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Sequence homology and structural similarity between cytochrome *b* of mitochondrial complex III and the chloroplast b_6–*f* complex: Position of the cytochrome *b* hemes in the membrane

(electron transport/energy transduction/hydropathy)

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ABSTRACT **The amino acid sequences of cytochrome *b* of complex III from five different mitochondrial sources (human, bovine, mouse, yeast, and *Aspergillus nidulans*) and the chloroplast cytochrome b_6 from spinach show a high degree of homology. Calculation of the distribution of hydrophobic residues with a "hydropathy" function that is conserved in this family of proteins implies that the membrane-folding pattern of the 42-kilodalton (kDa) mitochondrial cytochromes involves 8–9 membrane-spanning domains. The smaller 23-kDa chloroplast cytochrome appears to fold in five spanning domains that are similar to the first five of the mitochondria. Four highly conserved histidines are considered to be the likely ligands for the two hemes. The positions of the histidines along the spanning segments and in a cross section of the membrane-spanning α helices implies that two ligand pairs, His-82–His-197/198 and His-96–His-183, bridge the spanning peptides II and V, and the two hemes reside on opposite sides of the hydrophobic membrane core. In addition, the 17-kDa protein of the chloroplast b_6–*f* complex appears to contain one or more of the functions of the COOH-terminal end of the mitochondrial cytochrome *b* polypeptide.**

Although there are several hypotheses for the function of cytochrome *b* in complex III of mitochondria (1–3) and the b_6–*f* complex of chloroplasts (4–6) and a growing body of data on cytochrome structure (7, 8), there is little information available on the location of the cytochrome hemes in the membrane for either organelle system. The cytochrome *b* polypeptide of complex III has been found to be transmembranous (7, 8). Cytochrome *b* containing two hemes per polypeptide has been purified from complex III of yeast mitochondria (9), possibly providing an explanation (9) for the presence of two spectral forms, *b*-562 and *b*-566, in mitochondrial cytochrome *b* (10–12). Studies on the gene sequence show that the molecular weight of the yeast cytochrome is 42,000 (13). A 23-kilodalton (kDa) cytochrome b_6 polypeptide containing two hemes has been identified in the five-subunit b_6–*f* complex of chloroplasts (14, 15).

Additional molecular information on these cytochromes has recently become available, with the complete nucleotide sequence of cytochrome *b* of complex III having been obtained from five different sources: yeast (13), human (16), bovine (17), mouse (18), and fungal (19); the sequence of the chloroplast b_6 also has been determined (unpublished data).

RESULTS

Homology Between Mitochondrial Cytochromes. In order to construct a working model of the cytochrome *b* polypeptide as it spans the mitochondrial or thylakoid membrane, three major sources of information were used and compared: the primary amino acid sequences (Fig. 1), the position of the histidines in these sequences, and the distribution of hydrophobic residues calculated from these sequences. The distribution of hydrophobic residues along the yeast polypeptide chain, calculated according to ref. 20 with a sampling span of 11 residues, indicates 8 or 9 membrane-spanning regions (Fig. 2*A*): I (residues 32–52); II (residues 79–98); III (residues 114–134); IV (residues 143–168); V (residues 178–202); VI (residues 226–248); VII (residues 288–309); VIII–IX (residues 320–380).

Of the residues between positions 1 and 209, the region of overlap with the chloroplast cytochrome b_6, 44% are identical in all five of the proteins and 64% are identical in any four. Another test of similarity can be proposed for membrane proteins—calculation of the distribution of hydrophobic regions along the polypeptide chain (20–22). The results of such calculations, obtained with the hydropathy algorithm of ref. 20, are shown for yeast and human cytochrome *b* in Fig. 2 *A* and *B*. A model somewhat similar to those shown in Fig. 2, but indicating seven peaks of hydrophobicity, has been calculated (8) by using the transfer free energy values of Engelman and Steitz (22).

The hydropathy plots for the bovine, mouse, and *A. nidulans* sequences resemble those shown for yeast and human very closely. Calculation of a cross-correlation coefficient (23) between any two of the five hydropathy plots for the mitochondrial cytochromes demonstrates the high degree of mathematical homology. For the 10 pairs of cytochromes, the coefficient ranges from 0.91 for mouse–bovine to 0.69 for yeast–human, with an average of 0.78—all normalized by the auto-correlation coefficient of any function with itself being 1.00. Thus, many of the amino acid differences between the primary sequences of the cytochromes are conservative in preserving the distribution of hydrophobic regions along the polypeptide. That the homology between the hydropathy plots of the five cytochromes is not common for all membrane proteins can be seen from their correlation coefficients relative to other hydrophobic membrane proteins of similar size such as bacteriorhodopsin (24) and subunits I–III of the yeast cytochrome oxidase (25–27), which were 0.19 and −0.02 to +0.04, respectively. This conservation of sequence and structure between mitochondrial *b* cytochromes from such widely divergent sources implies that the hydropathy pattern, or pattern of folding in the membrane, is a structural signature for this group of proteins carrying out a common function. A model for the folding pattern of the mitochondrial *b* cytochrome in the membrane is shown for the yeast sequence (Fig. 3).

Homology with the Chloroplast Cytochrome b_6. The nucleotide sequence of spinach chloroplast cytochrome b_6 has

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Abbreviation: kDa, kilodalton.

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      1        10        20        30        40        50        60        70        80
Y:    MAFRKSNVYLSLVNSYIIDSPQPSSINYWWNMGSLLGLCLVIQIVTGIFMAMHYSSNIELAFSSVEHIIRDVHNGYILRY
B:    MTNIRKSHPLMKIVNNAFIDLPAPSNISSWWNFGSLLGICLILQILTGLFLAMHYTSDTTTAFSSVTHICRDVNYGWIIRY
H:    MTPMRKINPLMKLINHSFIDLPTPSNISAWWNFGSLLGACLILQITTGLFLAMHYSPDASTAFSSIAHITRDVNYGWIIRY
M:    MTNMRKTHPLFKIINHSFIDLPAPSNISSWWNFGSLLGVCLMVQIITGLFLAMHYTSDTMTAFSSVTHICRDVNYGWLIRY
A:    MRILKSHPLLKIVNSYIIDSPQPANLSYLWNFGSLLALCLGIQIVTGVTLAMHYTPSVSEAFNSVEHIMRDVNNGWLVRY
SP:   MIGSKNVSRFRRLRMIITSKYVPPHVNIFYCLGGITLTCFLVQVATGFAMTFYYRPTVTDAFASVQYIMTEVNFGWLIRS

      81        90        100       110       120       130       140       150       160
Y:    LHANGASFFFMVMFMHMAKGLYYGSYRSPRVTLWNVGVIIFILTIATAFLGYCCVYGQMSHWGATVITNLFSAIPFVGND
B:    MHANGASMFFICLYMHVGRGLYYGSY--TFLETWNIGVILLLTVMATAFMGYVLPWGQMSFWGATVITNLLSAIPYIGTN
H:    LHANGASMFFICLFLHIGRGLYYGSF--IYSETWNIGIILLLATMATAFMGYVLPWGQMSFWGATVITNLLSAIPYIGTD
M:    MHANGASMFFICLFLHVGRGLYYGSY--TFMETWNIGVLLLFAVMATAFMGYVLPWGQMSFWGATVITNLLSAIPYIGTT
A:    LHSNTASAFFFLVYLHIGRGLYYGSYKTPRTLTWAIGTVILIVMMATAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQD
SP:   VHRWSASMMVLMMILHVFR-V-YLTFKKPRELTWVTGVVLGVLTASFGVTGYSLPWDQIGYWAVKIVTGVPDAIPVIGSP

      161       170       180       190       200       210       220       230       240
Y:    IVSWLWGGFSVSNPTIQRFFALHYLVPFIIAAMVIMHLMALHIHGSSNPLGITGNLDKIPMHSYFIFKDLVTVFLFMLIL
B:    LVEWIWGGFSVDKATLTRFFAFHFILPFIIMAIAMVHLLFLHETGSNNPTGISSDVDKIPFHPYYTIKDILGALLLILAL
H:    LVQWIWGGYSVDSPTLTRFFTFHFILPFIIAALATLHLLFLHETGSNNPLGITSHSDKITFHPYYTIKDALGLLLFLLSL
M:    LVEWIWGGFSVDKATLTRFFAFHFILPFIIAALAIVHLLFLHETGSNNPTGLNSDADKIPFHPYYTIKDILGILIMFLIL
A:    IVEFIWGGFSVNNATLNRFFALHFLLPFVLAALALMHLIAMHDTGSGNPLGISANYDRLPFAPYFIFKDLITIFIFFIVL
SP:   LVELLRGSASVGQSTLTRFYSLHFVLPLLTAVFMLMHFLMIRKQGISGPL          MGHNYYWPNDLLYIFPVVILG

      241       250       260       270       280       290       300       310       320
Y:    ALFVFYSPNTLGHPDNYIPGNPLVTPASIDPEWYLLPFYAILRSIPDKLLGVITMFAAILVLLVLPFTDASVVRGNTFKV
B:    MLLVLFAPDLLGDPDNYTPANPLNTPPHIKPEWYFLFAYAILRSIPNKLGGVLALAFSILILALIPLLHTSKQRSMMFRP
H:    MTLTLFSPDLLGDPDNYTLANPLNTPPHIKPEWYFLFAYTILRSVPNKLGGVLALLLSILILAMIPILHMSKQQSMMFRP
M:    MTLVLFFPDMLGDPDNYMPANPLNTPPHIKPEWYFLFAYAILRSIPNKLGGVLALILSILILALMPFLHTSKQRSLMFRP
A:    SIFVFFMPNALGDSENYVMANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMFAAILALMVMPITDLSKLRGVQFRP
SP:   TIACNVGLAVLEPSMIGEPADPFATPLEILPEWYFFPVFQILRTVPNKLLGVLLMASVPAGLLTVPFLENNKFQNPFRRP

      321       330       340       350       360       370       380
Y:    LSKFFFFIFVFNFVLLGQIGACHVEVPYVLMGQIATFIYFAYFLIIVPVISTIENVLFYIGRVNK
B:    LSQCLFWALVADLLTLTWIGGQPVEHPYITIGQLASVLYFLLILVLMPTAGTIENKLLKW
H:    LSQSLYWLLAADLLILTWIGGQPVSYPFTIIGQVASVLYFTTILILMPTISLIENKMLKWA
M:    ITQILYWILVANLLILTWIGGQPVEHPFIIIGQLASISYFSIILILMPISGIIEDKMLKLYP
A:    LSKVVFYIFVANFLILMQIGAKHVETPFIEFGQISTIIYFAYFFVIVPVVSLIENTLVELGTKKNF
SP:   VATTVFLVGTVVAL-WLGIGATLPIDKSLTLGLF
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FIG. 1. Amino acid sequences of the *b* cytochromes. Y, yeast; B, bovine; H, human; M, mouse; A, *Aspergillus nidulans*; SP, spinach chloroplasts. SP residues 220–353 belong to the 17-kDa polypeptide. Deletions are assumed after residues 99 and 101 in SP, 106 in B, H, and M, and 334 in SP. Residues assumed to be inserted (not shown) in SP are G-G after T-105, T after H-183, G-E-P-A after Y-225, and V after N-310 and, in *A. nidulans*, V after T-204.

been obtained recently by one of us (unpublished data). In addition to the 23,000-kDa subunit containing two heme groups (15), the gel of the b_6–*f* complex defined three or four other polypeptides, including a 17-kDa polypeptide of unknown function (14). Comparison of the hydropathy function of the b_6 polypeptide (Fig. 2*C*) with that of the yeast (Fig. 2*A*), human (Fig. 2*B*), or other *b* cytochromes shows a detailed similarity of spanning segments I–V with respect to amplitudes and phases. The four histidines whose positions are conserved in these sequences are found in spanning segments II and V. The respective values of the cross-correlation coefficients with cytochrome b_6 are 0.70, 0.64, 0.61, 0.55, and 0.54 for the *A. nidulans*, yeast, human, mouse, and bovine cytochromes and 0.01 for bacteriorhodopsin. The amino acid sequence homology is smaller near the NH_2 terminus but is 31%, 36%, 37%, 38%, and 38%, respectively, between residues 50 and 209 for the cytochrome b_6 polypeptide and the cytochromes from yeast, human, mouse, bovine, and *A. nidulans*.

The Heme Binding Sites. An important aspect of the chloroplast b_6 sequence is that it contains only five histidines, which are at positions 25, 82, 96, 183, and 198 (197 in Fig. 1, where Thr-184 is not shown). The latter four histidines are shown to be conserved in all six cytochrome sequences and to occur in membrane-spanning regions of the polypeptide, whereas His-25 has neither property. By assuming that two histidines function as the ligands of each heme (28), the four conserved histidines are considered to be the likely heme ligands. Additional structural information was obtained from the location of the histidines in the hydrophobic domains. Of the four conserved histidine residues, two (82 and 96) were found to be in spanning segment II and two (183 and 197/198) were found in spanning segment V. Thirteen amino acids separate the two histidines in both spanning segments II and V of mitochondria. For the cytochrome b_6, there is an extra threonine inserted between position 183 and 184. There are three other locations where histidine is found in all of the mitochondrial sequences. There is a histidine at positions 53, 67, and 202 in all of the mitochondrial sequences but not at these or any adjacent position in the chloroplast b_6. These histidines, unlike the four in hydrophobic spanning segments II and V, are found in the hydrophilic phase (Fig. 3) and are located in the lipid polar head-group region or the aqueous phase. The polypeptide folding pattern shown in Fig. 3 shows the spatial relationship of the four histidine ligands in a plane perpendicular to the membrane surface. This relationship suggests that the two hemes are coordinated between spanning segments II and V by using the histidine pairs His-82(II)–His-197/198(V) and His-96(II)–His-183(V).

The model was tested further by examining the spatial relationship of these four histidines in the plane parallel to the membrane surface. It was assumed (*i*) that segments II–V consecutively span the membrane (Fig. 3), with the intervening hydrophilic regions forming the connections between the hydrophobic spanning segments, and (*ii*) that the hydrophobic spanning segments have an α-helical conformation, as has been argued to be thermodynamically probable (21, 22). The cross-sectional distribution of the histidines in segments II and V was studied through construction of helical wheels (29, 30) and through examination from the NH_2 side of the resulting amino acid distribution in cross section parallel to the plane of the membrane (Fig. 4 *A* and *B*). The rotation sense of helix V seen in this way is opposite to that of helix II. His-82 and His-96 on segment II are separated by 40° on the helical wheel (Fig. 4 *A* and *B*), as are His-183 and His-198 on spanning segment V of the chloroplast cytochrome (Fig. 4*B*). This leads to the possibility of ligand pairing without great strain by each of the two histidine couples, His-82(II)–

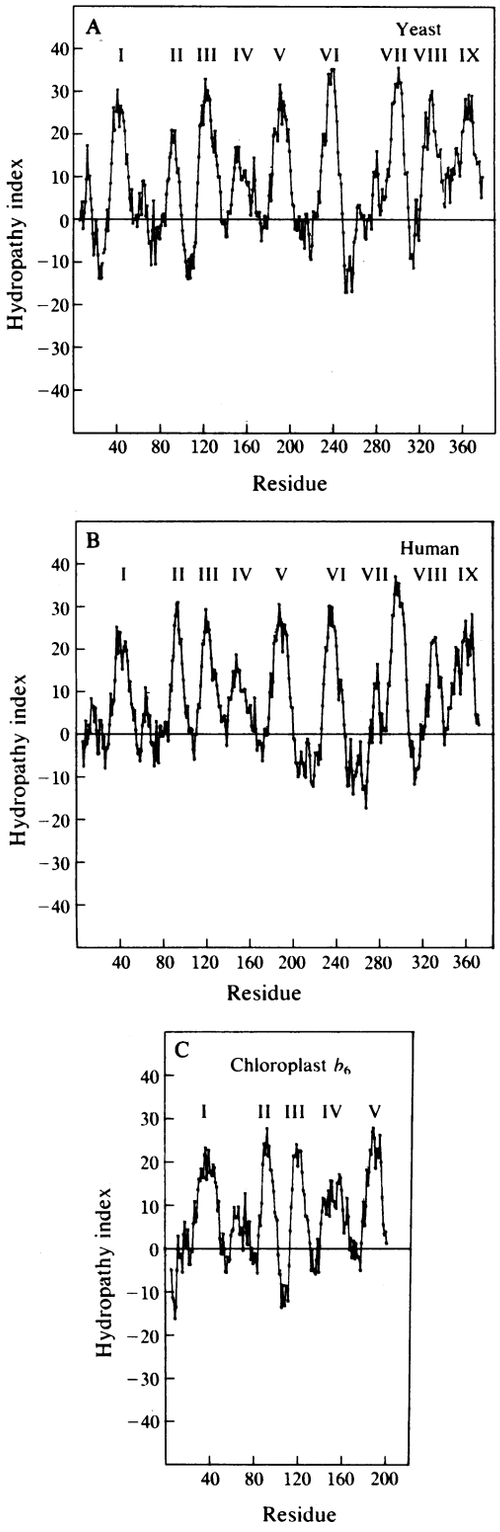

FIG. 2. Hydropathy plots of the mitochondrial *b* cytochromes from yeast (*A*) and human (*B*) mitochondrial complex III and spinach chloroplast cytochrome b_6 (*C*). The plots were generated after converting the algorithm of Kyte and Doolittle (20) to DEC BASIC by using a DEC LSI-11/23 computer interfaced to an incremental plotter.

His-198(V) and His-96(II)–His-183(V), assuming that spanning peptides II and V are close (Fig. 5). The absence of the extra threonine in the mitochondrial cytochrome sequence at position 184 leads to a separation of 40° in the opposite direction between His-183 and His-197 in helix V (Fig. 4*A*). In spite of this altered geometry, a similar pairing can occur for the pairs His-82(II)–His-197(V) and His-96(II)–His-183(V) in the mitochondrial cytochromes by rotating about the β bond of His-197 and/or tilting span V slightly from the vertical direction. The above pairings predict that the two hemes of the *b* cytochrome occupy positions near opposite sides of the nonpolar region of the membrane, with the heme planes more perpendicular than parallel to the membrane plane. Differential effects of an energized membrane on the two spectrophotometric forms of the *b* cytochrome in mitochondria have previously implied a transverse distribution (2, 12). EPR data indicated that both hemes are close to the cytoplasmic side of the mitochondrial membrane, although these results are dependent on inaccessibility of paramagnetic ions (31).

Similarity Between the Chloroplast 17-kDa Protein and the COOH-Terminal Region of Mitochondrial Cytochromes. The sequence of another polypeptide identified by Hauska and coworkers (14) from the b_6-*f* complex, one of 17 kDa (subunit 4), has been determined (unpublished data). The sequence of a 139-residue polypeptide included in this coding region is shown in Fig. 1 starting at position 220. Because of the disparity between the length of the mitochondrial and chloroplast cytochrome *b* gene products, a comparison was made between this sequence and the COOH-terminal end of the mitochondrial cytochromes. There is a 30% homology in the sequence between residues 260–353 of subunit 4 and at least four of the mitochondrial cytochromes. Furthermore, the functional mRNA for cytochrome b_6 and subunit 4 is dicistronic and decoded from b_6 to subunit 4 (unpublished data).

DISCUSSION

The high degree of homology and structural similarity between the chloroplast cytochrome b_6 and the mitochondrial *b* cytochromes implies that essential structural features of this protein, required for a similar function in electron transport and possibly proton pumping, are conserved in these proteins from widely divergent plant and animal sources.

The use of the hydropathy function to describe the membrane folding pattern of predominantly hydrophobic proteins has limitations. It is not capable, for example, of describing the membrane folding pattern of oligomeric amphipathic helices involved in channel formation. In the present case, however, it appears to be useful in describing the membrane folding pattern of a group of related proteins.

The combined use of comparisons of sequence homology and hydropathy correlation led to the conclusion that the two hemes of the *b* cytochromes are located near opposite sides of the hydrophobic membrane core, with the heme planes more perpendicular than parallel to the plane of the membrane. By using oriented membranes, the heme planes of the *b* cytochrome in pigeon breast mitochondria have been found by EPR to be approximately perpendicular to that of the membrane (32). The angle between the plane of the hemes and that of the membrane has been estimated to be approximately 50° for cytochrome b_6 (33). A construction of a physical model for the binding of the hemes by spanning regions II and V (not shown) indicates that the distance between the two heme edges is about 12Å. A number of conserved residues arise in the polar peptides linking spanning segments I–II, III–IV, and V–VI. These are Tyr-54, Ala-61, Phe-62, Ser-64, Ile-68, Val-72, Gly-75, Tyr- or Trp-76, and Arg-79 in the domain connecting segments I and II. Along with conserved residues His-202, Gly-205, Ser-206, Asp-208, Pro-209, and Gly-211, which are in the region linking segments V and VI of the mitochondrial cytochromes, these conserved residues may form a pocket around the heme coordinated by His-82 and His-197/198. Lys/Arg-99 and

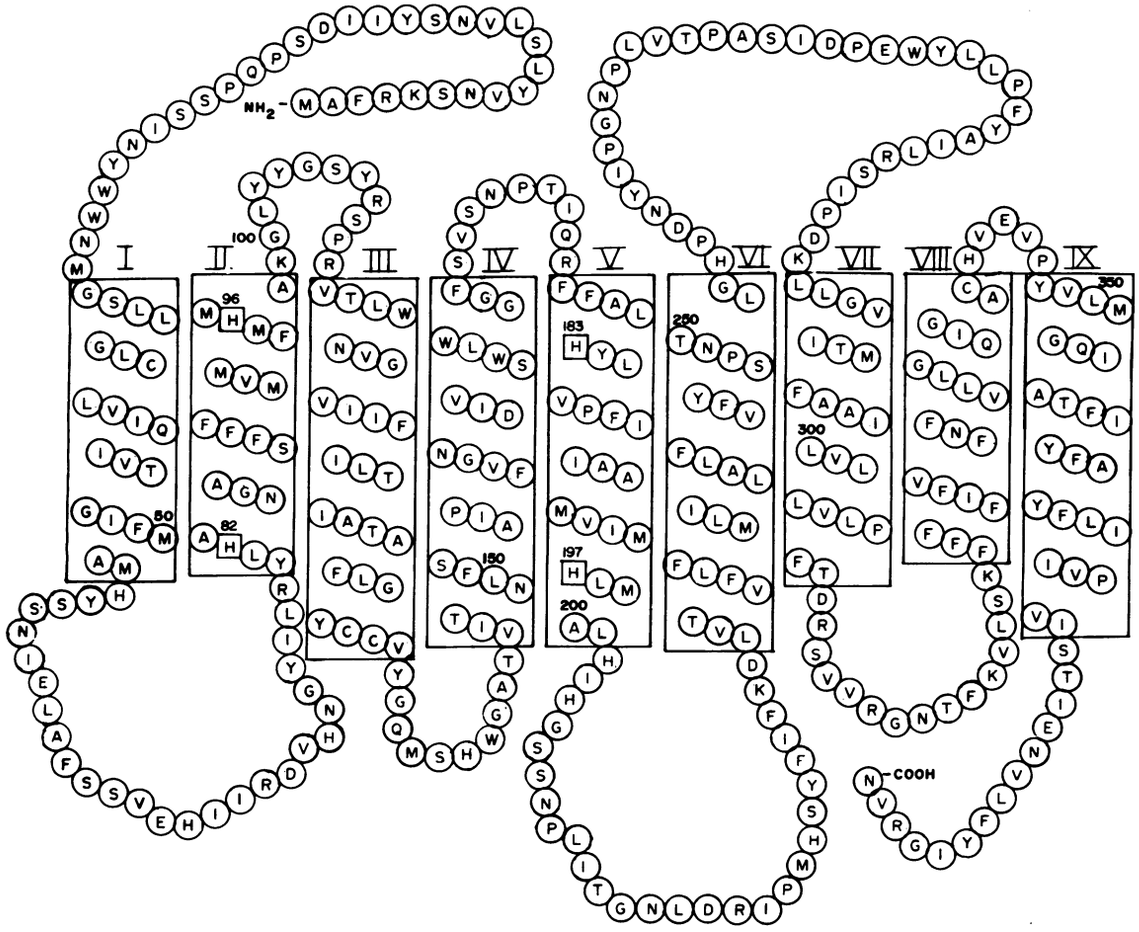

FIG. 3. Folding of the yeast mitochondrial cytochrome *b* in the inner mitochondrial membrane according to the hydropathy pattern of Fig. 2*A*. Spanning segments are numbered by the same convention as in Fig. 2. Alternatives at positions 253 and 270 are Q and V (13).

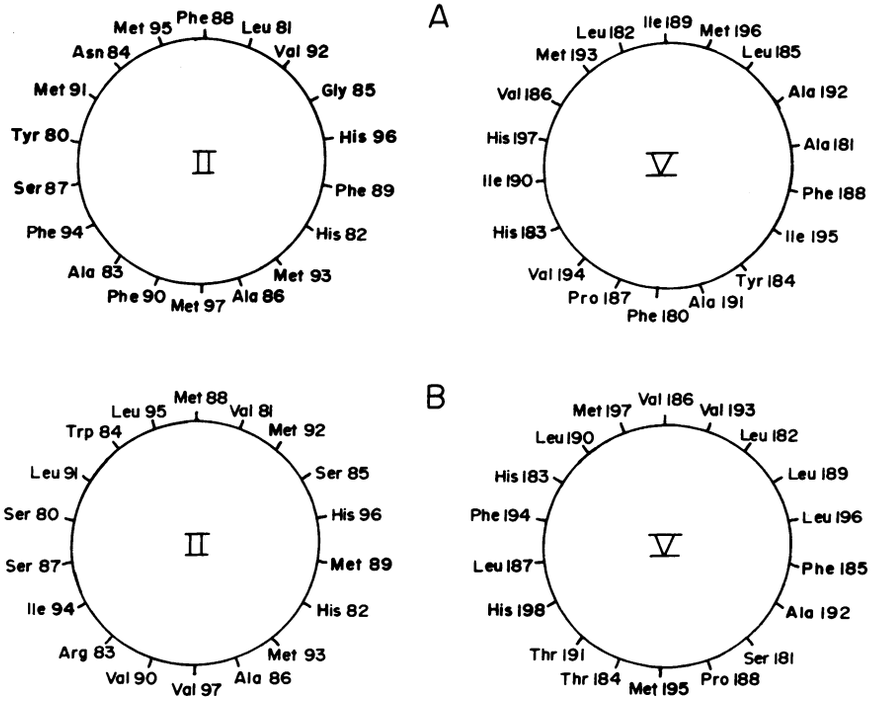

FIG. 4. A helical wheel diagram of spanning segments II and V of the yeast cytochrome (*A*) and the chloroplast cytochrome b_6 (*B*) assumed to be in an α-helical conformation.

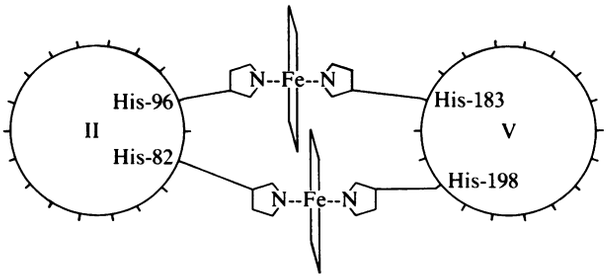

FIG. 5. A cross-section of the chloroplast cytochrome *b* in a plane parallel to the membrane surface, showing the coordination of the hemes by His-82–His-198 and His-96–His-183.

Arg-178 could form a salt bridge to the heme propionic acids extending to the aqueous phase on one side of the membrane and (Arg-79, His/Arg-202) a bridge to the two propionic acids from the heme on the other side of the membrane. The nonpolar vinyl side chains would then extend toward the center of the membrane.

The model for a transmembrane distribution of the cytochrome *b* hemes in the membrane provides a pathway across the membrane dielectric within this protein that is relevant to discussions of cytochrome *b* function (1–6, 12, 34–36) and to an explanation for the *in situ* splitting of absorption spectra and midpoint potentials observed for the mitochondrial cytochromes (2, 3, 10, 12, 37). The chloroplast cytochrome shows an intrinsic splitting of these properties *in vitro* (38). However, these splittings are not large enough *in situ* to be readily resolved (39, 40), although separation of the E_m values can be seen under some circumstances (39).

Finally, it is proposed that the single mitochondrial cytochrome *b* gene product of about 42 kDa in size is equivalent to two distinct chloroplast polypeptides of approximately 23 kDa and 17 kDa, arising from a split chloroplast gene. The 17-kDa polypeptide may be necessary for functions other than electron transport carried out by the cytochrome *b* complex in the membrane, such as binding of the Rieske Fe–S protein/PQ binding subunit and/or formation of a proton translocating complex in the membrane.

Note Added in Proof. A similar model and reasoning have been independently derived by Saraste and Wikström (41).

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