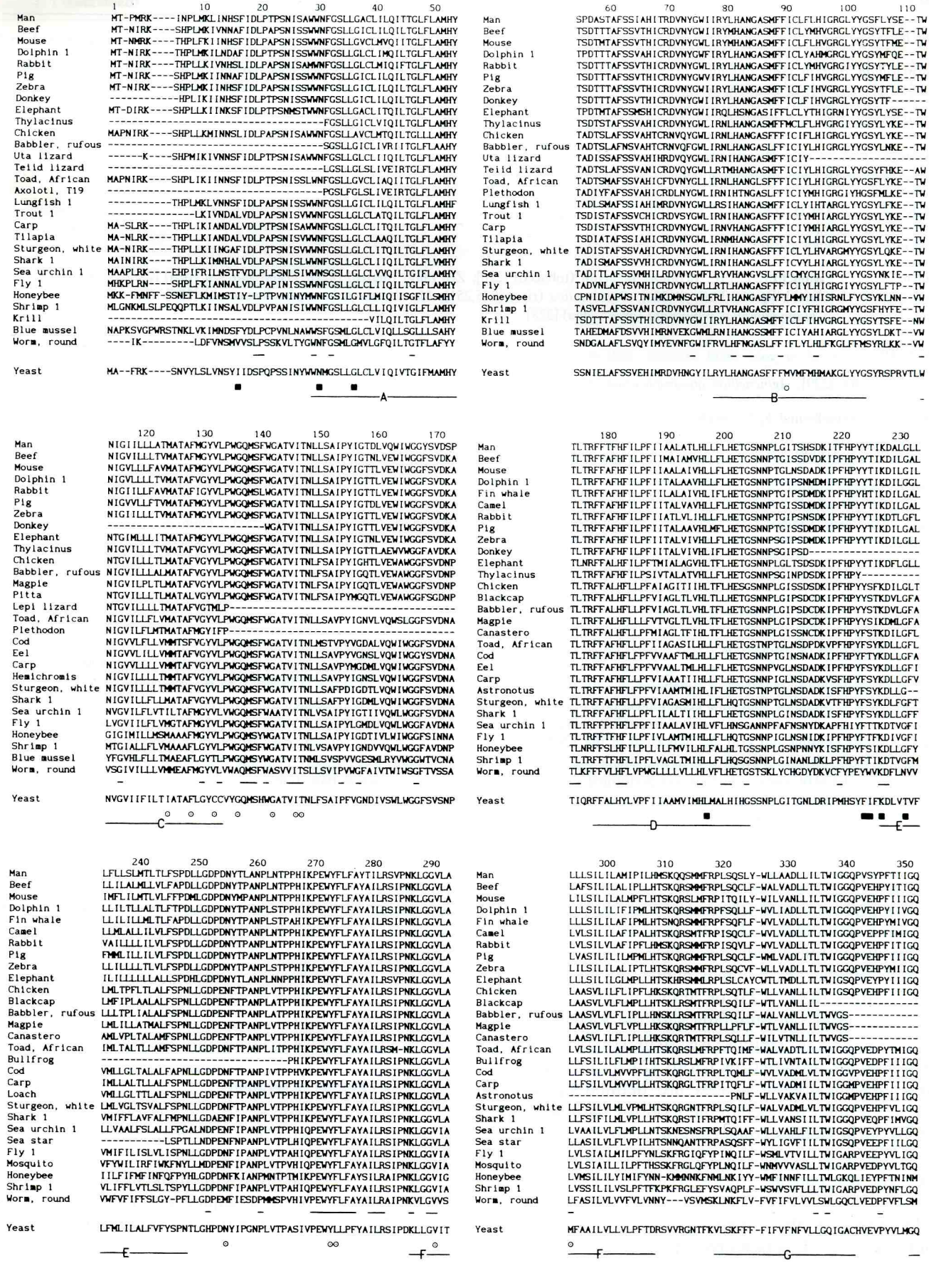
II-C. The two quinone reacting centers in cytochrome b

In addition to the two heme groups, the bc_1 complex contains two functionally-distinct sites at which ubiquinone interacts with cytochrome *b* in the reductase enzyme. The original designation of such sites as centers **i** (proton input) and **o** (proton output) proposed within the Q-cycle mechanism [13–15] is probably the most widely used, and we shall conform to it. Other common nomenclatures of the quinone centers are Q_i, Q_{in}, Q_c, Q_r or Q_n for center **i** and Q_o Q_{out}, Q_z or Q_p for center **o** [6–10,17–19,24–28].

III. Cytochrome *b* sequences

III-A. Source of sequences available

A series of scientific circumstances has rendered cytochrome *b* the most sequenced membrane protein today. The voluminous literature on the function of the bc_1 complex, the isolation of the complex from differ-


Man
Beef
Mouse
Dolphin 1
Rabbit
Pig
Zebra
Donkey
Elephant
Thylacinus
Chicken
Babbler, rufous
Uta lizard
Teiid lizard
Toad, African
Axolotl, T19
Lungfish 1
Trout 1
Carp
Tilapia
Sturgeon, white
Shark 1
Sea urchin 1
Fly 1
Honeybee
Shrimp 1
Krill
Blue mussel
Worm, round
Yeast
A
B
C
D
E
F
G

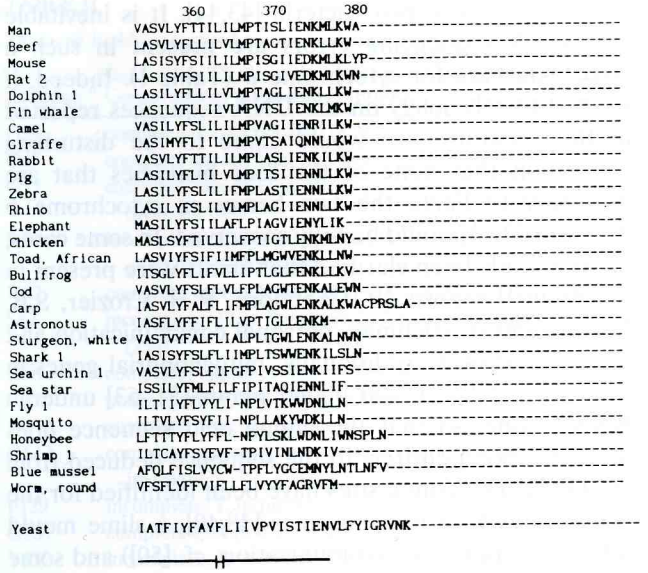


Fig. 1. Alignment of partial and complete sequences of mitochondrial cytochrome *b* proteins. Amino acid residues are aligned and numbered according to the protein from yeast (*S. cerevisiae* [37,41]). The sequences are shown in decreasing order of sequence conservation among the thirty species per alignment block and each block has a slightly different set of species (see Table I for the scientific name of the species and the references). The partial sequence of axolotl refers to individual 19 of *Ambystoma tigrinum* Ontario [61]. The uncertain N-terminus of the blue mussel protein [196] has been cut by ten residues. The 56 underlined residues are considered to be conserved in all other sequences of metazoan animals that have been analyzed thus far, including human variants [244,245]. Although the reported DNA sequences would show substitutions of some of these conserved residues, such substitutions consistently derived from single base changes and they were ignored on the basis of our criterion for removing plausible errors (see text and also [32,39,46]). This criterion was applied in the following cases in addition to those discussed in Table II. R79 → A in one salamander [61]; R99 → Q in one salamander [61] and R99 → P,W in two marsupials [252]; N115 → T in one Bajkal fish [187]; L122 → P in magpie [33]; S140 → A in one marsupial [252]; T145 → S in two birds [91] and T145 → I in one marsupial [252]; T148 → M in one marsupial [252]; G168 → S in one marsupial [252]; L193 → R in *Thylacinus* [252]; F275 → L in one shark [180]. The substitutions in Ref. [61,91] are indeed sequence errors (B. Hedges and A. Richman, personal communication). The lines at the bottom of the alignment define the predicted transmembrane helices (identified by capital letters, cf. [24]). The symbol ■ marks the positions where mutations induce resistance towards center **i** inhibitors (Table III) and the symbol ⊙ identifies the positions where mutations induce resistance towards center **o** inhibitors (Table IV). Only mutations changing a single residue are shown.

ent sources and the near ubiquity of the protein have stimulated studies to obtain sequence information on the protein. More recently, polymerase chain reaction (PCR) protocols that enable the sequencing of the cytochrome *b* gene from several individuals or species in studies of molecular evolution [29–33] have contributed to an enormous increase in the data base of this protein. With the aim of expanding the knowledge of the natural variation in cytochrome *b*, we present here new partial sequences obtained by these PCR procedures from the mitochondrial DNA of 10 animal species belonging to different phylogenetic groups (Table I). In order to compare these sequences with the others obtained by the same procedures and by conventional cloning, we have collected from the literature or through personal contacts sequence information of cytochrome *b* from about 900 species. Although the majority of such sequences are partial, over 140 000 amino acid residues have been determined for the cytochrome *b* protein in different species.

Nearly 200 complete sequences of cytochrome *b* are available and they have been taken almost equally from metazoans (multicellular animals) and all the other life forms (Table I). We present the alignment of the sequences in two parts: one containing partial and complete sequences – including ours – from metazoan species (Fig. 1), and the other containing only complete sequences of phylogenetically diverse species (Fig. 2).

III-B. Alignment of cytochrome b sequences

The crucial step in the analysis of any protein with many variants is to align the sequences in a way that maximizes the structural equivalence of homologous regions [6,11,34,35]. This seems to be relatively easy for the sequences of cytochrome *b* from animals, which generally show more than 50% identity [11,22,32], but it becomes much more difficult when other sequences are considered, particularly those from protozoans [6,11,27]. These sequences often have less than 25% identity with those of other taxa and present some unusual features that are difficult to interpret in the absence of a three-dimensional structure (cf. the globins [34]). To align the cytochrome *b* sequences, we have selected first the complete sequences which are less than 86% identical to each other [36]. This criterion was initially chosen to include the sequences from both *Leishmania* and *Trypanosoma* and to reduce the phylogenetically uneven representation of the species (Table I). The sequence of the yeast *S. cerevisiae* [37] protein

has been taken as a reference for consistency with previous studies [5,9,21,23,25–27,36].

Subsequently, the program CLUSTAL [38] has been applied to the selected sequences. This method is based upon the progressive clustering of related sequences and introduces many gaps to align the least homologous regions [38]. For cytochrome *b* sequences, the poorly homologous N and C-termini were thus aligned with a great number of gaps. However, application of the CLUSTAL procedure removed some mismatches that were present in previous manual alignments [6,11,12,26]. In particular, a rational alignment of the region 100–114 (yeast numbering) was obtained, with only two single gaps in metazoans vs. yeast (Fig. 1) and no gap in cytochrome b_6 at position 100 (Fig. 2).

The alignment has been refined further by employing the following approaches. First, a subset alignment of all the sequences from protozoans to those of yeast and plants has been carefully performed [39]. Secondly, consensus sequences [34] have been computed for the major groups of phylogenetically-related species (i.e., animals, yeasts/fungi, plants, protists, bacteria and chloroplast b_6) and they have been aligned to each other. Thirdly, the average hydropathy profile, the common sequence motifs, and the positions of the intron-exon junctions were concomitantly utilized as a guide for locating the gaps and insertions to yield minimal interruptions of the transmembrane helices [34,39,40]. Finally, all the available sequences were compared simultaneously to minimize insertions and deletions in the N- and C-terminal regions according to the principles outlined in Refs. 34, 35, 40.

Preliminary forms of the above alignment have been discussed previously [27,36,39]; the present alignment (Fig. 2) includes sequences that show less than 70% identity (except for the *bf* subunits) and belong to the most phylogenetically diverse species. For instance, the sequences of just one mammal and one nematode are included to represent animals. All the data in Figs. 1 and 2 have been carefully checked from the original and from the most recent references (quoted in Table I) in order to remove errors and to update the deduced amino acid sequences (e.g., position 69 in yeast is now known to be Met [41] and not Ile as in the first report [37]).

III-C. Discrepancies between DNA sequence and deduced protein sequence

The amino acid sequence of cytochrome *b* is, in most cases, deduced from the DNA sequence of its gene. The DNA sequence has been confirmed by direct sequencing of small peptides in beef [42], man (I.M. Fearnley and J.E. Walker (1987) Biochemistry 26, 8247–8251), potato (F.P. Braun and U.K. Schmitz, unpublished) and two bacteria [43,44]. It is inevitable that DNA sequencing errors are present in such a large data base for cytochrome *b* (Table I). Indeed, if we had to rely solely on the DNA sequences reported in the literature, we would come to the disturbing conclusion that none of the four histidines that are necessary to ligate the two hemes in cytochrome *b* [6–11,18,22,23] would be fully conserved. In some cases, it has already been clarified that errors were present in the original reports (P. Cantatore, R.H. Crozier, S.B. Hedges and A. Richman, personal communication and Refs. 32, 45–47). Additionally, mitochondrial genes in protozoans [48–50] and higher plants [51–53] undergo RNA-editing, so that the amino acid sequence does not correspond entirely to the sequence deduced from the DNA. The edited sites have been identified for the cytochrome *b* of trypanosomes [48,49], a slime mould (D. Miller, personal communication, cf. [50]) and some plants [51–53], and they consistently lead to more evolutionarily conserved amino acid sequences. The corrected sequences were not available in previous alignments, thereby leading to an incomplete evaluation of the conservation of certain residues such as the aromatic residue at position 94 (Fig. 2; cf. Refs. 6, 11, 21).

Having the above considerations in mind, we have adopted a parsimonious view of the possible variations in the primary sequence of cytochrome *b*. Whenever we encountered some very unusual substitutions in the aligned sequences, we analyzed whether such substitutions could be structurally 'implausible' (e.g., Refs. 34, 39). It would be implausible, for example, that only the nematode protein has the hydrophobic Phe at position 178 where all other species have the charged Arg or Lys (Figs. 1 and 2). However, the same type of substitution is seen at position 70 in African toad [55], position 79 in *Paramecium* [56] and position 288 in *S. pombe* [57], thereby indicating the occurrence of R(K) to F substitutions in regions of cytochrome *b* that are exposed at the positive side of the membrane.

Contrary to the above cases, the most frequently occurring odd substitutions of very conserved residues could be pinpointed to a single base change in the DNA codon. After consulting colleagues who reported sequences containing some of such substitutions, we learnt that they were errors in nearly all cases. The most efficient criterion for removing these random errors is the following. Any single base change leading to the substitution of an extremely conserved residue is ignored when this change is seen only in one of several related species. The applications of this criterion are listed in Table II and in the legend of Fig. 1 (see also below and [39]). In view of these corrections for plausible errors, the alignments in Figs. 1 and 2 represent our parsimonious picture of the cytochrome *b* sequences.

TABLE II

The most highly conserved amino acids in cytochrome b

Residue [a]	Conservation, notes and references
G33	complete, heme pocket [18,36]
G47	complete except E in one lizard [60], probably invariant [b]
G75	complete except D in *Paramecium* [56], probably invariant [39]
R79	incomplete: F in *Paramecium* [56] and H in some animals [252,254,255]
H82	complete except Q in flicker [33], probably invariant [b,c]
S87	incomplete: D in *Paramecium* [56] and T in some ants [254]
H96	complete, ligand of one *b* heme [65]
R99	probably complete as positively charged [b]
W114	complete, function unknown
G117	complete except A in one salamander [61], probably invariant [b]
F129	incomplete: L in pitta [33] and V in chloroplast b_6 [d]
G131	complete except E in giraffe [32], probably invariant [b,e]
Y132	incomplete: L in *Paramecium* [56] and T in one lizard [60]
S140	complete except G in chloroplast b_6
W142	incomplete: I in *Paramecium* [56] and F in *Bacillus* PS3 [82] [f]
T145	incomplete: V in nematodes [54] and K in chloroplast b_6
V146	incomplete: I in *Paramecium* [56] and chloroplast b_6
T175	complete except M in canastero [33], probably invariant [b]
H183	complete, ligand of one *b* heme
H197	complete except D in *P. lividus* [188], probably invariant [b,c]
H202	complete except R in chloroplast b_6
D229	complete except E in *Paramecium* [56], probably invariant [b,g]
I269	incomplete: V in *Paramecium* [56] and cod (C. Johansen, unpublished)
P271	complete, function unknown
E272	complete except H in *Paramecium* [56], probably invariant [b]
W273	complete except C in a deer [32], probably invariant [b]
L282	complete except F in one shark [180], probably invariant [b,h]
K288	incomplete: F in *S. pombe* [57] and H in *Paramecium* [56]
G291	complete except V in one alga [231], probably invariant [b]

[a] According to the alignment of Fig. 2 extended to all the available sequences (Table I) with the exception of SoxC of *Sulfolobus* – this protein binds heme *a* and belongs to a quinol oxidase [70] – and of the cytochrome *b* – like genes found in the nuclear DNA of some species (see Refs. 75, 162 and references therein). It should be noted that the residues G33 and S87 can be mutated to alanine without altering the activity and assembly of the reductase [18].

[b] After ignoring substitutions of extremely conserved residues that could be due to a single base change of the DNA (see text and Fig. 1 legend).

[c] This histidine is almost certainly conserved as the ligand of one *b* heme [8,65,71].

[d] Its mutation to L in myxothiazol resistant mutants [137] does not alter significantly the function of ubiquinol:cytochrome *c* reductase [36].

[e] Its mutation to S produces a failure of the protein assembly [151] and can be restored partially by secondary site mutations [76,152]. Recent sequencing of the giraffe gene confirms G131 (R.H. Crozier, personal communication).

[f] Its mutation to R induces respiratory deficiency in yeast, but function is restored by revertants having T or S at the same position [153].

[g] Its mutation to H or E induces antimycin resistance in *R. rubrum* and significant functional changes (A. Trebst, personal communication).

[h] Its mutation to F induces respiratory deficiency in yeast (D. Lemesle-Meunier, unpublished).

IV. Natural variation in the structure of cytochrome *b*

IV-A. Conserved residues

The comparison of the primary sequences of homologous proteins from distantly related species indicates the amino acids that are phylogenetically conserved. In principle, conservation arises from the requirement of specific amino acids for functional or structural properties of the protein [34,35,58], as illustrated for cytochrome *c* [58,59]. In eukaryotic cytochromes *c*, as in hemoglobins [34], the phylogenetically invariant residues generally form the heme binding pocket or lie in crucial positions within the three-dimensional structure of the protein [58,59]. A similar situation may be extrapolated to occur in cytochrome *b*, for which, at present, sequence comparison is the principal source of information to indicate important residues, since no atomic structure is available

In the first comparison of six sequences of mitochondrial cytochrome *b* 121 residues (31% of the total) appeared to be invariant [22]. This number decreased to 39 when an additional 12 mitochondrial and bacterial sequences were compared [11]. From our comparison of about 800 mitochondrial sequences (Figs. 1 and 2), we see only **9** invariant amino acids, and this number is not affected by the comparison with the bacterial sequences. An additional 10 residues may be invariant if we ignore unique substitutions that could be due to a single nucleotide error (see above and Ref. 39). Only two of the invariant residues are not conserved in chloroplast cytochrome b_6 (Table II).

Table II lists the invariant residues and those that appear to be conserved except for one or two species so far. From the comparison of the animal sequences of cytochrome *b*, several amino acid residues considered previously to be invariant [6,9,11,18,27,36] show substitutions in two unrelated species or arising from two base changes in the codon; hence, they can not be excluded by the consistent application of our criterion of error removal. These residues include: Q43 (A or E in reptiles and salamanders [60,61], R or K in some birds [33,62] – it is also M in *R. rubrum* [43]); the negatively charged residue at position 71 (N in birds [29,33,62,63] and also in *R. sphaeroides* [44]); F129 (L in one bird [33]); Y132 (T in one lizard [60] and L in

Paramecium [56]); T145 (V in nematodes [54]); the positively charged residue at position 178 (F in nematodes [54]); P187 (A in *Paramecium* [56], L in magpie [33]); T265 (N in elephant [32] and S in nematodes [54]); I269 (V in *Paramecium* [56] and cod – C. Johansen, unpublished); and P286 (missing in African toad [55]).

On the other hand, some reported changes are so unique or drastic, even if they do not occur at very conserved positions, that one may ask whether they are due to sequence errors. With the sequences presented here, we have demonstrated that two such cases are likely errors: S37 in one trout [64] – other trouts as well as all animals except chicken [63] have G37 – and L89 in *Julidochromis* [29] – other fishes have F and all animal sequences have an aromatic residue at this position (Fig 1).

The most important prediction advanced for the structure of cytochrome *b*, that the doublets of histidines in helices B and D are the ligands of the heme irons [22,23], was based on the evolutionary invariance of these residues and this conclusion still holds [65]. However, the assignment of the ligands to each heme had to be revised after the withdrawal of the former helix IV from the membrane [5,6,19,21,24–28,65]. Some of the previous speculations on the possible roles of other phylogenetically conserved residues are not fully sustained by the more extensive sequence alignments. For instance, the binding of the propionyl groups of the hemes has been proposed to involve the positively charged residues at position 79, 99, 178 and 202 [12,22,23]. Since R79 is not conserved in *Paramecium* [56] and some animals (Table II), nor R178 in nematodes [54], these four residues are not the only candidates for the heme propionyl interaction. Moreover, H202 may be too distant to bind a propionyl group of the b_H heme as deduced from protein modeling studies (data not shown).

In other hemeproteins the amino acids which bind the heme propionates are not strictly conserved [34,35,59]. Therefore, Y103 might be an alternative hydrogen bond donor to the propionates of the b_H heme, even if this residue is not conserved in some protozoans (Fig. 2). The involvement of specific residues in heme propionate binding could be tested by studying the pH dependence of the redox potential of the *b* hemes in species or mutants having substitutions of these residues. This pH dependence is influenced by the nature of the amino acids that are hydrogen bonded to the heme propionates of cytochromes (cf. Cai, M. and Timkovich, R. (1992) FEBS Lett. 311, 213–216).

From the spacing of four helical turns between the invariant histidines and spectroscopic information (see Refs. 21–23 and references therein), it is estimated that the edge to edge distance between the two hemes in cytochrome *b* may be around 1.2 nm [6–10,21–23]. This distance is sufficiently small to allow rates of electron transfer between the two hemes in the millisecond time range (i.e., the turnover of the enzyme, cf. R.A. Marcus and N. Sutin (1985) Biochim. Biophys. Acta 811, 265–322), but is also consistent with estimates of rates of electron transfer in the microsecond time range (cf. Refs. 7, 8, 14–16). If the latter is true, changes in the rate of electron transfer between the two hemes of roughly a factor of 1000, i.e., as long as this rate is not in the millisecond time range, would not be detected experimentally. We realize that, in the absence of a 3D structure for cytochrome *b*, comparison of many primary sequences does not add sufficiently strong arguments to discriminate between the two concepts regarding electron transfer in biological systems proposed in Ref. 66 and Ref. 67. Nevertheless, alignment of the primary sequences does indicate that the interheme distance is probably similar in all cytochrome *b* proteins, thereby constraining the electron transfer rates between these redox groups. More generally, the nature of the transmembrane amino acids appears to be relatively unimportant, an observation seemingly more compatible with Ref. 67.

On the other hand, the evolutionary invariance of a few ionizable residues in cytochrome *b* that lie near the lipid/water interphase of the membrane may reflect a crucial functional or structural role. In particular, the negatively charged residue that is conserved at each side of the membrane (D229 at the negative side and E272 at the positive side) could be involved in the protonation equilibria of ubiquinone at either center **i** or **o**. Preliminary results obtained by site-directed mutagenesis of these residues in *R. sphaeroides* appear to confirm their functional importance [8].

Several glycines are invariant or highly conserved in cytochrome *b* (Fig. 2 and Table II). By analogy with *c* cytochromes [58,59] and other hemeproteins [34,35], the evolutionary invariance of glycines in cytochrome *b* may be related to sites of severe steric constraint in the structure or to sites involved in the heme packing [18,36]. In particular, the four invariant glycines in cytochrome *b* that are separated by 13 amino acids each in helices A and C (Fig. 2) are remarkably symmetric to the two doublets of the ligand histidines, which are also separated by 13 residues [11,22,18,36]. This observation suggests that these glycines may contribute to the heme pocket [36]. Mutation of one of these glycines at position 33 destabilizes the protein and affects the *b* hemes [8,18], thereby supporting the predicted structural role. Furthermore, saturation mutagenesis of the highly conserved G143 residue has established that there are also severe steric requirements in the extramembrane regions of cytochrome *b* [68,69].

The symmetric motif of transmembrane glycines and histidines is characteristic of cytochrome *b* and is not

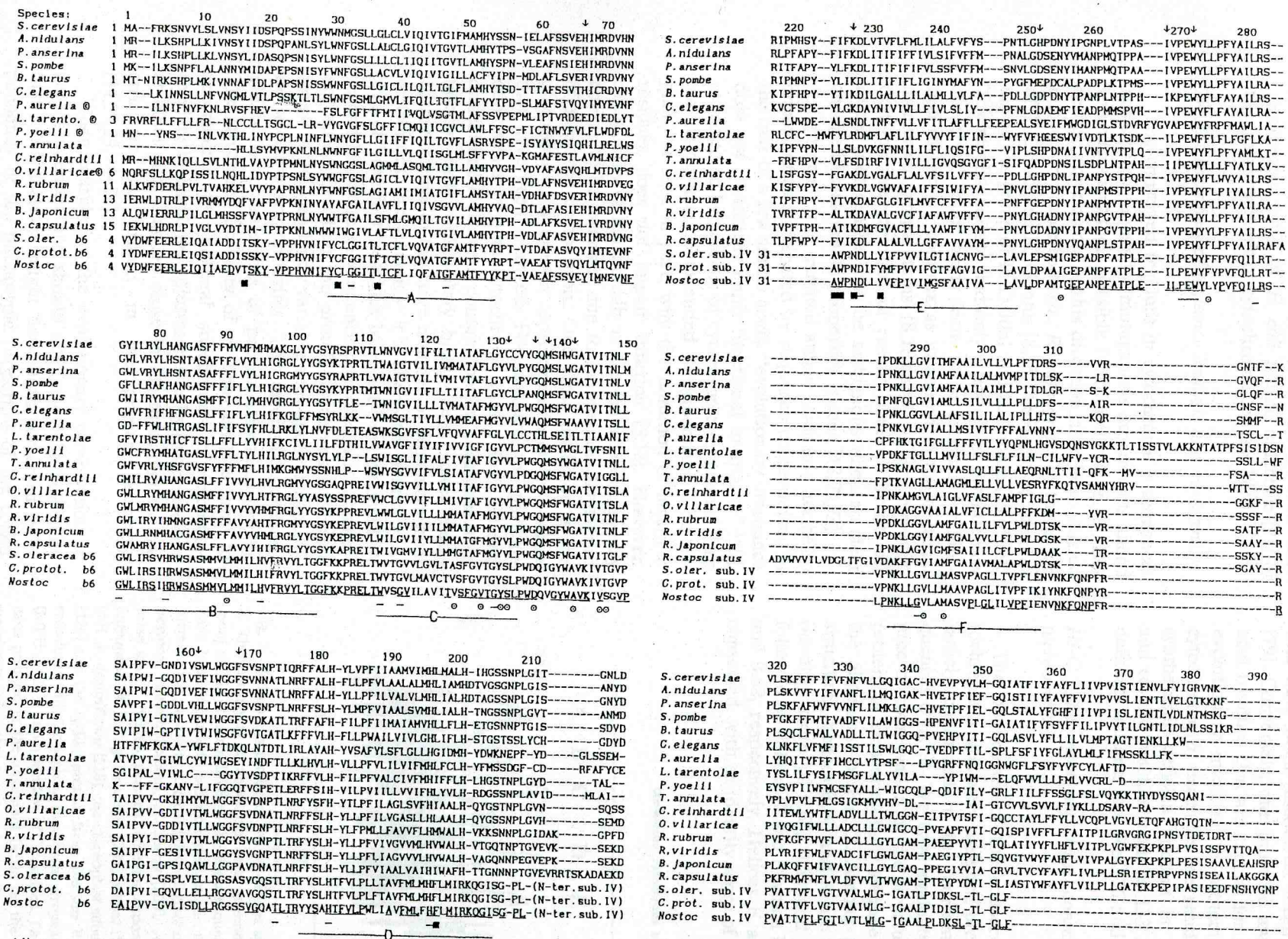


Fig. 2. Alignment of diverse sequences of cytochrome *b* (mitochondrial, bacterial and the chloroplast homologs) which represent the major lineages of living organisms (Table I and Ref. 39). The N-terminus of many sequences and the C-terminus of the bacterial sequences are partially cut off for a more compact presentation and normalization to yeast (see Refs. 11, 12 for alignments comprising N- and C-termini). The lines at the bottom of the alignment represent the predicted transmembrane helices and the symbols ■ and ⊙ locate inhibitors resistant mutations as in Figure 1. © identify sequences that have been corrected for RNA editing [49,51–53] or sequencing errors [47], and the sequence of *Paramecium*, in which unusual changes of otherwise invariant residues have been excluded on the basis of the parsimony criterion described previously [39] (see also Table II). The arrows on top of the alignment indicate the position of intron-exon junctions in *S. cerevisiae* [37,41] *A. nidulans* [199], *N. crassa* [200], *P. anserina* [201], *S. pombe* [57] and *C. smithii* [206]. The introns in liverwort [214] and in the chloroplast proteins [226,231] are not marked. The residues that are invariant or probably invariant (Table II) in all species are bold underlined at the bottom of the alignment. The light underlined residues below the sequence of *Nostoc* are conserved in all the b_6 and the subunit IV sequences available so far excluding the sequences of *Bacillus* PS3 [82] (see Table I for the list of species and their references).

seen, except for a subunit of *Sulfolobus* quinol oxidase [70], in other diheme membrane cytochromes (e.g., cytochrome *b*-558 of *E. coli* nitrate reductase [71] or the largest subunit of cytochrome oxidase [70]).

IV-B. Limitations of sequence analysis and mutagenesis

The conserved residues in cytochrome *b* sequences are obvious targets for mutagenesis experiments aimed to understand their possible functional role [8,18]. Recently, experiments in this direction have been carried out in two purple bacteria, *R. sphaeroides* by the group of T. Crofts and R. Gennis [8,18,44,65] and *R. capsulatus* by the group of F. Daldal and L. Dutton [19,68,69]. The results indicate that only a few of the evolutionary conserved amino acids seem to be essential for function of the bc_1 complex as measured in the bacterial membrane preparations [8,18,68,69]. Note, however, that the interpretation of results obtained by site-directed mutagenesis may be ambiguous when no clear change in measurable properties is seen [18,72,73].

Conversely, the mutated amino acids may not be so important as anticipated by sequence conservation. In the case of a few amino acids, "evolutionary invariance does not necessarily imply functional invariance" [59], as indicated by analysis of cytochrome *c* mutants. This conclusion may be extended to cytochrome *b* to explain, at least in part, why the mutation of invariant residues does not impair function [8,18]. However, catalytically or functionally non-essential residues may appear to be invariant due to the intrinsic limitations of sequence comparison. One clear limitation is the phylogenetically uneven representation of the species that have been analyzed. For instance, the phylogenetic series of both cytochrome *c* (see Ref. 59) and cytochrome *b* (Table I) contain too few sequences from taxa of early evolutionary history (e.g., lower metazoans) relative to the large number of sequences from vertebrates. Consequently, it is likely that natural variants of 'invariant' residues have not been detected. A second limitation of sequence comparison is its inherent assumption that the protein sequences are linear arrays of independently variable sites upon which natural selection acts uniformly. This assumption oversimplifies the complexities of protein structures [34,59]. The structural flexibility of proteins enables them to accomodate the unusual substitution of important residues by backbone adjustments or by compensations at other sites that are close in the three-dimensional structure but distant in the primary sequence [34,39,59,74–76].

When considering the evolutionary conservation of gene sequences, one should not ignore the possibility that DNA features might have been preserved independently of the phenotypic properties of the coded protein [77,78]. This may be the case for the non-functional but clearly homologous genes of cytochrome *b* that have been discovered recently in nuclear DNA [79]. Conversely, one must be aware also that amino acid residues that are crucial for function may not be evolutionary invariant in protein sequences [59]. In cytochrome *b*, examples are residue 143 for a photosynthetically-deficient mutant in *Rhodobacter* [19,68] and several residues such as 133 for yeast respiratory deficient mutants (see Ref. 76 and references therein).

III-C. The most and least conserved regions in cytochrome b

The boundaries of the conserved domains in cytochrome *b* that were assigned previously [6,27,33] are largely confirmed in the current alignment of sequences (Fig. 3). We have evaluated the different degree of conservation of the structural elements of the protein by measuring their average score of identity in the alignment of Fig. 2. Among the transmembrane structures, helices B, C and A are the most conserved (identity score of 0.63, 0.62 and 0.59, respectively), followed by helices D and F which have a score of 0.56 and 0.52, respectively. Helices E and H are the least conserved, with an identity score of about 0.4. Interest-

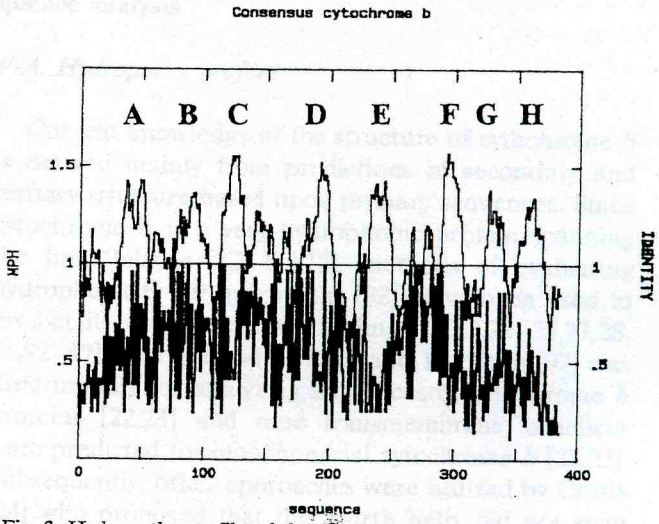


Fig. 3. Hydropathy profile of the consensus sequence of cytochrome *b*. This consensus sequence has been generated by using the alignment of Fig. 2 normalized to the yeast sequence (gaps or insertions have been excluded) and extended to the following sequences to increase the variation: *K. lactis* [198], *C. glabrata* (G.D. Clark-Walker, personal communication), *N. crassa* [200], *A. franciscana* (R. Garesse and F. Marco, personal communication), *T. brucei* [48,49], *S. purpuratus* [45], *P. gallinaceum* [205], wheat [51,209], *R. sphaeroides* [44], *P. denitrificans* [218], the chloroplast subunits of *M. polymorpha* [226] and the b_6-like protein from *Bacillus* PS3 [82]. The profile is calculated with the scale of membrane propensity for haemoproteins (MPH [94,95]) and a window of 7 residues. The fractional identity of each position in the alignment (scale on the right of the graph) is represented by the thick-marked histogram without smoothing (skyline plot [35]).

ingly, the profile of sequence identity does not vary significantly, either qualitatively or quantitatively, by permuting or increasing the compared sequences of cytochrome *b* except for the 100 residues towards the C-terminus. The variability of this part of the protein is much larger than previously calculated [6,11,27,28,36], because many more sequences can now be compared.

There is an uneven distribution of conservation in the regions of cytochrome *b* that are predicted to protrude at the two sides of the membrane, since only one third of the most conserved residues lie at the negative side (Table II and Refs. 6, 27). One reason for this may be the requirement of proper protein-protein contacts between cytochrome *b* and the 'Rieske' iron-sulphur subunit which plays a major role in ubiquinol oxidation at the positive side of the membrane [5,7,9,28,69,73,80,81].

IV-D. Cytochrome b and evolution

The occurrence of cytochrome *b* genes in nearly all eukaryotic organisms and in diverse prokaryotes indicates an early appearance during evolution [6,9,11]. It is found both in Gram-negative and Gram-positive eubacteria [9,82] and, though in a poorly conserved form, in one archaebacterium [70]. Thus, the ancestral gene must have existed before the separation of the major lineages of prokaryotes [83]. Speculations on the evolution of cytochrome *b* proteins have suggested fusions of different ancestral genes [6,11,71]. Since cytochrome *b* appears to have changed rather slowly during evolution [11], it is a useful molecule for deducing phylogenetic relationships among species [29–33]. Although a few regions of cytochrome *b* sequences tend to be more conserved [6], other regions exhibit considerable variability (Fig. 3) and thus are valuable for determining the phylogenetic distance among species [6,29,33].

The sequences of the bacteria *R. rubrum* and *B. japonicum* cytochrome *b* proteins show the highest amino acid identity and the minimal number of gaps or insert ions with respect to the proteins from algae and plants (Fig. 2). Cytochrome *b* of *R. rubrum* shows as much, or more, sequence identity to that of plant mitochondria (58.7% with liverwort) than to that of *R. capsulatus* (less than 57% identity), a purple bacterium belonging to a different phylogenetic group from *R. rubrum* [83]. This is consistent with other studies on the origin of mitochondria from purple bacteria (see [83,84] and references therein).

We note that among the gaps that are required for maximal matching of the sequences of cytochrome *b* (Fig. 2), that at yeast position 110–112 is shared by all metazoans and only one phylum of protozoans, the apicomplexa like *Plasmodium*. This difference might be related to the separation of the animal lineage from the vegetal/fungal lineage. If this hypothesis is valid, cytochrome *b* would retain in its sequence some features that are related to the early evolution of eukaryotes.

Some of the considerations just discussed led the group of Allan Wilson to use the gene of mitochondrial cytochrome *b* for establishing phylogenetic relationships among animal species [29–32], an approach which has been extended by many other investigators [31–33,60–62,64,85–91]. As for any other macromolecule used for such studies, cytochrome *b* offers both advantages and disadvantages (see Ref. 89 for a review). One disadvantage is the limited part of the cytochrome *b* sequence that is analyzed, since most studies have focused only on the region spanning helices A to C (Fig. 1 and Refs. 29–31, 64, 85–91). This particular region exhibits a high degree of conservation (Figs. 1 and 3) and thus offers a limited set of allowed changes that hampers resolution of close relationships among species [30,64,89]. The full potential of cytochrome *b* for the study of molecular phylogenetic relationships has not yet been exploited because its most variable regions, e.g., that spanning residues 210 to 250 (Fig. 3), have not been studied in detail except for groups of mammals [32], birds [33,91] and fish [90,180].

V. Structural deductions of cytochrome *b* from sequence analysis

V-A. Hydropathy profiles

Current knowledge of the structure of cytochrome *b* is derived mainly from predictions of secondary and tertiary structure based upon primary sequences. Since cytochrome *b* is a very hydrophobic protein spanning the lipid bilayer [1,3–6,9,12], methods of evaluating hydrophobicity (or hydropathy [92]) have been used to predict its transmembrane folding [10,12,22–25,27,28,71,92–97]. The method of Kyte and Doolittle [92] was used initially for analyzing sequences of cytochrome *b* proteins [22,23] and nine transmembrane α-helices were predicted for mitochondrial cytochrome *b* [22,23]. Subsequently, other approaches were utilized by Crofts [24] who proposed that the fourth helix did not span the membrane. This eight-helix model for cytochrome *b* is now widely accepted [5,6,8,9,12,19,73,76,94,98], primarily because it is consistent with the location of mutations producing resistance to center **i** and center **o** inhibitors [6,19,24–28,73]. Structural deductions by statistical methods of hydropathy [28,93–95], and experimental studies of membrane topology [8,12,21,36,65,98] sustain the same model.

The topology of the C-terminal part of cytochrome *b* comprising helices G and H is unclear. Since subunit IV of chloroplast *bf* complex, homologous to the C-terminal domain of cytochrome *b* [11,12,23], lacks helix

H (cf. Fig. 2), it has been suggested that mitochondrial cytochrome *b* may also be folded in seven transmembrane helices [12,99]. However, homologous subunits of redox complexes belonging to the same superfamily can have a different number of transmembrane helices as occurs for the largest subunits of quinol oxidase and of cytochrome *c* oxidase [70]. Moreover, extensive hydropathy analyses of the mitochondrial sequences (Fig. 4A, cf. Refs. 8, 27, 90, 94, 95) consistently indicate the transmembrane character of helix H. Helix G, rather than helix H, is the most weakly predicted of the eight putative transmembrane helices of cytochrome *b* (Fig. 3 and 4A, and data not shown). Nevertheless, the average hydropathy profile of cytochrome *b* sequences from the most diverse species shows that helices G and H have similar hydrophobicity, to each other and to other transmembrane helices (Fig. 4B). We continue to support, therefore, the eight-helix model for mitochondrial and bacterial cytochrome *b* proteins [24,26,19,28, 73,76,94,98].

No hydropathy method is satisfactorily accurate in predicting the termini of transmembrane α-helices [93–95,100,101]. Consequently, significant differences in the prediction of these termini in cytochrome *b* have been reported depending upon the sequences analyzed and upon the method employed [9,11,22,24,25,70,73, 76,90,93,94,96]. An improved procedure for predicting the termini of transmembrane helices is important for further deductions of cytochrome *b* structure [25,28,90] and we have therefore utilized several approaches to tackle this problem. These include: (i) comparison of the hydrophobicity profile of each sequence to the average hydropathy [40,94] of the most diverse species (Fig. 4B); (ii) location of the gaps in the alignment that maximize the homology with multiple sequences and overlap the regions containing intron-exon junctions, which generally occur in extrinsic loops [35,39,40,97]; (iii) similarity in the sequence motifs with the known transmembrane helices in the bacterial reaction centers [36,71,73,90,101–104]; and (iv) spectroscopic information on the membrane topology of the *b* hemes [7,36,105,106]. The termini of the transmembrane helices that resulted from the integration of the above approaches are shown in Figs. 1 and 2 and differ, considerably in some cases, from those suggested previously [6,11,18,21–27,70,73,76,94,96–99]. In particular, both helices B and D are extended three helical turns after the histidine ligands of the b_H heme, because this heme appears to be deeply embedded within the membrane dielectric [105,106] and a conserved GS/GG motif is seen at the C-terminus of both helices [36,90] (Figs. 1 and 2). Indeed, similar doublets of small residues, such as SS, GS and AT, are found at the termini of transmembrane helices in the photosynthetic reaction center [101–104].

V-B. Periodicity in the transmembrane helices

Given that the α-helix is the dominant conformation observed [21] and predicted in cytochrome *b* proteins [24–28], the periodicity profile approach of Eisenberg [107] may provide insights into the structure of cytochrome *b*. The profile of the helical periodicity of residue hydrophobicity can detect amphipathic α-helices [107,108]. Helical periodicity can be analyzed also by generating a profile of the amino acid variability (or mutability) moment of the residues in aligned sequences [28,102,103,108]. In the structure of the bacterial reaction center [101–104], the least conserved residues of the transmembrane helices face the lipids whereas the most conserved residues of the same helices face the interior of the protein. Hence, the maxima in the profile of the variability moment of membrane proteins correspond to helices exposed to the solvent, which can be either transmembrane helices largely surrounded by lipids or amphipatic helices [103,108]. The variability moment does not depend on a subjective choice of the hydrophobicity scale as does the hydrophobic moment [100,109], but only on the correctness of the alignment of the sequences [40,108].

Eisenberg and coworkers have applied the combined profile of the hydrophobic and variability moments [108] to the alignment of cytochrome *b* sequences reported by Hauska et al. [11]. This analysis indicated that helices A, C, F, G and loop cd (that was considered transmembrane) had strong variability moments [108]. Our alignment of Fig. 2 is substantially different from that of Hauska et al. [11] and includes a much wider set of phylogenetically different sequences (cf. Fig. 2 and Ref. 11). Hence, the periodicity analysis of the present alignment may provide further insights into the helical structures of cytochrome *b*. Helices A, B and, to a lesser extent, F and H show maxima in the profile of variability moment (data not shown) that are indicative of a lipid-exposed nature of one side of their transmembrane sector. The differences from previous analysis [28,108] derive from the more diverse set of sequences used here.

The profile of amphipathy of cytochrome *b* sequences shows features that are often coincident with those of the variability profile, especially for the sharp maxima in loops ab, cd and ef (Fig. 4B and Refs. 8, 28, 108). The regions around these maxima (at residues 63–65, 154–158 and 262–265, Fig. 4B) are predicted to have also a strong propensity for α-helix conformation (results not shown). Therefore, it is feasible that such regions of cytochrome *b* may form short amphipatic helices similar to those in the bacterial reaction center [101–104]. Indeed, the same computer analysis as that in Fig. 4B indicates that the extrinsic helices ab, cd, de and e in the L subunit of the reaction center are

associated with local maxima of the periodicity profiles (results not shown and Ref. 103).

We introduce here an alternative method of evaluating the amphipatic conservation of the residues along the transmembrane helices of cytochrome *b* (Fig. 5). In the helical-wheel representation we have inserted the

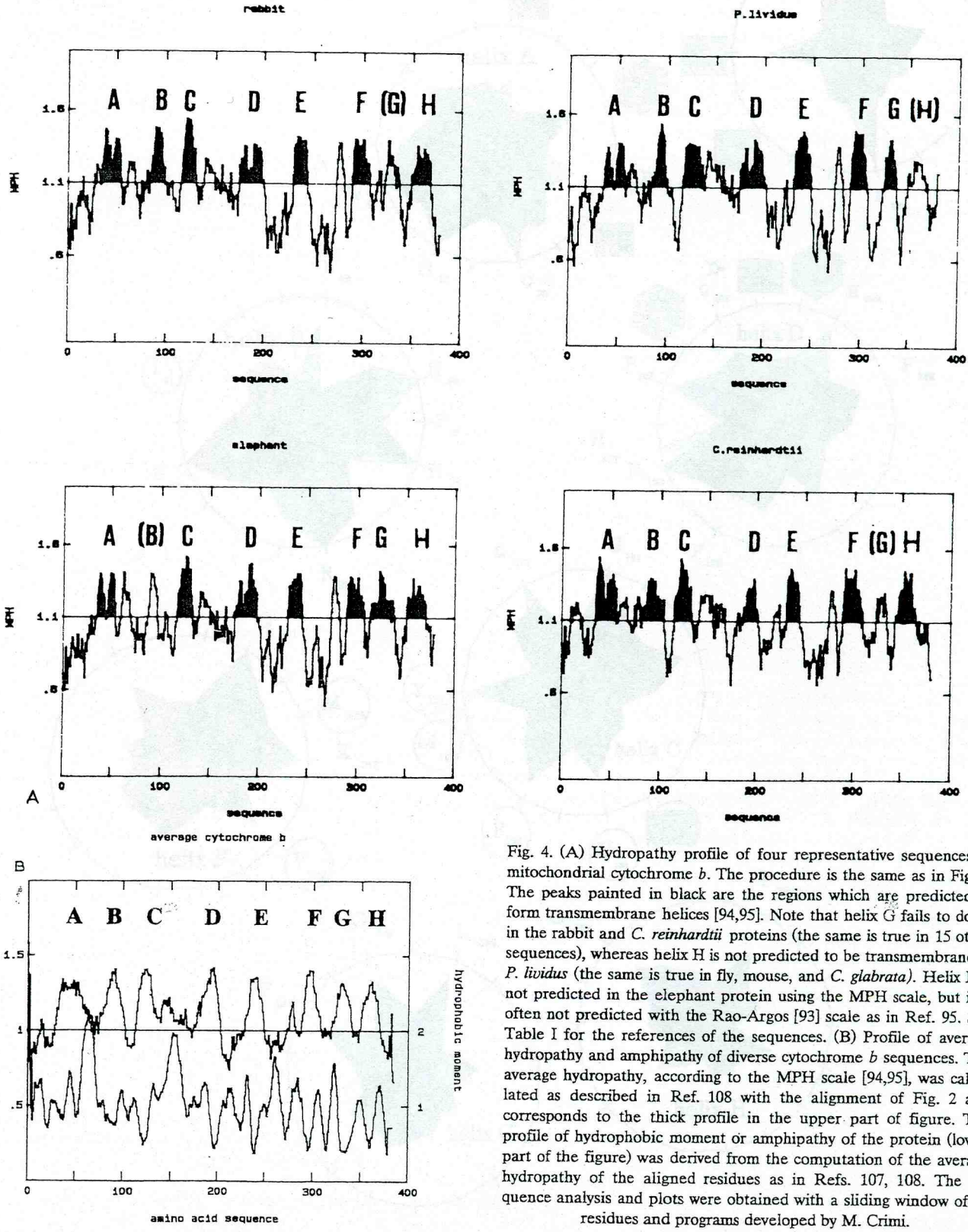
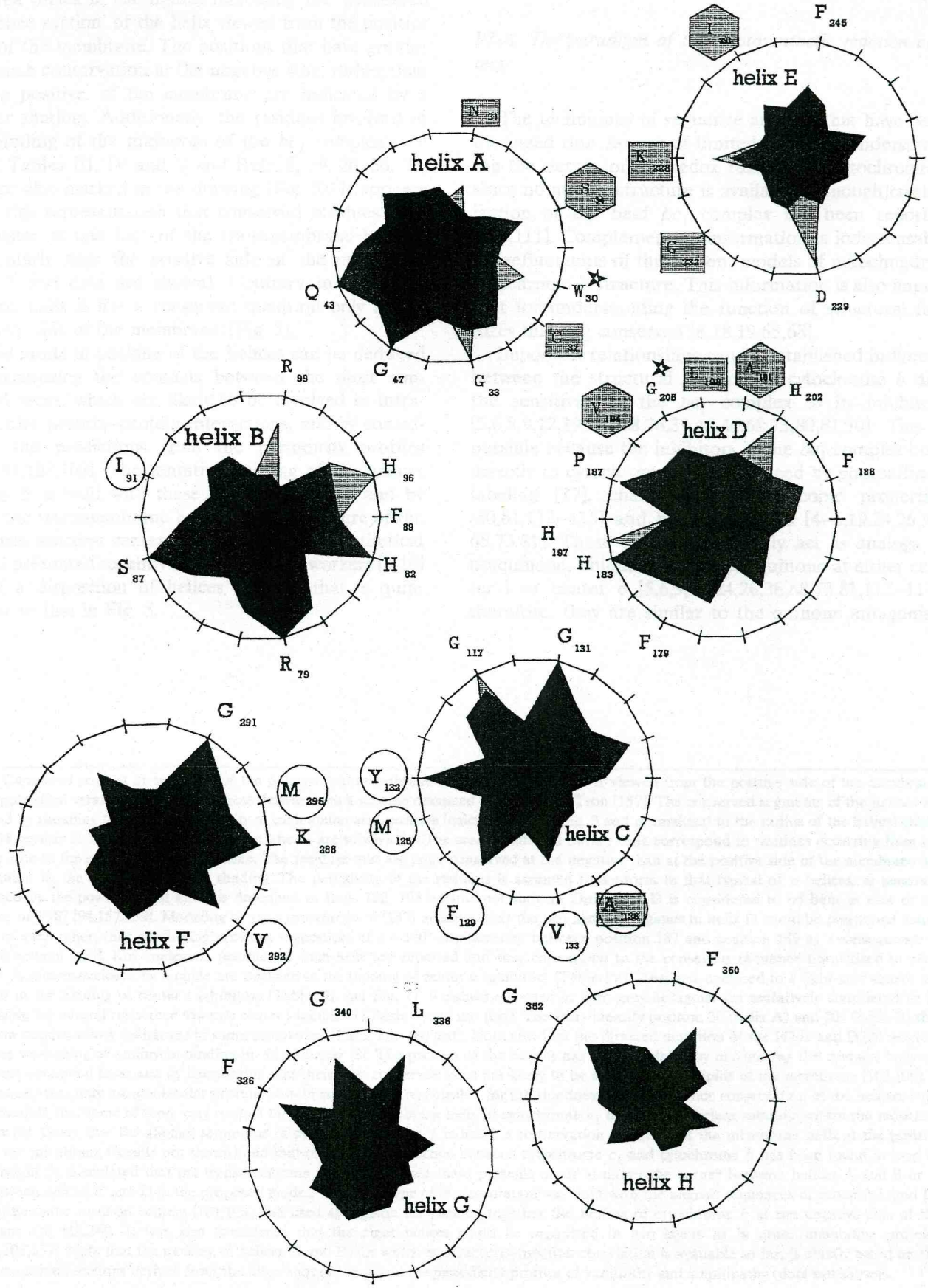


Fig. 4. (A) Hydropathy profile of four representative sequences of mitochondrial cytochrome *b*. The procedure is the same as in Fig. 3. The peaks painted in black are the regions which are predicted to form transmembrane helices [94,95]. Note that helix G fails to do so in the rabbit and *C. reinhardtii* proteins (the same is true in 15 other sequences), whereas helix H is not predicted to be transmembrane in *P. lividus* (the same is true in fly, mouse, and *C. glabrata)*. Helix B is not predicted in the elephant protein using the MPH scale, but it is often not predicted with the Rao-Argos [93] scale as in Ref. 95. See Table I for the references of the sequences. (B) Profile of average hydropathy and amphipathy of diverse cytochrome *b* sequences. The average hydropathy, according to the MPH scale [94,95], was calculated as described in Ref. 108 with the alignment of Fig. 2 and corresponds to the thick profile in the upper part of figure. The profile of hydrophobic moment or amphipathy of the protein (lower part of the figure) was derived from the computation of the average hydropathy of the aligned residues as in Refs. 107, 108. The sequence analysis and plots were obtained with a sliding window of 11 residues and programs developed by M. Crimi.


helix A
helix B
helix C
helix D
helix E
helix F
helix G
helix H
N 25
S 34
W 30
Q 43
G 47
G 33
R 99
I 91
H 96
F 89
S 87
H 82
R 79
I 241
F 245
K 228
G 232
D 229
G 205
L 198
A 181
H 202
V 184
P 187
F 186
H 197
H 183
F 179
G 117
G 131
Y 132
M 129
F 129
V 133
A 128
G 291
M 295
K 288
V 292
G 340
L 336
F 326
F 360
G 352

degree of conservation per each position, which defines an area (black in the figure) indicating the 'conserved sequence section' of the helix viewed from the positive side of the membrane. The positions that have greater sequence conservation at the negative side, rather than at the positive, of the membrane are indicated by a lighter shading. Additionally, the residues involved in the binding of the inhibitors of the bc_1 complex (see later, Tables III, IV and V and Refs. 8, 19, 26, 36, 73, 76) are also marked in the drawing (Fig. 5). It appears from this representation that conserved residues tend to cluster at one face of the transmembrane helices, particularly near the positive side of the membrane (Fig. 3 and data not shown). Contrary to the other helices, helix E has a conserved quadrant only at the negative side of the membrane (Fig. 5).

The mode of packing of the helices can be deduced by maximizing the contacts between the most conserved faces, which are likely to be involved in intramolecular protein–protein interactions, and by considering the predictions from the periodicity profiles [8,18,34,103,108]. The tentative packing of the helices in Fig. 5 is built with these features in mind and by using the transmembrane helices in the structure of the bacterial reaction center as a model. The hypothetical model presented recently by Crofts and coworkers [8,18] shows a disposition of helices A to F that is quite similar to that in Fig. 5.

VI. Structure versus function in cytochrome *b*

VI-A. The paradigm of the photosynthetic reaction centers

The techniques of sequence analysis that have been discussed thus far are of limited value for understanding the details of the redox function of cytochrome *b* since no atomic structure is available, although crystalization of the beef bc_1 complex has been reported [110,111]. Complementary information is indispensable for refinements of the present models of mitochondrial cytochrome *b* structure. This information is also important for understanding the function of structural features that are conserved [8,18,19,65,68].

Important relationships can be established indirectly between the structural features of cytochrome *b* and the sensitivity of the bc_1 complex to its inhibitors [5,6,8,9,12,19,24–28,36,39,44,46,68,73,80,81,90]. This is possible because the inhibitors of the bc_1 complex bind directly to cytochrome *b* as evidenced by photoaffinity labeling [17], changes in spectroscopic properties [80,81,112–115] and genetic analysis [4–6,19,24,26,36, 68,73,81]. These inhibitors basically act as analogs of ubiquinone, ubiquinol or ubisemiquinone at either center **i** or center **o** [5,6,9,19,24,26,36,68,73,81,112–114]; therefore, they are similar to the quinone antagonists

Fig. 5. Conserved regions or segments of the putative transmembrane helices of cytochrome *b* viewed from the positive side of the membrane. The hypothetical arrangement of the helices derives from a scheme discussed previously by Tron [157]. The conserved segments of the helices are obtained by summing the fractional identity at each amino acid position (calculated as in Fig. 3 and normalized to the radius of the helical circle) in the 18 sectors of 20 degrees into which the wheels are subdivided. The areas shaded in darker tone correspond to residues occurring from the positive side to the middle of the membrane. The residues that are more conserved at the negative than at the positive side of the membrane are represented by the areas with lighter shading. The periodicity of the residues is assumed to conform to that typical of α-helices, as generally confirmed by the power helical analysis described in Refs. 102, 103 (results not shown). Only helix D is considered to be bent in view of the presence of P187 [94,157,159]. Modeling of yeast cytochrome *b* [157] indicates that the two histidine ligands in helix D could be positioned nearly on top of each other, thus confirming previous suggestions of a $\simeq 140°$ displacement between position 187 and position 188 as a consequence of the proline-bent [159]. Key conserved residues of each helix are reported and they correspond to the consensus sequence normalized to yeast (Fig. 2). Residues enclosed by a circle are involved in the binding of center **o** inhibitors (Table IV). Residues enclosed in a light-grey square are involved in the binding of center **i** inhibitors (Table III and Fig. 1). Residues enclosed in light-grey hexagons are tentatively considered to be responsible for natural resistance towards center **i** inhibitors (Table V and see text). The stars identify position 30 (helix A) and 205 (helix D) that may show compensatory exchanges in some protozoans (Fig. 2 and see text). Note also that the directed mutation of the H202 and D229 residues produces weakening of antimycin binding in *Rhodobacter* [8]. The packing of the helices has been modeled by maximizing the contacts between their most conserved faces and by considering that their least conserved faces are likely to be exposed to the lipids of the membrane [103,108]. It was assumed that only intramolecular protein-protein contacts are responsible for the sidedness in the sequence conservation of the helices, even if it is possible that some of them may contact the single transmembrane helix of cytochrome c_1 or of other nuclear subunits within the reductase complex [9]. Given that the aligned sequences of cytochrome c_1 and *f* indicate a conservation quadrant of the membrane helix at the positive side of the membrane (results not shown) and that one site of interaction between cytochrome c_1 and cytochrome *b* has been found in loop cd [69], it might be speculated that the transmembrane contacts between these proteins occur at either the corner between helices A and B or at that between helices C and D in the proposed model. The same type of representation was built with the aligned sequences of subunits L and D_1 of photosynthetic reaction centers [101,103] and used as a guide for packing together the helices of cytochrome *b* at the negative side of the membrane (cf. [18,24]). It was also considered that the eight helices could be organized in two layers as in other membrane proteins [75,101,103,157]. Note that the packing of helices G and H, for which no structure–function correlation is available so far, is chiefly based on the conserved sections derived from the alignment of Fig. 1 and the periodicity profiles of variability and amphipathy (data not shown).

(herbicides) that bind to the Q_B site in photosynthetic reaction centers [8,19,24,71,73,101,116–121]. Several compounds are inhibitors of **both** the photosynthetic systems and cytochrome *c* reductase: hydroxy quinoline *N*-oxide (HQNO), diuron, 6-undecyl-5-hydroxy-2,3-dioxobenzothiazole (UHDBT), stigmatellin and myxothiazol [113,117,121–123]. Furthermore, reactions at center **i** of the bc_1 complex such as semiquinone stability are similar to those of the photosynthetic Q_B site [7,24,28,73,106,115,117,124,125]. Additionally, mutants resistant to inhibitors are available for both the photosynthetic systems (reviewed in Refs. 116–118, 120) and cytochrome *b* (see below and Refs. 6, 9, 19, 24, 26, 36, 73).

TABLE III

Sensitivity points towards center i inhibitors

Residue	Change	Species	Ref. [a]	Relative inhibitor titre (I_{50}) [b]				Notes [c]
				Antimycin	Funiculosin	HQNO	Diuron	
I17	→ F	*S. cerevisiae*	[26]	1	1	yes	yes	[129]
N31	→ K	*S. cerevisiae*	[129,234]	1	0.6	19	20	[133] d
N31	→ K	*K. lactis*	[130]	yes	–	67	32	[132] e
N31 + I44	→ K + → T	*K. lactis*	[130]	yes	–	–	1	[132] e
S34 + G37 + G232	→ F + → F + → N	*Paramecium* *	[56]	4.5	> 2000	22	≥ 1	[39] f
G37	→ V	*S. cerevisiae*	[26,235]	16 (4)	5 (4)	(11)	(0.3)	[129] g
G37	→ V	*M. musculus*	[134]	< 1000	< 100	2.6	–	h
A37	→ V	*S. pombe*	[131]	yes	–	–	yes	–
A37	→ G	*S. pombe*	[131]	yes	–	–	yes	–
G37 + A61	→ V + → V	*S. cerevisiae*	[26,235]	yes	–	–	–	[129]
A126	→ T	*S. cerevisiae*	[91,141]	(1)	(2)	–	–	f
L198	→ F	*S. cerevisiae*	[136]	(1.5)	8 (2)	(5)	(0.7)	e
S206 + N208	→ L + → Y,K	*S. cerevisiae*	[156]	1	5–7	12	1	f
F225	→ S	*S. cerevisiae*	[129,234]	1	3	24	20	[133] d
F225 + I226	→ L + → F	*S. cerevisiae*	[26,234]	1	0.6	9	17	[133] d
K228	→ M	*S. cerevisiae*	[26,236]	7	1	–	–	[235] g
K228	→ M	*K. lactis*	[130]	yes	–	–	1	[132] e
deletion230 + deletion231 + T232	→ S	*K. lactis*	[130]	yes	–	30	2	[196] e
G232	→ D	*M. musculus*	[128]	2–14	10	21	–	[135] f
G232	→ N	*A. suum* *	[54]	yes	–	5	–	[127] d
G232 + A194	→ T + → V	*S. cerevisiae* *		1	6	1	0.1	[90] f

[a] Original reference describing the isolation and/or sequencing of the mutants and their properties with regard to inhibitors resistance.

[b] The relative titre of the inhibitor is the ratio between the I_{50} in the mutant and that of the wild type or of sensitive species after normalization to equivalent contents of cytochrome *b* [36]. 'Yes' indicates that resistance has been observed without any quantitative data being reported, whereas the dash indicates that no information is available. When several mutants carrying the same genotipic mutations have been reported, data are shown only for one of them. Number in parenthesis are the titres obtained in the specific assay of mitochondrial ubiquinol:cytochrome *c* reductase measured as described in [36,39,46] (Tron, T., Ghelli, A., Coppèe, J.Y, Colson, A.M., Bruel, C., Lemesle-Meunier, D. and Degli Esposti, M., unpublished data). Note that different titres are often obtained for the same mutant depending upon the type of assay employed [132,135,237]. The list does not include the yeast respiratory deficient mutant M221 → L, which binds antimycin with low affinity [150], and mutants recently obtained in bacteria (A. Trebst, personal communication and Refs. 8, 69).

[c] Additional reference and type of assay employed for the data shown.

[d] Assay of NADH respiration of mitochondria [133].

[e] Assay of succinate respiration in mitochondria [130,136].

[f] Assay of the ubiquinol-2:cytochrome *c* reductase in isolated mitochondria [39,90]. Species considered to be naturally resistant to one or more inhibitor (see text and also [36,39,90]) are marked by an asterisk. These data and those in parenthesis are directly comparable with the levels of natural resistance obtained here (Table V).

[g] Assay of ethanol respiration in whole cells [235]. Clear discrepancies between the inhibitor titrations obtained in this type of assay and those at the mitochondrial level are commonly seen in yeast mutants (cf. [130,237]).

[h] Assay of succinate:cytochrome *c* reductase [134].

The similarities in quinone redox chemistry and protein topology of the resistance loci suggest that the structure of the quinone-binding sites of the bacterial reaction centers may be a valuable model for the quinone reacting sites in cytochrome *b* [8,24,28,36,71, 73,124,125]. In particular, by analogy with the resistance to herbicides in plants and photosynthetic bacteria [116–120], it is likely that the inhibitor resistance loci in cytochrome *b* contribute to the structure of the quinone binding sites [5,6,9,12,18,19,24–28,36,68,73, 81,115].

One difficulty in extrapolating the present information on herbicide resistant mutants to inhibitor resistance mutants in cytochrome *b* regards the differences between the Q_B center and center **o** [115]. Whereas center **i** is formed structurally from cytochrome *b* alone [9], as the L subunit forms the Q_B site [101,103,120], center **o** is formed by cytochrome *b* plus the 'Rieske' iron-sulphur protein [7–9,15,80,81,115,126]. The latter is necessary for the oxidation of ubiquinol [7–9,80,81,113] and the binding of the inhibitor stigmatellin [126]. Moreover, at center **o** there seem to be two sites for inhibitors [115,126] and possibly for ubiquinone/ubiquinol as well [81].

VI-B. Cytochrome b residues involved in binding of center i inhibitors

Functionally, the center **i** inhibitors block the reoxidation of cytochrome *b* and destabilize the bound ubisemiquinone [8,9,80,106,125]. Antimycin is the most powerful of these compounds (see Ref. 113 for a review), but it is by no means a universal inhibitor. This antibiotic, in fact, is not potent in the bc_1 complex of parasitic nematodes [127] and of the protozoan *Tetrahymena* [39], and quite ineffective in chloroplast *bf* complexes [3,121].

Table III lists all known mutations affecting the sensitivity towards center **i** inhibitors in mitochondrial cytochrome *b*. The mutated residues consistently lie within transmembrane helices A, D and E and lead to an increase in the volume of the exchanged residue (Fig. 2 and Table III, cf. Refs. 6, 8, 12, 90, 128–136). With the exception of the mouse mutant G232 → D [135], the mutations do not significantly alter the turnover of the reductase and, in general, produce a limited increase in the titre of the inhibitors (Table III and [26,128–136]). These properties of the mutations leading to resistance towards center **i** inhibitors are similar to those exhibited by the herbicide-resistant mutations that map within the transmembrane helices of the photosynthetic subunits [116–118]. Such similarities are useful for suggesting which amino acid residues confer natural resistance towards center **i** inhibitors [19,39,90]. Antimycin resistance in *Paramecium* (Table III cf. Ref. 39), for instance, is likely due to the dramatic increase in volume side chain by the substitution G37 → F of cytochrome *b* (Fig. 2 cf. Refs. 39, 56). The same substitution is seen in some b_6 sequences (Fig. 2). In addition, the chloroplast counterparts of cytochrome *b* show replacements of other residues which induce resistance to antimycin in yeasts and mouse (namely N31 → C, K228 → N and G232 → Y, Fig. 2). Cumulatively, these substitutions are likely to contribute to the low sensitivity to antimycin ($I_{50} \simeq 10^{-5}$ M [121]) of the *bf* complex [6,19,26,73,134], but it should be noted that they also occur in some protozoan cytochrome *b* proteins (Fig. 2), in which the inhibitor has a much higher affinity ($I_{50} < 10^{-9}$ M [39]). So, the replacement of H202, which is the only residue conserved in mitochondrial cytochrome *b* but not in its chloroplast counterparts at the negative side of the membrane (Fig. 2 and Table II), may be also involved in conferring the antimycin insensitivity of the *bf* complex.

VI-C. Cytochrome b residues involved in binding of center o inhibitors

A large variety of compounds act as center **o** inhibitors (see [113] for a review). Although they all block reduction of the 'Rieske' iron-sulphur protein and prevent cytochrome *b* reduction in the presence of antimycin [9,15,80,94,106,112–115], they can be subdivided into three types depending upon their effect on the metal groups at center **o** [113]. The methoxyacrylates, including myxothiazol, do not substantially alter the mid-point potential or the EPR line shape of the Rieske iron-sulphur cluster, but alter the electronic absorption spectra of the cytochrome *b* hemes [80,81, 113–115]. The hydroxyquinones, such as UHDBT, specifically alter the cluster and its redox equilibrium with cytochrome c_1 and ubiquinol [9,73,81,113,114]. The chromone inhibitors, including stigmatellin, alter both the EPR spectra and the midpoint potential of the iron-sulphur cluster, and the optical spectra of the cytochrome *b* hemes (Refs. 81, 113, 126 and references therein). The latter are universal center **o** inhibitors, since they are potent inhibitors of the the *bf* complex [12,121] as well as of the Q_B site in photosynthetic reaction centers [121,123].

A detailed characterization of mutants resistant towards center **o** inhibitors is available from studies of both mitochondrial [36,134,137,138] and bacterial systems [8,19,44,68,69,73,81] (Table IV). The results indicate that different positions within the cytochrome *b* protein are critical for the binding of myxothiazol and stigmatellin. Apparently, chloroplast *bf* complex is insensitive to myxothiazol but quite sensitive to stigmatellin [3,121]. This can be correlated with the fact that some mutations affecting myxothiazol sensitivity in cytochrome *b* resemble the natural substitutions in the

chloroplast sequences (e.g., F129 → V), whereas those affecting stigmatellin are not altered in the chloroplast sequences (e.g., T148, Fig. 2 and Refs. 6, 19, 73, 134, 137). Hence, multiple sites for the binding of inhibitors may coexist at center **o**, probably reflecting its complex quaternary structure [9,36,73,81,113,115,126,138].

TABLE IV

Sensitivity points towards center o inhibitors

Residue	Change	Species	Ref. [a]	Relative inhibitor titre (I_{50}) [b]				Notes [c]
				Myxothiazol	Mucidin	UHDBT	Stigma	
I91	→ P	*R. capsulatus*	[19]	8	yes	0.6	4	[73] d
M125	→ I	*R. capsulatus*	[19]	5	–	6.0	–	[73] d
F129	→ L	*S. cerevisiae*	[137,238]	930	11	(0.5)	1	[36] e
F129	→ L	*R. capsulatus*	[19]	530	1	11	1	[73] d
F129	→ L	*C. reinhardtii*	[139]	400	10	–	–	f
F129	→ S	*R. capsulatus*	[19]	530	yes	0.9	1	[73] d
F129	→ S	*R. sphaeroides*	[44]	28	–	–	–	d
Y132	→ C	*C. reinhardtii*	[239]	yes	yes	–	–	f
C133 A126	→ D → T	*S. cerevisiae*	[152]	(3)	–	(0.3)	–	[141] e
A126	→ T	*S. cerevisiae*	[141,152]	(2)	–	(1.1)	–	[141] e
G137	→ R	*S. cerevisiae*	[137,237]	4	4	–	1.5	[138] g
G137	→ E	*S. cerevisiae*	[149]	20	–	–	–	g
G137	→ V	*S. cerevisiae*	[149]	4	–	–	–	g
G137	→ S	*R. capsulatus*	[19]	37	yes	1.1	2	[73] d
G137	→ S	*C. reinhardtii*	[239]	yes	yes	–	–	f
W142	→ T,K	*S. cerevisiae*	[153]	5–10	yes	–	–	e
G143	→ A	*M. musculus*	[134]	2000	yes	–	1–4	i
G143	→ A	*P. lividus* *	[188]	1990	147	–	–	[46] e
G143	→ D	*R. capsulatus*	[19]	10000	–	–	–	[240] e
G143	→ S,A	*R. capsulatus*	[68]	yes	yes	–	–	[19] f
G137 G143 N256	→ T → T → F	*Paramecium* *	[39]	22000	yes	–	1	e
I147	→ F	*S. cerevisiae*	[137]	1	1	–	20	[36] e
I147	→ F	*Leishmania* *	[39]	1	–	–	40	e
T148	→ A	*R. capsulatus*	[19]	3	1	3.3	6	[73] d
T148	→ M	*M. musculus*	[134]	1	–	–	6	i
N256	→ Y	*S. cerevisiae*	[137,241,242]	6	11	–	2	[138] g
Y274 L275	→ N → F	*S. cerevisiae*	[137,237]	yes	yes	–	–	h
L275	→ S	*S. cerevisiae*	[137,238]	5.5	5	–	–	[242] h
L275	→ T	*S. cerevisiae*	[137,241]	4	yes	–	–	h
V292	→ A	*R. capsulatus*	[19]	5	1	2.4	7	[73] d
L295	→ F	*M. musculus*	[134]	4	1	–	5	i

[a] Original reference describing the isolation and/or sequencing of the mutants and their properties with regard to inhibitors resistance.

[b] The relative titre of the inhibitor corresponds to the ratio between the I_{50} in the mutant and that of the wild type or sensitive species after normalization to equivalent contents of cytochrome *b* [36]. 'Yes' indicates that resistance has been observed without any quantitative data being reported, whereas the dash indicates that no information is available. When several mutants carrying the same genotipic mutations have been reported, data are shown only for one of them. Numbers in parenthesis are the titres obtained in the specific assay of mitochondrial ubiquinol:cytochrome *c* reductase measured as described in [36,39,461 (Tron, T., Ghelli, A., Coppèe, J.Y, Colson, A.M., Bruel, C., Lemesle-Meunier, D. and Degli Esposti, M., unpublished data). Stigma. indicates both stigmatellin and its tridecyl analog (cf. [36]).

[c] Additional reference and type of assay employed for the data shown.

[d] Pre-steady-state assay with flash-induced reduction of cytochrome *b* in bacterial chromatophores [44,73,106].

[e] Assay of the ubiquinol-2:cytochrome *c* reductase in isolated mitochondria [36,39]. Species considered to be naturally resistant to one or more inhibitor (see text and also [36,39,90]) are marked by an asterisk. These data and those in parenthesis are directly comparable with the levels of natural resistance obtained here (Table V).

[f] Assay in vivo based on cell growth [19,139].

[g] Assay of succinate respiration of isolated mitochondria [138,149].

[h] Assay of NADH respiration in isolated mitochondria [237,238,242].

[i] Assay of succinate:cytochrome *c* reductase [134].

The positions affecting the sensitivity towards center **o** inhibitors are concentrated in two conserved domains of cytochrome *b* (Fig. 2). The first domain spans helix C and the adjacent part of loop cd and contains two-thirds of the resistance loci (Table IV and Refs. 6, 19, 36, 73, 76, 134, 137, 139). The beginning and the end of loop ef and the adjacent helix F form the second domain [6,19,73,134,137], where mutations show, in general, a level of resistance lower than those in the first domain (Table IV). Outside the above two domains there is the single bacterial mutant L91 → P, in which the altered amino acid lies in the middle of helix B [19,73]. Presumably because of this location, this mutant is affected in reactions occurring at the center **i** site [73]. Contrary to the mutations conferring resistance to center **i** inhibitors (Table III), those inducing

TABLE V

Relative titre of inhibitors of the bc_1 complex in different species

Species and preparation [a]	Cytochrome *b* residues [b]	Relative I_{50} [c]			
		Funiculosin [d]	HQNO [e]	UHDBT [f]	Myxothiazol [g]
Beef heart	M191 [e]	1	2.3	1	1.2
Rat liver	–	1.5	1	1	1.7
Pig liver	–	1.4	2.0	0.8	1
Rabbit heart	V194 [d]	60	2.0	1.3	1.1
Horse heart and Donkey heart	V194 [d]	80	1	1	1
Chicken heart	T194 [d]	10	1.1	1	0.9
Sturgeon liver	M126 [dfg], F231 [de]	21	2.8	2	3.6
Tilapia liver	M126 [dfg], T194 [d]	75	1.4	≥ 2	2.8
Drosophila	L191 [e], T194 [d]	8	2.1	–	1.2
Wheat germ [h]	G31 [e], V126 [dfg]	230	0.2	3.6	2.7
Paramecium	F34 [d], F37 [de], V126 [dfg] N232 [de], L132 [g], T137 [g], I142, T143 [fg], F256 [g]	> 3000	22.0	2000	22000
Crithidia or *Leishmania*	F34 [d], V126 [dfg], I191 [e] I194 [d], F231 [e], L232 [de], T137 [g], M138 [g], S256 [g]	6900	17.1	3.3	849
Rhodobacter	I34 [d]; V194 [d]	440	0.7	1.5	0.6
Rhodospirillum	M43 [fg]	–	–	0.1	8.0

[a] Mitochondria were prepared from heart, liver or whole organisms as described previously [36,39,46,90]. The concentration of the bc_1 complex was estimated by either the antimycin titre [90,143] or from the content of cytochrome *b* of the preparation [36,39]. The enzyme purified from *Rhodobacter capsulatus* Ga was kindly provided by N. Gabellini and the data for the purified enzyme from *Rhodospirillum rubrum* are taken from Ref. [243].

[b] Residues in the sequence of cytochrome *b* that might be responsible for the alteration in the titre of one or more of the inhibitors (specified by the letters). These residues are hypothesized to be involved in inhibitor binding by a combination of sequence analyses (Fig. 5 and Ref. 90) with inhibitor titrations carried out in several species whose cytochrome *b* shares one or more amino acid substitutions with the resistant species. In the case of animals, several other species were studied for such a scrutiny (man, fox, cat, sheep, turkey, toad, salamander and many fishes, results not shown).

[c] Relative titre of inhibition of the ubiquinol-2 (10–15 μM): cytochrome *c* (10 μM) reductase assayed with 1–3 nM of bc_1 complex as described previously [36,39,46,90]. Except for HQNO, which was routinely added to the assay cuvette, the inhibitors were incubated for ca. 2 min with the mitochondrial preparation dissolved in 0.25 M sucrose, 0.03 M Tris-Cl, pH 7.4 at 0.5–2 μM cytochrome *b* [39,90]. The titres, calculated as in Table III and IV after normalization to the content of the bc_1 complex, are the average of two or more separate titrations and are underlined when they are significantly different from the respective average titre. Although the specific titre of the inhibitors is about two-fold higher in mitochondria than in the isolated reductase, its relative ratio in different species remains constant (results not shown). Inhibitors concentration was measured as described in Ref. [113].

[d] Funiculosin, a generous gift from Sandoz, Basel, was dissolved in slightly basic ethanol and incubated over 2 min with the preparations [90,143]. The average titre of the most sensitive species, e.g., beef, was 3 mol per mol of bc_1 complex and has been taken as the reference ('1').

[e] HQNO from Sigma was added to the cuvette and had an average titre of 250 mol per mol of bc_1 complex in several animal species and yeast wild-type strains. Separate experiments were performed by incubating the inhibitor with the concentrated preparations or the isolated bc_1 complex to assess, in particular, the hypersensitivity of plants.

[f] UHDBT was purchased from B.L. Trumpower and exhibited an average titre usually around 20 mol per mol of bc_1 complex in mammals.

[g] The average titre of myxothiazol in mitochondria of most animals and several wild-type yeast strains was 1.1 mol per mol of bc_1 complex. This value is taken as the reference for calculating the relative titre.

[h] Similar results were obtained in other plant preparations like maize and pea hypocotiles (results not shown) and crude bc_1 complex from Jerusalem artichoke tubers [148].

resistance towards center o inhibitors often lie in extramembrane loops and lead to high levels of resistance towards the inhibitors [19,36,73].

VI-D. Natural resistance as a source of new structure–function relationships

Can we exploit the natural variation of cytochrome *b* to determine structure–function relationships? Some speculations on naturally occurring sites of altered sensitivity towards bc_1 inhibitors have already been made [6,19,26,39,46,90,129–137]. In order to substantiate speculations of this kind, a thorough investigation of the sequence to property relationship must be undertaken in several variants of a protein [72,140]. The numerous sequences of cytochrome *b* that are available and the possibility of measuring inhibitor binding from the titrations of the cytochrome *c* reductase activity in mitochondria [36,39,46,90,115,133–136,138] have allowed us to systematically carry out this investigation. Natural resistance has been found in many species (Table V), thus providing cases of altered properties that could be related to structural changes in the natural variants of cytochrome *b*.

The problem is to correlate changes in the titre of one inhibitor with one (or few) specific residue substitutions that naturally occur in the sequence of cytochrome *b*. This problem is complicated by the absence of a known three-dimensional structure of the protein and by the difficulty of finding a strict one-to-one relationship. Two considerations, however, mitigate the problems. (1) The detailed knowledge of the available resistant mutants can be used as a guide for locating the protein regions or deducing the type of amino acid replacement that may cause a given inhibitor response [9,19,26,39,73,90,129,134,137]. (2) The natural variants of a protein are stable and fully functional [75,140]. In contrast, mutated proteins generally have major functional derangements [59,72,75] (see [8,18,68,76,133,135,137,141] for cytochrome *b* mutants). Moreover, the 'element of surprise' [140] in the natural amino acid variation can provide multiple substitutions for assessing the role of specific residues.

Correlations between an unusual response to one bc_1 inhibitor and the sequence of the cytochrome *b* protein are most convincing when they combine sequence analysis in related species with the information derived from mutants. In the case of the natural resistance of fish to funiculosin [90], the results of a selected screening of funiculosin sensitivity in animal mitochondria suggested that the substitution of the conserved alanine 126 with the bulky methionine in the fish protein (Fig. 1) is probably responsible for a substantial increase in the titre of this inhibitor relative to normally sensitive species (Table V and Ref. 90). Natural resistance in the plant mitochondrial bc_1 complex [142] could also be correlated with the exchange of A126 with the bulkier V in the cytochrome *b* sequence (Fig. 2 and Ref. 90). The buried location of position 126 within the transmembrane sector of helix C (Fig. 1) may account for the 'hybrid' effects of funiculosin, which effects both center i and center o [90,114,143–146]. Note that the proteins having a bulky amino acid at position 126 also show resistance to UHDBT (Table V), which is a center o inhibitor that shares with funiculosin the property of effecting both quinone sites [113,114,143,144].

An interesting property of funiculosin is its remarkable species specificity, even among mammals [143,147]. The volume pattern and the comparison of the sequences of sensitive and resistant species suggested previously that position 194 may also be involved in funiculosin binding [90]. By inspecting the aligned sequences, we noticed that the rabbit protein shows the substitution of alanine 194 with a bulkier valine residue (Fig. 1). Hence, rabbit mitochondria were expected to be quite resistant to funiculosin, which would explain why rabbits are resistant to this drug in vivo [147]. This is indeed the case, since the inhibitory potency of funiculosin on the ubiquinol : cytochrome *c* reductase activity is about 60-fold lower for mitochondria isolated from rabbit than those from sensitive mammals (Table V). The cytochrome *b* proteins of zebra and donkey also have valine at position 194 (Fig. 1 and Ref. 32) and differ from that of pig, a species fully sensitive to funiculosin [90,147], in a dozen residues within the predicted transmembrane regions at the negative side of the membrane, where center i inhibitors bind [9,26,90,136]. With the exception of the replacement A194 → V, these residue changes are seen in other mammals whose mitochondria are as sensitive to funiculosin as those of pig but over 40-times more sensitive than those of donkey (or its close relative horse, results not shown and Table V). We propose, therefore, that position 194 and/or its surrounding region is involved in the binding of funiculosin (see Ref. 90 for further discussion of this proposal).

The screening of the responses to HQNO revealed several cases of significant alterations of its sensitivity (Table V), the most striking of which is represented by the hypersensitivity of the reductase in plant mitochondria [148]. The sequence of plant cytochrome *b* shows the unusual change N31 → G (Fig. 2) that we consider responsible for HQNO hypersensitivity for two reasons. First, when N31 is mutated to a bulkier residue such as K [26,129,132], resistance to HQNO is observed [130,133]. Since the increase in volume of the residue is the major theme in resistance mutations of center i inhibitors (Table III), one would expect that the considerable decrease of the volume by exchanging

an asparagine for a glycine would facilitate the binding of the quinone antagonist. Secondly, the almost opposite mutation of glycine232 to aspartate induces HQNO resistance [128].

Although the cytochrome *b* protein of trypanosomes also shows the substitution N31 → G, mitochondria from these protozoans are highly resistant to HQNO (Table V). This does not necessarily contradict the above correlation because several unusual substitutions in the transmembrane helices A, D and E of trypanosomal cytochrome *b* lead to an increase in the protein volume at the negative side of the membrane (Figs. 2 and 5). By combining different sequence analyses, we hypothesize that the substitutions A191 → I, L231 → F and G232 → L in the cytochrome *b* of *Leishmania* may contribute to HQNO resistance in trypanosome mitochondria (Table V).

Positions 30, 31, 34 (helix A), 103 (helix B), 191, 194, 205 (helix D), 228, 231 and 232 (helix E) are concomitantly altered in trypanosomes and *Paramecium* cytochrome *b* with respect to most other species (Fig. 2). This observation suggests that such residues are mutually related in the protein structure, in agreement with the helical packing shown in Fig. 5. Moreover, the cumulative substitution of these residues probably alters the normal properties at center i and thus explains the strong natural resistance to both HQNO and funiculosin in the mitochondria of these protozoans (Table V). Interestingly, some of these positions are substituted in a few species that also show natural resistance to either HQNO or funiculosin: (1) S34 → I occurs in *R. capsulatus* (Fig. 2), which is more resistant to funiculosin than equides even though they all have the substitution A194 → V (Table V). The substitution S34 → F is seen in trypanosomes and *Paramecium* (Fig. 2) which have the strongest resistance towards funiculosin (Table V). Hence, position 34 may also be critical for funiculosin binding. (2) Position 191 is usually A, and changes to the bulky M in the beef and L/I in the trypanosomal proteins (Figs. 1 and 2). Since the cytochrome *c* reductase of beef appears to be partially resistant to HQNO as compared to that of most animals (Table V), position 191 may be another residue influencing sensitivity to this inhibitor. (3) Position 231 is specifically changed to the bulkier F in the sturgeon protein (Fig. 1) as in trypanosomes (Fig. 2). The cytochrome *c* reductase of sturgeon mitochondria shows a significantly higher titre of HQNO than that in mitochondria of other fish and most animals (Table V), thereby suggesting that position 231 is also involved in the binding of this inhibitor (this is further supported by the triple mutant of *K. lactis* at position 230–232 that is antimycin and HQNO resistant [130,132]).

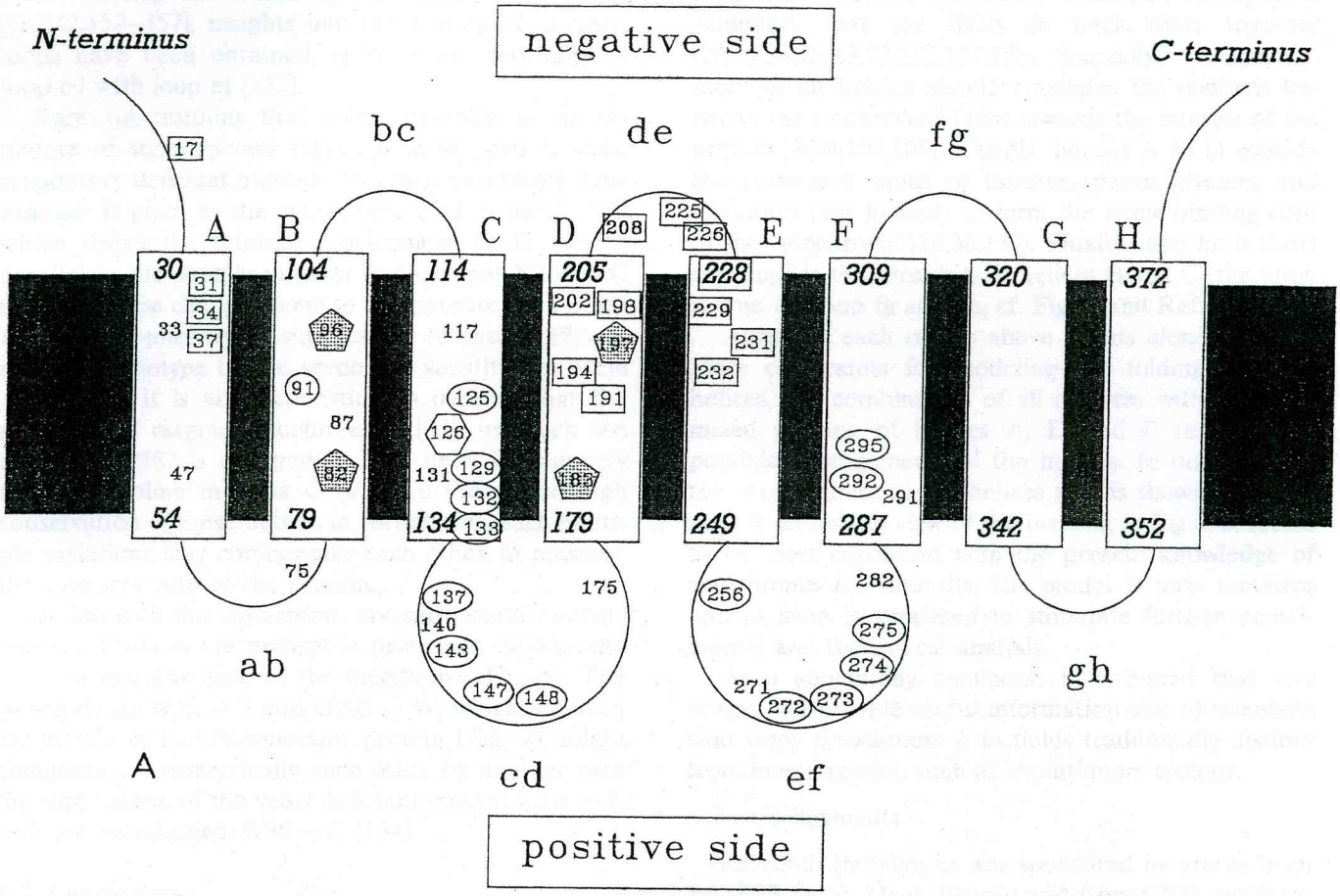
The possible location of the sensitivity positions towards inhibitors binding to cytochrome *b* is illustrated in Fig. 6A.

VI-E. Other information relevant for structure versus function

Table IV also lists data on functionally deficient mutants of cytochrome *b*. The photosynthetically deficient mutant G143 → D of *Rhodobacter* (see Ref. 19 and references therein) occurs at a position that is very critical for the binding of myxothiazol [19,36,46,68,69, 134]. Three yeast respiratory deficient mutants which map around position 143 show only a partial decrease of the ubiquinol:cytochrome *c* reductase activity in vitro and also display a slight resistance to myxothiazol (Table IV and [149–152]). Interestingly, the mutation C133 → Y [151] produces a loss in the specificity for the quinol ring (T. Tron, A. Ghelli, J.Y. Coppée, A.M. Colson, C. Bruel, D. Lemesle and M. Degli Esposti, unpublished data), since mitochondria of this mutant are more active with plastoquinol than with ubiquinol analogs. These results, previous deductions based on resistance towards methoxy-acrylate inhibitors [19,36, 73,134,137], and comparisons with the sequences of cytochrome b_6 suggest that the region comprising the end of helix C and the beginning of loop cd may play a specific role in the binding of the methoxy groups which distinguish ubiquinol from plastoquinol. This region also contains the only residue that is conserved in cytochrome *b*, but not b_6, at the positive side of the membrane, namely S140 (Fig. 2). Moreover, the cytochrome *b* of *Ascaris*, an organism possessing the rare ubiquinone analog, rhodoquinone, in which one methoxy of the ring is substituted by an amino group [127], shows the substitution of T145, conserved except in b_6 (Table II).

Although it is difficult to extrapolate common functions from sequence similarities with the reaction center subunits (see above section V-I), it is worth noting that there is a statistically relevant homology between the conserved peptide W142GATV(I) in loop cd of cytochrome *b* and the conserved peptide H215GATV(I) in the chloroplast D_2 subunit and its homologous M subunit of the bacterial reaction center (cf. Fig. 2 and Refs. 101, 102). In the crystal structure of the reaction center, either the histidine (H217 in *R. viridis* [101]) or the threonine (T222 in *Rhodobacter* [102,104]) are hydrogen-bonded to one carbonyl of the Q_A molecule. It is tempting to speculate that either W142 or T145 in cytochrome *b* might be hydrogen-bond donors to ubiquinone and methoxy-acrylate inhibitors. Recent results obtained in revertants of the yeast respiratory deficient mutant W142 → R suggest that this residue is involved in the binding of myxothiazol [153] (see also Table IV).

Important information has been recently obtained by the screening of secondary-site revertants of yeast respiratory deficient cytochrome *b* mutants [76,141, 152–157]. Some substitutions in these revertants occur



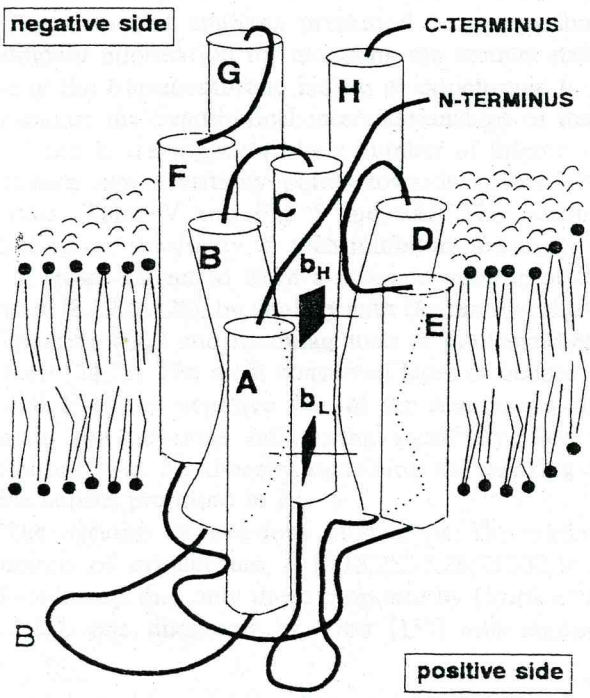


Fig. 6 (A) Overall view of the positions (yeast numbering, cf. Fig. 2) of key residues in the eight-helices model of cytochrome *b*. Positions predicted to start or end each transmembrane helix are shown in bold italic. The four heme ligands are identified by the shaded pentagons. Positions corresponding to amino acids that are conserved in all sequences, excluding the chloroplast subunits of the *bf* complex (Table II), are in bold roman type. Positions affecting the sensitivity towards center **i** inhibitors, including the new positions inferred here on the basis of natural resistance (see text and Table V), are surrounded by squares. Positions affecting the sensitivity towards center **o** inhibitors are surrounded by circles. Position 126, which may be involved in the binding of funiculosin [90] and UHDBT (Table V), is surrounded by a hexagon. The dark-shaded area corresponds to the lipid membrane. (B) Tentative representation of the overall folding of cytochrome *b*. The arrangement of the helices corresponds to that shown in Fig. 5, but it is viewed from the negative side of the membrane and consequently it appears upside down. The filled squares represent the two heme groups. The extrinsic loops cd and ef at the positive side of the membrane cross each other and have an overall Ω structure as suggested by several suppressor mutants in yeast [76,141,152]. The same is true for loop de at the negative side of the membrane [26,152,154,157].

far away in the primary sequence from the mutation leading to loss of function, thereby indicating contacts between different segments of cytochrome *b* in the tertiary structure [152,154]. Though the distribution of the suppressor mutations generally overlaps the regions altering the sensitivity to center **o** inhibitors [76,142,152–157], insights into the folding of extrinsic loops have been obtained, such as the proximity of loop cd with loop ef [152].

Rare substitutions that occur naturally in the sequence of some species resemble those seen in yeast respiratory deficient mutants plus their revertants. One example is given by the cytochrome *b* of *S. pombe* [57] which shows the unusual replacement G137 → N in parallel to the equally unusual replacement N256 → C (Fig. 2). These changes seem to compensate each other as with the phenotypic suppression of the G137 → E mutant phenotype by the secondary substitution N256 → K [152]. It is also interesting to observe that the sequence of magpie cytochrome *b* [33], in which the conserved P187 is changed to L in helix D, uniquely shows a proline in helix C (Fig. 1). Given the high conservation of these helices in vertebrates, such multiple variations may compensate each other to preserve the core structure of the protein.

In line with this discussion, several naturally occurring substitutions are present in protozoan cytochrome *b* at the negative side of the membrane (Fig. 5). The substitutions W30 → S and G205 → W, which are seen exclusively in the *Paramecium* protein (Fig. 2), might compensate volumetrically each other by analogy with the suppression of the yeast deficient mutant S206 → L with the substitution W30 → C [154].

VII. Conclusions

The data and analyses presented here contribute additional information for modeling the tertiary structure of the transmembrane helices of cytochrome *b*. In particular, the hypothetical interrelationships of helix A, D and E are supported by a number of inferences, including new sensitivity points towards center **i** inhibitors (Table V and Fig. 5 and 6A). The residues affecting the sensitivity to such inhibitors are likely to be in close contact to form a common volume of the protein [8,19,24–28], by analogy with the binding site of ubiquinone (Q_B) and its antagonists in photosynthetic systems [24,73]. The most conserved faces of helices A, D and E at the negative side of the membrane also contain the positions influencing sensitivity towards inhibitors (Fig. 5), thereby sustaining the packing of these helices proposed in Fig. 5.

The scrutiny of previous models for the tertiary structure of cytochrome *b* [8,18,25,27,28,73,152,157–159] indicates that only those proposed by Crofts et al. [8,18,28], one discussed by Tron [157] and another advanced by Degli Esposti et al. [158] are consistent with the most likely arrangement of helices A, D and E (cf. Fig. 5). Other points should be also considered for deducing the possible associations of the transmembrane helices of cytochrome *b*. First, helices B, C and F contain residues influencing sensitivity to center **o** inhibitors that are likely to pack close together [18,19,24,25,28,73,152,157,158]. Secondly, the arrangement of all helices should maximize the contacts between their conserved faces towards the interior of the protein [8,28,103,108]. Thirdly, helices A to D contain the conserved motif of thirteen-spaced glycines and histidines that is likely to form the heme-binding core of the cytochrome [18,36,157]. Finally, loop bc is short and implies the proximity of helices B and C (the same is true for loop fg and gh, cf. Fig. 2 and Ref. 25).

Although each of the above points alone provides loose constraints for modeling the folding of eight helices, the combination of all of them with the optimized packing of helices A, D and E restricts the possible arrangements of the helices. In our opinion, the arrangement of the helices that is shown in Fig. 5 and, in an overall view of the protein, in Fig. 6B, seems to be most consistent with the present knowledge of cytochrome *b*. Naturally, the model is only tentative and as such is proposed to stimulate further experimental and theoretical analysis.

As a concluding comment, it is hoped that this review will provide useful information also to scientists who study cytochrome *b* in fields traditionally distinct from bioenergetics, such as evolutionary biology.

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