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Review

Prediction and comparison of the haem-binding sites in membrane haemoproteins

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Contents

Abbreviations: COI, II, III, subunit I, II and III of cytochrome oxidase; DMSO, dimethylsulfoxide; EPR, electron paramagnetic resonance; HALS, highly anisotropic low-spin EPR signal; HQNQ, 2-heptylhydroxyquinoline *N*-oxide; HS, high-spin EPR signal; MPH,l membrane propensity for haemoproteins; Q, either ubiquinone or menaquinone; QH_2, either ubiquinol or menaquinol; RLS, relaxed low-spin EPR signal; SER, specifically enriched residue in one position of a peptide that binds a Q-reacting membrane iron.

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Summary

This article contains a comparative review of the structural properties of membrane haemoproteins, with particular emphasis on the possible similarities of the haem-binding peptides. A procedure is suggested for identifying the peptides which may bind membrane-buried haems on the basis of the primary sequences of the proteins. The integration of this procedure with the information deduced by refined hydropathy analysis indicates that the basic structural model for the haemoproteins which interact with quinones may be a transmembrane helical bundle containing the haem(s) at its centre.

Structural similarities exist in the sequence of hydrophobic segments that are predicted to bind the membrane-buried haems of *b*-cytochromes which interact with quinones. The predicted haem-binding sites show similarities also with the peptides that bind the non-haem iron in the bacterial reaction centres, and this may be correlated to the common function of interacting with quinones and their intermediates.

The analysis of the amino-acid composition of the proposed ligand peptides in the membrane haemoproteins examined has provided a molecular rationale for explaining the highly anisotropic low-spin EPR signal which is characteristic of many membrane-bound *b*-cytochromes.

I. Introduction

b-type cytochromes are probably the most common prosthetic groups of the membrane-bound enzymes that interact with quinones (Qs) in energy-conserving electron transfer chains. *b*-cytochromes are found in quinone reductase complexes (e.g., succinate : Q reductase or Complex II [1,2]), quinol oxidoreductase complexes (e.g., QH_2 : cytochrome *c* reductase or bc_1 complex [3,4]), and ubiquinol oxidase complexes such as the *bo* complex [5,6].

The polypeptides that bind the *b*-cytochromes in all these systems traverse the membrane several times and have their metal centre embedded in the lipid bilayer. Such a physical characteristic enables these haemoproteins to interact directly with the quinones and quinols that diffuse within the membrane [1,3]. The transmembrane nature of the *b*-cytochromes seems to be also essential for anchoring other redox subunits of the complexes to the membrane [1,2,7,8].

Various sequences are now available for the apoproteins of the *b*-cytochromes that interact with quinones. Superficially, the proteins that belong to different families of quinone-reacting complexes do not show regions of clear homology, e.g., cytochrome *b* of the bc_1 complex and cytochrome *b*-558 of the succinate : Q reductase in *B. subtilis* [2,9]. However, it is likely that the haem-binding sites of these proteins do possess common structural features because they are all involved in redox reactions with quinones and/or the stabilization of their semiquinone intermediates [1,3,10]. A similarity in the physical properties of the *b*-haems is also inferred from common features in their EPR spectra that are generally indicative of a strained, bisimidazole coordination [11,12].

The molecular organization of membrane-bound *b*-cytochromes is not known because no resolved crystal structure is yet available. Therefore, the integration of the present genetic, spectroscopic and biochemical information with deductive procedures based on sequence analysis is indispensable for understanding the structure of membrane haemoproteins. This work pursues this aim by bringing together the spectroscopic and structural information which has been recently accumulated for a variety of membrane haemoproteins.

I-A. General features of the b-cytochromes that interact with quinones

The various *b*-cytochromes that interact with quinones can be grouped into a single class of membrane haemoproteins which is distinguishable from other membrane haemoproteins by two main characteristics: (i) physical (redox) interaction with quinones or quinols; (ii) membrane folding organized in at least three transmembrane segments. This class includes several families of *b*-cytochromes, each of which is encoded by a distinct gene and belongs to a specific quinone-reacting complex. The basic properties of these cytochromes are listed in Table I.

2-Alkyl-*N*-hydroxyquinoline (HQNO or NQNO) is the most general inhibitor of the quinone-reacting *b*-cytochromes (Table I). This compound is capable of inhibiting quinone reductases (e.g., formate reductase [13]) and nearly all the quinol oxidoreductases/oxidases, with the notable exception of the fumarate reductase of *W. succinogenes* (Table I). The structure of HQNO is reminescent of that of physiological quinones [14] and this suggests that its binding pocket may partially overlap quinone-interacting sites in the vicinity of the *b*-cytochromes exposed at the negative side of the membrane [10,14–16].

TABLE I

Various properties of membrane b-cytochromes that interact with quinones

b-haem,	per mol [a]	Species	$\lambda_{max\ \alpha\text{-band}}$ room T (nm)	EPR [b]	CD [c]	Mass (kDa)	E_m (mV)	Interaction with HQNO [d]	Ref.
1. Family of formate : Q reductase									
b	1	*W. succinogens*	562	–	–	25	−224	yes	13
b-556	1	*E. coli*	559	–	–	20	−110	yes	8
2. Family of succinate : Q reductase (Complex II)									
b-556	1	*E. coli*	558	HALS, HS	–	14 & 13 [e]	36	yes [f]	6, 18, 37, 73
b-558	1	*P. denitrificans*	558	–	–	14 & 13	–	?	38
b-558	1–2	*B. subtilis*	558	HALS	–	23 [e]	ca. 50 [f]	yes [f]	2, 9, 12
b-559	1	*N. crassa* (mitos) [g]	559	–	–	14	–	?	38
b-558	1–2	*A. suum* (mitos)	558	HALS, HS	–	15 & 14 [h]	–	?	74
b-562	1	mung bean (mitos)	562	–	–	15 & 13	–	?	75
b-560	1	beef (mitos)	560	HALS, HS	–	14 & 13	−114, −180	?	1, 76, 77
3. Family of QH_2 : fumarate reductase									
b-563	2	*W. succinogenes*	563	–	i	30 [e]	−20, −180	no	29
–	–	*E. coli*	–	–	–	15 & 13 [e]	–	yes	7, 26
–	–	*P. vulgaris*	–	–	–	15 & 13 [e]	–	yes	7, 27
4. Family of QH_2 : nitrate reductase									
b-556	1–2	*E. coli*	558	–	k	26 [e]	+20, +110	yes	8, 31, 78
b-557	2	*P. denitrificans*	557	–	k	20	+95, +210	yes	32, 79
5. Family of QH_2 : DMSO reductase									
b-559	1	*R. capsulatus*	559	–	–	–	ca. 0	?	33, 80
(*b*?)	–	*E. coli*	–	–	–	31 [e]	–	?	34
6. Family of QH_2 : cytochrome *c* reductase (bc_1 complex or Complex III)									
b_h, b_l	2	*R. capsulatus* [l]	561, 566	HALS	c	48 [e]	−120, +150	yes	4, 81–83
b_h, b_l	2	beef (mitos) [m]	562, 566	HALS	c	42 [e]	−30, +120	yes	3, 4, 82, 84
7. Family of plastoquinol (QH_2) : plastocyanin reductase (b_6f complex)									
b_6	2	spinach [n]	563	HALS	c	23 [e]	+5, −120	yes	4, 51, 83
8. Family of *o*-type ubiquinol oxidase (*bo* complex)									
o	2	*E. coli*	555, 562	RLS, HS	–	74 [o,e]	+125, +160	yes	5, 6, 19, 37
o	2	*G. suboxidans*	558, 562	–	–	76 [o]	–	yes	85
o	2	Vitreoscilla	561, 564	–	–	74 [o]	+165	yes	86
9. Family of *d*-type ubiquinol oxidase (*bd* complex)									
b-558	1	*E. coli* [p]	560	HS, HALS [q]	–	58 [e]	+180	yes	5, 19, 37, 87, 92
10. Family unknown:									
b-561	2	*E. coli*	561	–	–	20 [e]	+20	?	6, 37, 88, 89
b-562	1–2	*R. sphaeroides*	562	HALS, HS	–	17 [e]	−100, +60	?	35, 36

[a] Usual name of the *b* haem(s) and mol of haem equivalents per mol of protein.

[b] Type of EPR signal; the high-spin (HS) signals are generally seen after partial denaturation consequent to the isolation of the protein [1,37,74]. See Table VI for the values of the HALS signals.

[c] CD studies available so far [59–62].

[d] The question mark indicates that the effect of HQNO has not been reported.

[e] Complete sequence known as deduced from the gene.

[f] Kita, K. (*E. coli*) and Hederstedt, L. (*B. subtilis*), personal communications.

[g] See Refs. 2, 38 for information on these and other succinate : Q reductases.

[h] Partial sequence known [74].

[i] Recent CD studies provide evidence for two interacting haems in this system (Degli Esposti, M., Kortner, C. and Kröger, A., unpublished results).

[k] See Ref. 90 for the CD characterization of the assimilatory (soluble) nitrate reductase from *Chlorella vulgaris*.

[l] The sequence of a mutant of *Rhodobacter capsulatus, Paracoccus denitrificans* [4], *Rhodopseudomonas viridis* (Gabellini, N., personal communication) and *Rhodobacter sphaeroides* (Gennis, R., personal communication) are also known.

[m] Over 20 sequences of mitochondrial cytochrome *b* are now available (see Refs. 4, 65, 66).

[n] The sequence of cytochrome b_6 is also known from tobacco, liverworth and Nostoc [4].

[o] The *bo* complex is formed by at least three other subunits of approx. 47 (cyoC), 29 and 22 kDa [5,19,86,91].

[p] A *bd* complex has been purified also from *P. phosphoreum* (see Ref. 6 and references therein).

[q] The EPR spectrum of the *b*-558 haem is low-spin in situ and resembles that of bacterial cytochrome c_1 [92,93]. It is now demonstrated that subunit I also binds the haem of cytochrome *b*-595 (Gennis, R., personal communication, and Ref. 94).

A variability exists in the optical maxima of the reduced alpha band among members of the same family of cytochrome *b* (Table I). Hence, there is no strict correlation between λ_{max} of the optical spectra (the parameter which is commonly used in the identification of the cytochromes [6,8]) and the function or structure of the *b*-cytochromes. A more homogeneous spectroscopic property of the *b*-cytochromes that interact with quinones is the highly anisotropic low-spin signal (usually called the HALS signal, see below).

The molecular mass of the majority of the *b*-cytochromes or membrane-anchoring subunits is in the region of 20 kDa. Larger molecular weights are seen only in the *b*-cytochromes of the bc_1 complex and the quinol oxidases (Table I). The sequences now available for the *b*-cytochromes of the bc_1 and b_6f complexes largely outnumber those of other quinone-reacting membrane haemoproteins (Table I). The alignment and comparison of these sequences reveals that only four histidines are conserved that may acount for the coordination of their two haems [4,11,17]. These histidines are located in predicted transmembrane segments of the proteins and can be reasonably assumed to form the axial ligands of the haem irons [4,10,11,17] – no methionine, the alternative ligand that may be compatible with the spectroscopic properties of the haems [11], is conserved in the aligned sequences [4]. This could well be exploited for deducing the location of the histidine ligands in other membrane haemoproteins.

However, standard methods have to date failed to indicate significant homologies between the proposed haem-binding sites in the cytochrome *b* of the bc_1 complex and histidine-containing peptides in other *b*-cytochromes [1,2,9,18,19]. Alternative statistical procedures are required, therefore, for identifying the haem-binding sites of membrane haemoproteins [20].

II. Procedures for identifying the possible ligands of the haems in membrane-bound *b*-cytochromes

II-A. The 'histidine index'

As discussed above, the search of the likely ligands of the haems in membrane *b*-cytochromes can be restricted, with good approximation, to histidine residues. Besides the *b*-cytochromes of the bc_1 and b_6f complexes, the histidine ligands of chloroplast cytochrome *b*-559 can be also deduced clearly from the sequences [21]. The potential usefulness of the several sequences now available for all these cytochromes is in principle weakened by their homology [4]. This problem can be reduced, at least in part, by using a 'learning' data base [22] that includes also proteins which are non-homologous to these cytochromes but show some functional analogy with them [20]. These proteins are the L and M subunits of the bacterial reaction centres [23] and their related D_1 and D_2 subunits in green plants [24], which possess a membrane-buried non-haem iron that is coordinated to histidine residues. These residues and their surrounding peptides are directly involved in the binding of ubiquinone and ubisemiquinone [23], a function that is essentially analogous to that fulfilled by the HQNO-sensitive *b*-haem (b_H [10]) in the bc_1 complex [3,10,14,20].

110 deduced or known ligand heptapeptides of all the above proteins have been used for deriving the simple parameter of the 'histidine index' [20]. This index measured the cumulative distribution of probability of structural similarity with such peptides by the sum of the frequencies of the residues in the heptad surrounding any histidine of a sequence [20]. All the positions from −3 to +3 are considered, because the conformation of the peptides is not known a priori. In no case do the known ligands within and outside the data base exhibit a 'histidine index' lower than 0.3 (results not shown, see also Ref. 20).

II-B. The SER parameter

An alternative parameter for the identification of ligands is the number of specifically enriched residues (hereafter abbreviated as SER), i.e., the residues occur-

TABLE II

Specifically-enriched residues (SER) in the position of the nonapeptides containing at their centre the proposed ligands of the haem irons in cytochrome b, b_6, b-559 and of the non-haem irons in photosynthetic reaction centres

Specifically enriched residues are considered those amino acid that exhibit a frequency at least 20% higher than their frequency of occurrence in all the transmembrane regions of cyt *b*, b_6, *b*-559, the L and M subunits of all the bacterial reactions centres [24] and the homologous D_1 and D_2 subunits of green plants. These transmembrane frequencies are very similar to those calculated in all the bacterial haemoproteins examined here (M. Degli Esposti, unpublished results). The data base used includes the following proteins: cytochrome *b* from beef, yeast, maize, *L. tarantolae*, *P. denitrificans* bc_1 complex (maximal sequence homology less than 55% [4]); cytochromes b_6 [4] and *b*-559 [21] from spinach; the L and M subunits of the bacterial reaction centres listed in Ref. 24; the D_1 subunits from spinach, *E. gracilis*, *A. hybridus*, tobacco and the D_2 subunit from spinach and *Synechococcus* (see Ref. 63 for the pertinent references). The set of SER derived only from the cytochrome sequences is very similar (but not identical) to the above [20,54].

Position from the ligand	Specifically enriched residues (SER)
−4	C, F, I, L, M
−3	E, F, R, S
−2	A, G, S, Y
−1	I, L, M
+1	A, F, G, I, R
+2	A, G, L, N, W
+3	A, G, L, R, T
+4	A, G, L, P, W

ring in a given position around the ligands of the data base at a frequency significantly higher than that expected from their random distribution in the sequences (Table II). The SER parameter is derived from an analysis which is different from that used previously [20] in two points: (i) the sequences showing strong homology are excluded in order to reduce biases in the predictions; (ii) the residues in position −4 and +4 are also considered, as they may also be in contact with the haems in typical helical structures [25]. There is an approx. 80% probability of identifying the iron-binding peptides in proteins that interact with quinones when the number of SER is equal or higher than 4 (Fig. 1).

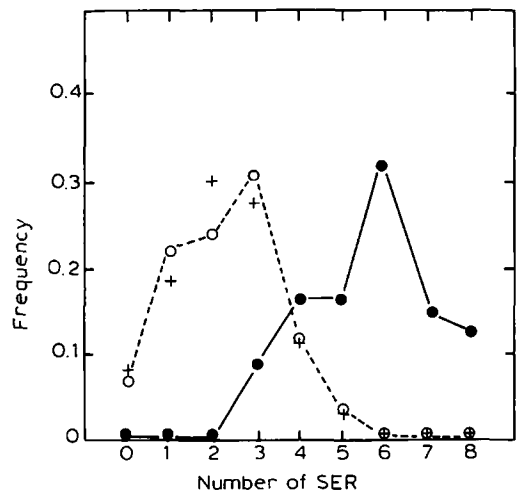

Fig. 1. Distribution of the specifically enriched residues (SER) in different histidine-containing nonapeptides. ●———●, 80 peptides that are proposed to bind the haems in cytochrome *b* and b_6 which are not included in the mixed data base of Table II. All of these peptides have a histidine index higher than 0.33. o — — — o, about 300 peptides that do not bind iron centres in the following membrane proteins with redox function: several sequences of cytochrome *b* [4]; all the available sequences of the chloroplast D_1 and D_2 subunits (see Ref. 63 and references therein); sequences of COII, COIII and the unidentified reading frames listed in Refs. 64–66; many sequences of cytochromes *P*-450 [52]; sequences of various cytochromes c_1 and cytochromes *f* [4]; sequences of various Rieske proteins [4]. Over 80% of these non-ligand peptides have a histidine index higher than 0.3. The frequency of having at least 4 SER is lower than that found in these proteins when the analysis is extended to membrane proteins which have no redox function, such as opsins, ion pumps and ion channels (results not shown). + — — — +, over 200 ligand peptides of soluble iron-proteins or of membrane anchored haemoproteins having the haem in a hydrophilic domain that were taken from the following proteins: haemerythrin and myohaemerythrin [48]; various cytochromes b_5 [67]; cytochrome *c* (only the histidine ligand) from chimpanzee, horse, yeast and *C. crusei* [68]; all the cytochromes c_1 and cytochromes *f* listed in Ref. 4; all the cytochromes *P*450 (cysteine ligand) listed in Ref. 52; cytochrome *b*-562 (both the histidine and the methionine ligands) from *E. coli* [47]; all the ligands of several transferrins [69]; multihaem *c*-cytochromes [70]; the cytochrome subunit of the reaction centre in *R. viridis* (histidine and methionine ligands [71]); several of the globins reported in Ref. 72. The ligand peptides of soluble haemoproteins that show 4 or more SER generally belong to hydrophobic helices, such as those of leghaemoglobin or cytochrome *c* peroxidase.

Interestingly, the histidine ligands of the non-haem iron in bacterial reaction centres, which are known from the crystal structure [23], are correctly identified by the largest SER derived only from the non-homologous *b*-cytochromes (Table III).

II-C. Local sequence similarities related to the interaction with quinones

Correct predictions like those obtained with photosynthetic proteins (Table III and results not shown) suggest that the above statistical parameters recognize structural similarities in the quinone-reacting peptides of apparently non-homologous proteins. So, these parameters can be exploited for predicting the possible haem-binding peptides and/or peptides that are likely to interact with quinones in the various systems of Table I. In view of the importance of local protein similarity, the predictions should be combined with an independent method of measuring segmented sequence homology. The similarity matrix of Levin and Garnier is used here due to its simplicity and accuracy [22]. The sequence similarity is systematically searched without gaps against 100 non-identical peptides of the *b*-cytochromes utilized previously [20], of other *b*-cytochromes of bc_1 complexes that have been recently sequenced (Gallerani, R., Saccone, C. and Gabellini, N., personal communications) and of the proteins examined here. These peptides are collectively defined the QB (quinone-*b*-cytochromes) data base and are listed in Appendix 1.

II-D. A procedure for predicting the histidine ligands of b-cytochromes

The selection based on the following criteria yields the maximal accuracy in identifying the known histidine ligands of membrane-buried irons interacting with quinones, as illustrated in Table III.

(1) Histidine-containing peptides that exhibit a 'histidine index', calculated as in Ref. 20, lower than 0.3 are excluded.

(2) The histidine-containing peptides that show 4 or more SER are likely to bind membrane-buried irons and/or to interact with quinones.

(3) The peptides that show less than 4 SER can be selected as potential haem- or quinone-binding sites by ranking their highest score of sequence similarity with the QB data base, calculated by the matrix in Ref. 22.

(4) When the above criteria do not clearly select sufficient ligands for the haem(s), preference is given to the histidine-containing peptides that show the largest number of significant sequence similarities (i.e., the scores above 6 [22]) with the QB data base.

This procedure does not generally identify haem-binding sites in globular proteins or membrane proteins

TABLE III

Application of statistical parameters to the L and M subunits of the reaction centre of Rps. viridis

Histidine peptide	Function [a]	(1) histidine [b] index	(2) SER [c]	(3) Highest score [d] of similarity	(4) Number of [e] similarities
A. Subunit L					
H116	none	0.352	1	6	2
H144	none	0.395	1	0	0
H153	none	0.305	0	6	1
H168	none	0.354	4	6	2
H173	Mg ligand	0.042	0	0	0
H190	Fe ligand	0.944	6	9	4
H211	none	0.311	2	0	0
H230	Fe ligand	0.843	7	9	3
B. Subunit M					
H16	none	0.471	2	10	2
H78	none	0.318	2	6	2
H108	none	0.878	3	8	3
H143	none	0.383	2	0	0
H162	none	0.344	3	0	0
H180	none	0.249	2	6	1
H200	Mg ligand	0.200	2	0	0
H217	Fe ligand	0.410	6	7	4
H264	Fe ligand	0.533	6	11	2
H299	none	0.396	3	6	1

[a] Derived from the known crystal structure [23].
[b] Index calculated from position −3 to position +3 of 90 *b*-cytochromes [54].
[c] Calculated from position −4 to position +4 using the peptides of the QB data base (cf. Table II and Appendix 1).
[d] Calculated from position −4 to position +4 against all the peptides of the QB data base as in Ref. 22. 0 means any score under 6.
[e] Scores of similarities ⩾ 6 with the QB data base.

without redox function (see Fig. 1). However, care must be taken in evaluating the predictions obtained in membrane haemoproteins, as with any other method of structural predictions [22]. The membrane topology of the histidines, which can be deduced by other methods, is also useful for discriminating among ambiguous predictions (see the following sections).

III. The putative haem-binding sites in various *b*-cytochromes interacting with quinones

III-A. The b-cytochromes of succinate : Q reductase

The primary sequence of the membrane-anchoring subunits of the succinate : Q reductase is known from *E. coli* (usually called cytochrome *b*-556 [1,6]) and from *Bacillus subtilis* (usually called cytochrome *b*-558 [2,9,12]. The two hydrophobic polypeptides that bind cytochrome *b*-556 are also similar to the hydrophobic subunits of the fumarate reductase complex in *E. coli* [18,26] and *P. vulgaris* [27], consistent with the functional similarity of the two enzymes [1,2,7].

Table IVA shows the application of the prediction procedure for identifying the histidine ligands of the membrane-buried haems in the sequences of cytochrome *b*-556. H30 in the SDHC subunit and H71 in the SDHD subunit are predicted on the basis of criterion 2. However, it is to be noted that H84 in the SDHC subunit shows the highest similarity score and the largest number of significant similarities with the QB data base. All these residues belong to transmembrane segments and are located near the negative side of the membrane (Fig. 2A,B). Consequently, it is unclear whether the *b*-556 haem is bound to histidines of separate subunits [1], or is bound only to the SDHC subunit [6].

The latter possibility is apparently supported by the comparison with the predictions obtained for cytochrome *b*-558 of the succinate : Q reductase from *B. subtilis* (Table IVB). H13 and H70, which belong to the first and second transmembrane segment, respectively (Fig. 2C,D), are the predicted ligands if the haem of cytochrome *b*-558 is exposed at the negative side of the membrane as in the succinate : Q reductase of *E. coli* (Fig. 2A,B) and mitochondria [1]. In the case that cytochrome *b*-558 contains also a second haem [2], H28 and H155 are selected as its possible ligands, since these residues are predicted to lie at the same positive side of the membrane (Fig. 2C,D). H113 is unlikely to be a haem ligand (Table IVB) also for its location in a hydrophilic region (Fig. 2C,D; see also Ref. 2). This is in agreement with the results of mutagenesis of H113

TABLE IV

Application of the prediction procedure for the haem- and/or quinone-binding sites in the membrane-anchoring subunits of bacterial succinate:Q reductase and QH_2:fumarate reductase

Protein [a]	Histidine peptide	(1) Histidine [b] index	(2) SER [c]	(3) Highest score [d] of similarity	(4) Number of [e] similarities	Predictions and criteria
A. Cytochrome *b*-556 *E. coli*, succinate:Q reductase						
SDHC	H30 [f]	0.784	4	8	7	ligand, (2), (3)
SDHC	H84 [f]	0.451	2	9	12	ligand?, (3), (4)
SDHC	H91 [f]	0.138	0	7	4	excluded
SDHD	H14 [f]	0.306	2	8	3	excluded
SDHD	H71 [f]	0.477	4	7	6	ligand?, (2)
B. Cytochrome *b*-558 *B. subtilis*, succinate:Q reductase						
SDHA	H13 [f]	1.203	6	10	14	ligand, (2), (3) (4)
SDHA	H28 [g]	0.430	1	9	7	ligand, (3), (4)
SDHA	H47 [g]	0.412	3	0	0	excluded
SDHA	H70 [f]	0.412	2	9	2	ligand, (3)
SDHA	H113 [f]	0.452	3	8	2	ligand?, (3)
SDHA	H155 [g]	0.329	2	6	6	ligand, (4)
C. *E. coli* QH_2:fumarate reductase						
FRDC	H83 [f]	0.684	3	9	3	Q-binding, (3)
FRDD	H82 [f]	0.562	3	6	3	–
FRDD	H84 [f]	0.379	2	8	8	Q-binding, (3), (4)
FRDD	H85 [f]	0.385	2	0	0	–
FRDD	H88 [f]	0.649	3	6	1	–
FRDD	H93 [f]	0.471	3	7	7	–
D. *P. vulgaris* QH_2:fumarate reductase						
FRDC	H83 [f]	0.314	3	7 [h]	4	Q-binding, (4)
FRDD	H81 [f]	0.812	3	6	5	–
FRDD	H84 [f]	0.452	3	8	3	Q-binding, (3)
FRDD	H85 [f]	0.573	2	0	0	–
FRDD	H88 [f]	0.665	1	7	1	–
FRDD	H93 [f]	0.498	2	0	0	–

[a] Names of the genes codifying the protein subunits [2,7,8,9,18,27].
[b] Index calculated from position −3 to position +3 as in Ref. 20.
[c] Calculated from position −4 to position +4 (Table II).
[d] Calculated from position −4 to position +4 against all the peptides of the QB data base (except those of the protein examined) as in Ref. 22.
[e] Scores of similarities at least 6 with the QB data base.
[f] Predicted to be located near or at the negative side of the membrane by hydropathy analysis (Fig. 2 and Refs. 1, 2, 9, 18, 27).
[g] Predicted to be located near or at the positive side of the membrane.
[h] This shows a score of 11 with H83 in the *FRDC* subunit of *E. coli*.

that exlude its involvement in haem coordination [2,28]. The exclusion of H47 as a potential haem ligand (Table IVB) is also consistent with recent genetic data (Hederstedt, L., personal communication).

III-B. The membrane-anchoring subunits of QH_2:fumarate reductase

No histidine-containing peptide of the membrane-anchoring subunits of the fumarate reductases from *E. coli* and *P. vulgaris* shows more than 3 SER (Table IVC,D). This may be correlated with the lack of evidence for a *b*-haem associated with these systems (Cecchini, G. and Ingledew, J., personal communication; see also Ref. 7). However, the fumarate reductase of other Gram-negative bacteria is known to possess a functionally active *b*-cytochrome, as in *W. succinogenes* [29]. In view of this intriguing discrepancy, the prediction procedure developed here is useful essentially for identifying structural similarities which may be related to the interaction with quinones in the membrane (see Table III). Indeed, H83 in the FRDC subunit of *E. coli* shows the largest similarity score with the QB data base (Table IVC) and is involved in the redox communication with QH_2 as revealed by studies of its mutagenesis [30]. H84 in the FRDD subunit ranks second in the sequence similarity with the QB peptides amongst all the other histidine-containing regions in both *E. coli* and *P. vulgaris* (Table IVC,D).

Cytochrome *b*-563 of the fumarate reductase from *W. succinogenes* is bound to a single subunit [29], the sequence of which (Kortner, C. and Kröger, A., per-

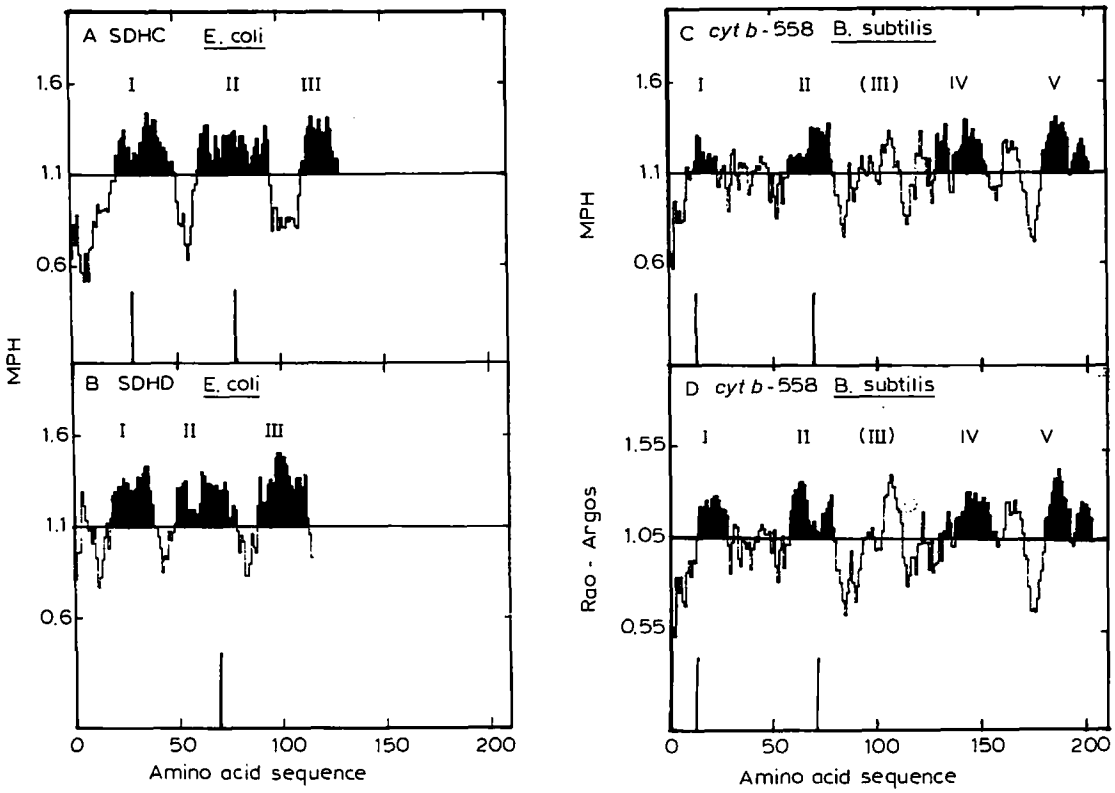

Fig. 2. Hydropathy plots of the membrane-anchoring subunits of bacterial succinate:Q reductase. The MPH hydropathy plots [42] of the SDHC and SDHD subunits of the succinate:Q reductase from *E. coli* [18] are shown in A and B. The N-terminus domain contains a net positive charge in both subunits [18], and is consequently predicted to be located at the negative (cytoplasm) side of the membrane [1,44]. The hydropathy plots of cytochrome *b*-558 from *B. subtilis* [9] obtained with the MPH method [42] and the Rao-Argos method [43] are shown in C and D, respectively. Both methods predict that the former helix III [2,9] is actually unlikely to transverse the membrane, because it does not fulfil the criterion of length. The short N-terminus domain of cytochrome *b*-558 possesses a net positive charge [9]. Consequently, it is predicted to be located at the negative (cytoplasm) side of the membrane [44]. The nomenclature of the transmembrane segments has been taken from Refs. 9, 18. The bars in the bottom of the plots indicate the histidines that are predicted to be the ligands of the haems (Table IVA,B).

sonal communication) shows similarities with that of *B. subtilis* cytochrome *b*-558, particularly in the predicted location of the haem at the negative side of the membrane.

III-C. The b-cytochromes of QH_2: nitrate reductase and QH_2: DMSO reductase

The QH_2: nitrate reductase complex of *E. coli* possesses a membrane-bound *b*-cytochrome, also called *b*-556, that is coded by the *narI* gene [8]. The sequence of this gene has been recently reported [31] and the deduced protein shows four segments which are consistently predicted to span the membrane by several methods (results not shown, see also [31]). Among the five histidines that are predicted as potential haem ligands (Table VA), H150 may be excluded owing to its location in a very hydrophilic region of the protein [31]. The other predicted ligands are located in the first and fourth transmembrane segment near each side of the membrane, thereby allowing the coordination of two *b*-haems. Potentiometric studies do indicate that two distinct *b*-haems are present in the nitrate reductase of both *E. coli* [8] and *P. denitrificans* [32].

The membrane-anchoring subunit of the QH_2: DMSO reductase contains a quinone-reacting *b*-cytochrome in photosynthetic bacteria [33]. The sequence of this protein has been deduced from the DMSC gene of *E. coli* [34]. H65 and H278 show 4 SER, high scores of similarities with the QB data base (Table VB) and are predicted to be located in transmembrane segments near the negative side of the membrane [34]. Hence, these residues are the possible ligands of the *b*-haem that may be present also in the DMSO reductase of *E. coli*.

III-D. Cytochrome b-562 from Rhodobacter sphaeroides

Table VIC shows the predictions obtained for cytochrome *b*-562 of *R. sphaeroides* [35,36]. There are several reasons to suspect that this cytochrome is structurally related to the *b*-cytochromes of bacterial succinate:Q reductase, even if purified from a preparation of bc_1 complex [35]: (i) the molecular mass of 17 kDa is

TABLE V

Application of the prediction procedure for the haem- and/or quinone-binding sites in the membrane-bound b-cytochromes of bacterial complexes interacting with QH_2

Protein [a]	Histidine peptide	(1) Histidine [b] index	(2) SER [c]	(3) Highest score [d] of similarity	(4) Number of [e] similarities	Predictions and criteria
A. Cytochrome *b*-556 *E. coli*, QH_2: nitrate reductase						
narI	H56 [f]	0.421	3	8	7	ligand, (3), (4)
narI	H66 [g]	0.653	2	9	5	ligand, (3), (4)
narI	H74 [g]	0.194	1	7	2	excluded
narI	H150 [g]	0.444	4	7	3	ligand?, (2)
narI	H170 [g]	0.545	3	8	3	excluded
narI	H176 [g]	0.551	2	0	0	excluded
narI	H187 [f]	1.088	5	11	29	ligand, (2), (3), (4)
narI	H206	0.744	4	8	9	ligand, (2), (4)
B. Membrane-anchoring subunit *E. coli*, QH_2: DMSO reductase						
DMSC	H6 [f]	1.213	4	0	0	excluded
DMSC	H65 [f]	0.812	4	12	16	ligand, (3), (4)
DMSC	H210 [f]	0.304	2	6	3	excluded
DMSC	H278 [f]	0.565	4	11	21	ligand, (3)
C. Cytochrome *b*-562 from *Rb. sphaeroides* (complex II?)						
b-562	H14 [f]	0.630	3	8	3	ligand?, (3)
b-562	H49 [g]	0.332	1	7	2	excluded
b-562	H52 [g]	0.840	3	9	8	ligand?, (3), (4)
b-562	H120 [g]	0.545	4	6	1	ligand?, (2)
b-562	H134 [f]	0.440	3	10	29	ligand, (3), (4)
b-562	H141 [f]	0.203	1	9	9	excluded
D. Subunit I (*b*-558) *E. coli, bd* complex						
cydA	H19 [f]	0.524	2	11	10	ligand, (3) [h]
cydA	H86 [f]	0.535	2	0	0	excluded
cydA	H126 [f]	0.083	0	0	0	excluded
cydA	H186 [g]	0.351	1	9	14	ligand, (3), (4) [i]
cydA	H134 [g]	0.278	2	8	2	excluded

[a] Names of the genes codifying the protein subunits [5,6,19,31,34,36].

[b] Index calculated from position −3 to position +3 as in Ref. 20.

[c] Calculated from position −4 to position +4 (Table II).

[d] Calculated from position −4 to position +4 against all the peptides of the QB data base (except those of the proteins examined in this table) as in Ref. 22.

[e] Scores of similarities at least 6 with the QB data base.

[f] Predicted to be located near or at the negative side of the membrane by hydropathy analysis [1, 2, 9, 18, 27, 87].

[g] Predicted to be located near or at the positive side of the membrane.

[h] It has been recently demonstrated by site-directed mutagenesis that this histidine binds the haem of cytochrome *b*-595 which is also bound to subunit I (Gennis, R., personal communication, and Ref. 94).

[i] This histidine forms one of the two axial ligands of cytochrome *b*-558 as demonstrated by its site-directed mutagenesis [19]. The second axial ligand may be a methionine located near the positive side of the membrane, as deduced by local sequence homology with the methionine ligands of some cytochrome c_1 (Degli Esposti, M., unpublished results).

similar that that of the largest subunit of the *b*-cytochrome in Complex II (Table I); (ii) the sequence of cytochrome *b*-562 has no cysteine and a high content of glycine [36] as in both cytochrome *b*-556 or *E. coli* and cytochrome *b*-558 of *B. subtilis* [2]; (iii) the EPR properties of *b*-562 are more similar to those of the *b*-cytochromes of succinate: Q reductase than to those typical of the *b*-cytochromes of the bc_1 complex [12,37]. It is therefore possible that cytochrome *b*-562 of *R. sphaeroides* corresponds to the largest hydrophobic subunit of the succinate: Q reductase of this bacterium, for most of the preparations of the bc_1 complex are largely contaminated by Complex II (see Ref. 38 and references therein). H134, which may be located within the membrane and close to the negative side (results not shown), is the ligand that is most clearly predicted by the present procedure in this cytochrome (Table VC). Only H14 may form the second histidine ligand at the same side of the membrane (Table VC).

IV. Structural similarities between the putative haem-binding sites in other membrane haemoproteins

IV-A. Bacterial quinol oxidases

E. coli and other bacteria possess two terminal oxidases that oxidize ubiquinol utilizing molecular

TABLE VI

Correlation between the HALS EPR signal and the residues present in some 'critical' positions of the proposed ligand peptides of membrane haemoproteins

Haem	Enzyme	Species	EPR g_z	Ref.	Residue in position [a]			
					−4	−1	+1	+4
b_H [b]	bc_1	beef	3.44	82	C	**M**	V	L
					I	V	L	L
b_L [b]	bc_1	beef	3.78	82	I	**M**	A	A
					F	**F**	**F**	P
b_H [b]	bc_1	yeast	3.60	84	V	**M**	**M**	G
					M	M	L	L
b_L [b]	bc_1	yeast	3.76	84	L	L	A	A
					F	L	**Y**	P
b_{6H} [b]	b_6f	spinach	3.5	51	**M**	L	V	G
					F	**M**	**F**	I
b_{6L} [b]	b_6f	spinach	3.5	51	I	V	**R**	A
					F	L	T	L
b-556 [c]	succinate : Q reductase	*E. coli*	3.57	[d]	A	I	**R**	G
					A	**Y**	V	G
b-560	succinate : Q reductase	beef	3.46	77	unknown			
b-558	succinate : Q reductase	*B. subtilis*	3.5	12	**F**	L	S	G
					P	**Y**	A	G
b-559	Photosystem II	spinach	3.08	57	**Y**	I	S	I
					W	I	G	V

[a] According to the alignment of the sequences and/or the predictions obtained here. The residues in bold are long and bulky enough for exerting a steric strain on the imidazole coordination of the haem in the 'critical' position (see text for further details).
[b] According to the folding model proposed in Refs. 10, 42 (cf. Fig. 3).
[c] Assuming that the haem is bound to H30 and H84 in the *SDHC* subunit (Table IV, see also Refs. 6, 37).
[d] Ohnishi, T., personal communication.

oxygen as the electron acceptor, the *bo* complex and the *bd* complex [5,6]. Each of these enzymes contains two *b*-type haems that are involved in either the oxidation of quinol or the reduction of oxygen and are bound to hydrophobic subunits. Such subunits are therefore membrane haemoproteins that have a common function to either cytochrome *b* of the bc_1 complex or cytochrome a_3 of cytochrome oxidase [5,6,19,39]. From this hybrid function it can be inferred that the haem-binding sites of these quinol oxidases may have properties similar to either those of the *b*-cytochromes that interact with quinones or those of other haemoproteins that interact with oxygen.

The largest subunit of the *bd* complex in *E. coli* is known to bind cytochrome *b*-558, which is directly involved in quinol oxidation [5,19]. Recently, it has been demonstrated that this subunit also binds the *b*-595 haem, which reacts with oxygen (Gennis, R., personal communication). On the basis of criteria 3 and 4 of the prediction procedure, H19 and H186 are the most likely haem ligands in subunit I of the *bd* complex (Table VD). This prediction is entirely in agreement with the site-directed mutagenesis of all the histidines of the protein which has established H186 as one of the two axial ligands of cytochrome *b*-558 [19] and H19 as the axial ligand of cytochrome *b*-595 (Gennis, R., personal communication). These two residues are predicted to lie at the opposite sides of the membrane (results not shown, see also Ref. 19), consistent with their likely topological arrangement [5].

The application of the prediction procedure to the sequences of the subunits in the *bo* complex (Gennis, R., personal communication) indicates that only two histidine-containing peptides of the cyoC subunit show 4 SER (results not shown). This subunit shows some weak similarity with COIII of mitochondrial and bacterial cytochrome oxidase [19]. However, the largest subunit of the *bo* complex, cyoB, is a much better candidate for binding the haems than the cyoC subunit, in view of its clear similarity with COI [19,39]. Several histidine-containing peptides of cyoB show a significant sequence homology with some peptides of the QB data base (results not shown), but the most striking homologies are seen between six histidines and the conserved histidines in positions 61, 240, 290, 291, 368 and 376 of bovine COI (results not shown). It is likely that these residues are important in the coordination of the copper(s) and haems in both the cyoB subunit of the *bo* complex and COI of cytochrome oxidase. Clearly, the possible assignment of the ligands of each metal centre is extremely uncertain due to the fact that either the copper or the haem irons are coordinated to histidines

[5,39]. Hopefully, the site-directed mutagenesis of the histidines of cyoB will identify the ligands of some metal centres.

IV-B. The a-haems in COI of cytochrome oxidase

Although many sequences of COI are now available, the identification of the ligands of the two *a*-haems and of the copper (CuB) that are bound to this subunit of cytochrome oxidase [39] is far from being assessed. Two recent models have proposed different ligands for haem a_3 and similar coordinations for haem *a* [20,39]. However, both models are probably incorrect because they are based on structural predictions which have not considered all the sequences available now. If the sequence of the cyoB subunit of the *bo* complex is compared with the sequences of mitochondrial COI, only the six residues mentioned above appear to be conserved in equivalent hydrophobic positions of all systems. This restricts the number of possible combinations that may be compatible with the known spectroscopic features of the metals. However, the modelling of the metal-binding sites is severely complicated by the uncertainties of the folding deductions obtained for COI by different methods and in different species [20,39]. The systematic search of local sequence similarity with haemoproteins of known structure that have some functional or structural relationship with cytochrome oxidases, e.g., they react with oxygen as cytochrome $P\text{-}450_{cam}$ [20] or bind copper as haemocyanin, may help the identification of the residues that bind each metal of COI.

IV-C. Cytochrome b-561 of chromaffin granule vesicles

Cytochrome *b*-561 of mammalian chromaffin granules has been recently sequenced [40] and constitutes a unique type of membrane haemoprotein, since it reacts with ascorbate and is not associated with other redox subunits (see Ref. 40 and references therein). There is now conclusive spectroscopic evidence for the presence of two haems in this cytochrome [41]. Four histidine residues can be easily selected as the likely ligands of the two haems by the procedure described here and their predicted transmembrane location is compatible with one *b*-haem exposed to each side of the chromaffin vesicles membrane [41].

V. Folding and evolution of membrane haemoproteins

V-A. Common folding patterns in membrane haemoproteins

Information on the transmembrane folding of membrane haemoproteins can be obtained from an accurate analysis of the hydropathy profile of their sequences [10,17,19,20,39,41,42]. As discussed before, the prediction of the possible iron-binding sites must be integrated with a careful estimation of the number of transmembrane segments, as only the histidines that can be located at the same face of the membrane or within the same half of the lipid bilayer can be selected as potential ligands of one haem [2,17,39]. The statistical methods of membrane propensity for haemoproteins (the MPH method [42]) and of Rao and Argos [43] should be preferred to other procedures because they are more precise in excluding from the membrane hydrophobic segments that are unlikely to transverse the bilayer, such as helix IV of cytochrome *b* of the bc_1 complex [10,42].

The membrane sidedness of the hydrophilic segments connecting the transmembrane shafts can be estimated also from the distribution of charged residues at the two sides of the membrane, which should conform to the membrane potential of the energy-transducing membranes (negative inside and positive outside [10,24,44]). The alpha-helix is certainly the preferred conformation of the transmembrane segments [23,24,39,42–45], even if distortions may be present along the helix axis [42]. Presumably, the transmembrane helices are packed in an antiparallel bundle [42,45], a common structural motif of all-alpha-helix proteins [45–49]. This folding maximizes the helix–dipole interactions that may be particularly strong in the low-dielectric medium of the lipid membrane [45,46]. The haems are sandwiched between the helices with their planes close to the membrane normal, as deduced from several spectroscopic studies [1,17,50,51].

The model of the four-helical antiparallel bundle which is deduced from the above considerations for chloroplast cytochrome b_6 (Fig. 3) may be extended to other dihaem cytochromes that react with quinones, as shown in Fig. 4. A bundle similar to those in Figs. 3 and 4B is seen in the core of the bacterial reaction centre and is formed by interlocking helices of the L and M subunit that bind the cofactors and the quinones [23]. An analogous heterodimer structure may be present in the membrane-anchoring subunits of succinate : Q reductase and QH_2 : fumarate reductase [1]. The connections of the helices may follow different patterns within the bundles, as in soluble proteins with this motif [45–49]. For instance, the two haems of cytochrome *b*-556 of nitrate reductase from *E. coli* may be bound to two antiparallel helices, whereas those in cytochrome b_6 to two parallel helices (cf. Figs. 3 and 4).

V-B. Have the quinone-reacting haemoproteins evolved from a common ancestor?

From the predictions of the likely haem-ligands and the above guidelines, it appears that the transmembrane helices I and/or II of the haemoproteins examined here

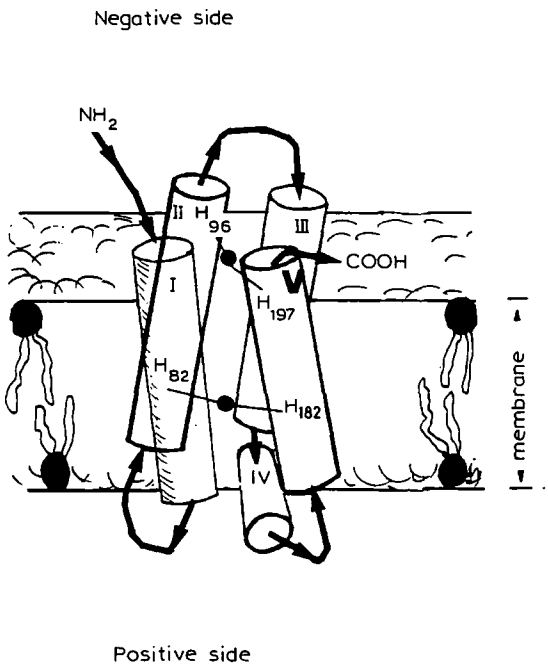

Fig. 3. Model for the folding of cytochrome b_6 from plant chloroplasts. The sequence of cytochrome b_6 from spinach [4] has been evaluated by statistical hydropathy plots which predict only four transmembrane segments [10,42,43]. The transmembrane segments, assumed to be in alpha-helix conformation [10,43], are considered to form an antiparallel helical bundle [42,45–49]. The two black circles represent the irons of the two haems of this cytochrome and are believed to be bound to H82, H96, H182 and H197 [4,10,42].

consistently contain one haem-binding site, generally at the negative side of the membrane (Tables IV, V and Figs. 2–4). In particular, the N-terminal region around the first transmembrane helix shows common structural features which can be summarized in the following points: (i) the hydrophilic loop at the N-terminal side of helix I is enriched in aromatic residues, especially W; (ii) helix I starts with a glycine or another small residue (P or S) that corresponds to the conserved G33 in mitochondrial cytochrome *b* (using the yeast sequence as the reference [42]); (iii) three helical turns towards the membrane interior, there is a predicted histidine ligand (e.g., H56 in cytochrome *b*-556 of nitrate reductase, Table VA) that corresponds to the conserved Q43 of mitochondrial cytochrome *b* [4] and Q18 in *E. coli* DMSC [34] – note that the codons for H and Q differ only for the third base; (iv) one turn further inside the lipid core, there is a glycine that corresponds to the conserved G47 in mitochondrial cytochrome *b*. It is worth noting that several mutations leading to inhibitor (e.g., antimycin) resistance in the bc_1 complex map in this region of cytochrome *b* [15,16], thereby indicating its important functional and/or structural role [3,10,15,16].

Such a local conservation of the protein structure appears to be comparable to that seen in other *b*-type haemoproteins, e.g., cytochome *P*-450 [52], and suggests that the quinone-reacting *b*-cytochromes might derive from a common ancestral protein. The intriguing question then arises of how they have evolved to the *b*-cytochromes of today (Table I). A pattern of divergent evolution requires the existence of simple examples which could be related to an ancestral protein [53]. The gene of this protein might then have evolved by processes of multiplication, diversification and fusion [53–55].

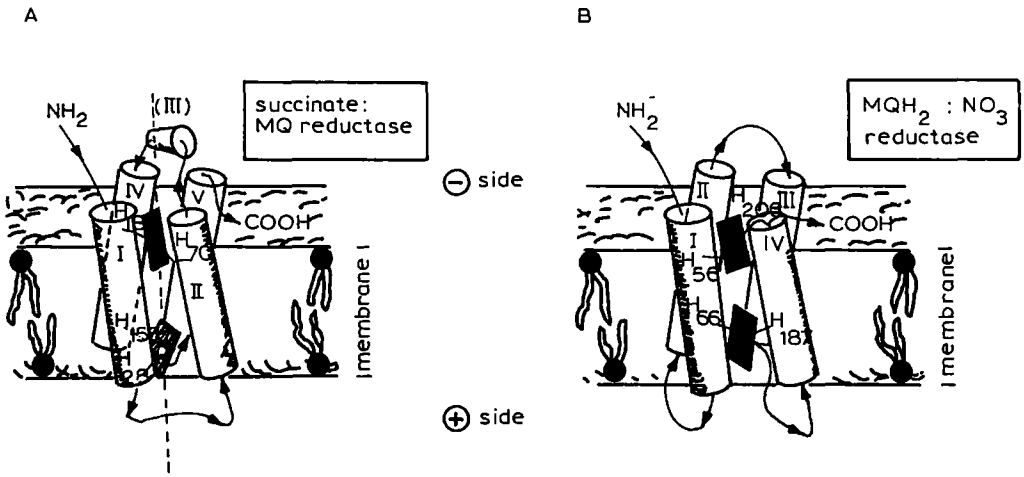

Fig. 4. Models for the folding of quinone-reacting *b*-cytochromes from bacteria. (A) Cytochrome *b*-558 of *B. subtilis* succinate : Q reductase [9]. The model is based on the hydropathy plots of Fig. 2C,D and on the predictions of the most likely histidine ligands of the haem iron (Table IVB). The four predicted transmembrane segments, corresponding to the former helices I, II, IV and V [9], are packed in a transmembrane helical bundle analogous to that proposed for cytochrome b_6 (Fig. 3). The black square represents the haem near to the negative side of the membrane and is proposed to be bound to H13 in helix I and to H70 in helix II. The second haem iron that may be present in cytochrome *b*-558 [2] (hatched square) is tentatively proposed to be bound to H28 in helix I and to H155 in helix IV. The dashed line in the middle of the bundle represents a possible axis of symmetry in the protein, by analogy with other iron-proteins with known structure [23,48,53]. (B) Cytochrome *b*-556 of *E. coli* QH_2 : nitrate reductase [31]. Four transmembrane helices are predicted also in this haemoprotein, the hydropathy profile of which is very similar to that of cytochrome b_6 (cf. Refs. 31 and 42). The black squares represent the two haems that may be bound to the protein [8,32] according to the predictions of Table VA.

The simplest membrane-bound *b*-cytochromes are *b*-561 (dihaem) and *b*-556 (SDHC [6]) of *E. coli*, both of which appear to be organized in three transmembrane segments (Fig. 2A, see also Refs. 1, 6, 18). Hence, cytochrome *b* of the mitochondrial bc_1 complex may have evolved through a triplication and fusion of an ancestral gene somehow related to the *b* cytochrome of succinate : Q reductase. This idea derives from the observations that: (i) nine hydrophobic regions are present in mitochondrial cytochrome *b* [4,17] (even if the fourth is now excluded to span the membrane [10,15,16,42]); (ii) the intron–exon junctions in the cytochrome *b* gene of yeast and fungi are clustered between helix III and IV and between helix VI and VII [15,16,56], the expected joining regions of three similar proteins, each of which has three helices [53,54]; (iii) the genes of *E. coli* SDHC and SDHD are overlapping [18] and their hydropathy profile, if joined together, matches well that of the haem-binding domain of mitochondrial cytochrome *b* [54]; (iv) cytochrome b_6 is associated, in the b_6f complex, with a protein that is homologous to the C-terminus region of mitochondrial cytochrome *b* comprising helices VII, VIII and IX [4] and shows a hydropathy profile which is nearly superimposable to that of *E. coli* SDHC [54].

The comparison of the hydropathy plots is useful for deducing evolutionary relationships, since the hydrophobicity profile is much more conserved than the sequence in distantly related proteins [42,43,52,53]. For instance, the good match of the hydropathy profile of cytochrome *b*-561 from chromaffin granules [40] with that of a duplicated *E. coli* SDHC [54] suggests that this haemoprotein may have evolved via a process similar to that postulated above.

VI. EPR spectroscopy and the structure of the haem binding sites

EPR spectroscopy is the biophysical technique most extensively applied to the study of the structure of the metal centre in membrane haemoproteins (Table I, see also Refs. 2, 11, 12). The *b*-cytochromes that interact with quinones generally show a characteristic highly anisotropic low-spin EPR signal (HALS) [11,12]. The unusually large g_z values of these HALS signals (typically $g_z = 3.4–3.8$ [11]) is considered to arise from a strained bisimidazole coordination of the haem iron in which the two histidines are forced to be nearly perpendicular to each other [11]. The most favourable coordination of bisimidazole haems has, instead, the two ligand histidines in a (nearly) coplanar configuration, as in the known structure of cytochrome b_5 [11]. This 'relaxed' coplanar geometry produces signals having a g_z value around 2.95; similar g_z values are also found in the EPR spectra of some membrane haemoproteins such as *b*-559 [57] and the purified, denatured bovine cytochrome *b* [45,58]. The molecular reasons for the strained configuration of the histidine ligands in native cytochrome *b* and in other cytochromes are still obscure [11,12,45,58].

A possible explanation for the HALS signal is that the local environment of the ligand peptides induces a steric hindrance on the imidazole coordination of the haem, for instance through the contact with bulky side-chains of neighbouring residues [58]. Fig. 5 compares the average residue volume from position −4 to position +4 around the proposed ligands of cytochrome *b* and b_6 with that of the same positions around the known ligands of soluble haemoproteins that exhibit a 'relaxed' EPR signal [11]. There are significant differences in the average volume, particularly in position −1 and +1, in which the *b*-cytochromes of the bc_1 and b_6f complexes possess much bulkier residues (Fig. 5). Protein modelling studies indicate that only some bulky residues are *long* enough to produce a sterically hindered conformation of a histidine bound to a haem iron (Degli Esposti, M., unpublished results). These residues are F, K, H, M, R, W and Y in position −1, and F, K, M, R and Y in positions +1, −4 and +4, owing to the geometry of the α-helix [25]. Scrutiny of the sequences of the *b*-cytochromes exhibiting the HALS signal (Table VI) shows that these long bulky residues are present in at least one of the two proposed binding peptides for each *b*-haem.

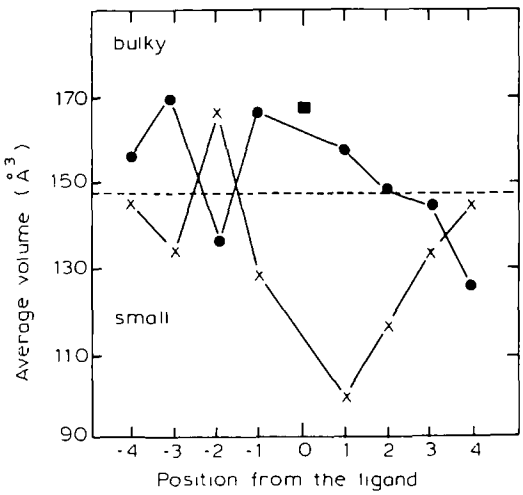

Fig. 5. Average residue volume in the ligand nonapeptides of cytochrome *b* of mitochondrial bc_1 complex and in soluble haemoproteins with known structure and EPR properties. ●———●, average volume of the residues surrounding the proposed ligands in cytochrome *b* and b_6 (Appendix 1), obtained by multiplying the specific volume of the residues in the crystal structure of proteins [25] and their frequency in each position of the nonapeptides. ×——×, average volume for the residues surrounding the known ligands of soluble haemoproteins that exhibit a relaxed EPR signal [11], selected from those examined in Fig. 1. The dashed line indicates the average volume obtained using the transmembrane composition of the residues in the haem-coordinating domain of cytochrome *b* [42]. The black square represents the specific volume of histidine [25], corresponding to position 0.

From these observations, the following molecular explanation of the HALS signal is proposed: the unusually large g_z values derive from the steric hindrance imposed on one of the two ligand histidines by long bulky residues, such as F, K, M, R, W and Y, that are located in one or more of the 'critical' positions, −4, −1, +1 and +4, of the ligand peptide in the α-helix conformation. The loss of α-helix conformation consequent to protein denaturation removes these physical constraints, and may allow the relaxing of the imidazole coordination, as in isolated cytochrome *b* from mitochondrial bc_1 complex [45,58]. The correlation existing between the structural properties of the haem-binding peptides and the shape of their EPR spectra (Table VI) supports the above explanation.

In spite of its clear usefulness in studying membrane proteins, EPR spectroscopy suffers of some drawbacks, since it requires high concentrations of haem and is applicable only to the ferric iron [11]. The latter problem renders this technique unsuitable for studying redox-linked conformational changes in haemoproteins.

VII. The potential of CD spectroscopy for studying the structure of membrane haemoproteins

CD spectroscopy has been applied, so far, to a few membrane haemoproteins (Table I). Only the bc_1 complex [59–61] and mitochondrial cytochrome oxidase [62] have been extensively studied with this technique. Valuable information, however, can be obtained from the CD spectra in the visible region of haemoproteins, where the dichroic signals are due only to the interactions of the haem with its protein environment [62]. The information includes: (i) detection of redox-linked conformational changes [59,62]; (ii) presence of haem–haem interactions in the dihaem or multihaem system [41,60–62]; (iii) identification of surrounding aromatic residues that may be important in electron-transfer process [59]; (iv) probing the membrane sidedness of a haem on the basis of the interactions with aromatic residues [61].

Clearly, the application of CD spectroscopy is particularly useful when the sequence of a haemoprotein is known and folding models can be derived from it [59]. For instance, the molecular interpretation of the CD spectra of oxidized cytochrome *b* in various bc_1 complexes [61] favours the folding model of Crofts [10] rather than that of Saraste [17]. Another example of the usefulness of CD spectroscopy is offered by the theoretical interpretation of the CD spectra of dihaem systems such as the *b* cytochromes of the bc_1 complex [60] and the *a*-cytochromes in COI [39]. In the former system, the intense bisignate CD spectra in the reduced state are indicative of haem–haem interactions that can be rationalized assuming an interhaem distance of about 2 nm with the planes of the haems in a chiral configuration [60]. A chiral configuration of the *a*-haems is also postulated in Ref. 39, where the interhaem distance is considered to be less than 1.6 nm. This geometry theoretically implies that the CD spectra of cytochrome oxidase should exhibit a very strong signal derived from haem–haem coupling, by analogy with the model of cytochrome *b*. However, the CD spectra of cytochrome oxidase do not show this signal (Palmer, G., personal communication; see also Ref. 62 for a review on this subject). This indicates that either the two *a*-haems are not in a chiral configuration, e.g., nearly parallel to each other along the membrane normal, or their distance is much more than 1.6 nm [20].

VIII. Perspectives of research on membrane haemoproteins

Until the three-dimensional structure of one or more membrane haemoproteins is elucidated, the molecular aspects of these important proteins can be studied only by an integration of the information derived from complementary approaches. At the present stage, the models which can be derived from the analysis of the sequences are quite tentative because the amount of integrated information is still scarce. More and more sequences for membrane haemoproteins are needed for deducing with more accuracy the possible folding and the location of the haem-binding sites. However, the models suggested herein provide demarcated hypotheses to be experimentally tested by utilizing biochemical, spectroscopic and genetic techniques. In particular, it is hoped that the deductive procedure described here will facilitate screening of target residues for site-directed mutagenesis aimed to locate the haem-binding sites.

It may be expected that when all the entries in Table I are filled with solid data, our knowledge of the structure and function of the membrane haemoproteins that interact with quinones will be profound.

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Appendix 1

QB data base

Peptide	Histidine	Enzyme	Species
CLYMHVGRG	H 96	QH_2:cyt *c* reductase	*Bos taurus*
IAMVHLLFL	H197	QH_2:cyt *c* reductase	*Bos taurus*
IRIMHANGA	H 82	QH_2:cyt *c* reductase	*Bos taurus*
FFAFHFILP	H183	QH_2:cyt *c* reductase	*Bos taurus*
CLFLHIGRG	H 96	QH_2:cyt *c* reductase	*Homo sapiens*
LATLHLLFL	H197	QH_2:cyt *c* reductase	*Homo sapiens*
IRYLHANGA	H 82	QH_2:cyt *c* reductase	*Homo sapiens*
FFTFHFILP	H183	QH_2:cyt *c* reductase	*Homo sapiens*
CLFLHVGRG	H 96	QH_2:cyt *c* reductase	*Ractus ractus*
LAIVHLLFL	H197	QH_2:cyt *c* reductase	*Ractus ractus*
FFAFHFILP	H183	QH_2:cyt *c* reductase	*Ractus ractus*
CIYLHIGRG	H 96	QH_2:cyt *c* reductase	*Xenopus laevis*
ASILHLLFL	H197	QH_2:cyt *c* reductase	*Xenopus laevis*
IRNLHANGL	H 82	QH_2:cyt *c* reductase	*Xenopus laevis*
FFAFHFLLP	H183	QH_2:cyt *c* reductase	*Xenopus laevis*
VVHLHIFRG	H 96	QH_2:cyt *c* reductase	*Zea mays*
LRYVHANGV	H 82	QH_2:cyt *c* reductase	*Strobilocentrotus purpureus*
FFPFHFLFP	H183	QH_2:cyt *c* reductase	*Strobilocentrotus purpureus*
CMYCHIGRG	H 96	QH_2:cyt *c* reductase	*Strobilocentrotus purpureus*
LAVIHLVFL	H197	QH_2:cyt *c* reductase	*Strobilocentrotus purpureus*
MTMIHLLFL	H197	QH_2:cyt *c* reductase	*Drosophila yakuba*
LRTLHANGA	H 82	QH_2:cyt *c* reductase	*Drosophila yakuba*
FFTFHFILP	H183	QH_2:cyt *c* reductase	*Drosophila yakuba*
VMFMHMAKG	H 96	QH_2:cyt *c* reductase	*Saccharomyces cerevisiae*
MVIMHLMAL	H197	QH_2:cyt *c* reductase	*Saccharomyces cerevisiae*
LRILHANGA	H 82	QH_2:cyt *c* reductase	*Saccharomyces cerevisiae*
FFALHYLVP	H183	QH_2:cyt *c* reductase	*Saccharomyces cerevisiae*
FLYLHIGRG	H 96	QH_2:cyt *c* reductase	*Schizosaccharomyces pombe*
LSVMHLIAL	H197	QH_2:cyt *c* reductase	*Schizosaccharomyces pombe*
LRAFHANGA	H 82	QH_2:cyt *c* reductase	*Schizosaccharomyces pombe*
FFSLHYLMP	H183	QH_2:cyt *c* reductase	*Schizosaccharomyces pombe*
LVYLHIGRG	H 96	QH_2:cyt *c* reductase	*Aspergillus nidulans*
LALMHLIAM	H197	QH_2:cyt *c* reductase	*Aspergillus nidulans*
VRYLHSNTA	H 82	QH_2:cyt *c* reductase	*Aspergillus nidulans*
FFALHFLLP	H183	QH_2:cyt *c* reductase	*Aspergillus nidulans*
LVYLHIGRG	H 96	QH_2:cyt *c* reductase	*Neurospora crassa*
LVLMHLIAL	H197	QH_2:cyt *c* reductase	*Neurospora crassa*
FFALHFVLP	H183	QH_2:cyt *c* reductase	*Neurospora crassa*
ASLLHLAAL	H197	QH_2:cyt *c* reductase	*Zea mays*
LRYMHANGA	H 82	QH_2:cyt *c* reductase	*Zea mays*
FFSLHHLLP	H183	QH_2:cyt *c* reductase	*Zea mays*
LRYMHASGA	H 82	QH_2:cyt *c* reductase	*Oenothera berteriana*
VVYLHVFPG	H 96	QH_2:cyt *c* reductase	*Helianthus annuus*
LLYIHIFKS	H 96	QH_2:cyt *c* reductase	*Trypanosoma brucei*
ILILHLFCL	H197	QH_2:cyt *c* reductase	*Trypanosoma brucei*
IRSVHICFT	H 82	QH_2:cyt *c* reductase	*Trypanosoma brucei*
LHVLHVLLP	H183	QH_2:cyt *c* reductase	*Trypanosoma brucei*
LLYVHIFKS	H 96	QH_2:cyt *c* reductase	*Leishmania tarantolae*
VIFMHLFCL	H197	QH_2:cyt *c* reductase	*Leishmania tarantolae*
IRSTHICFT	H 82	QH_2:cyt *c* reductase	*Leishmania tarantolae*
FFAFHFLFP	H183	QH_2:cyt *c* reductase	*Paracentrotus lividus*
AVYAHTFRG	H 96	QH_2:cyt *c* reductase	*Rhodopseudomonas viridis*
VVMLHVWAL	H197	QH_2:cyt *c* reductase	*Rhodopseudomonas viridis*
IRYIHMNGA	H 82	QH_2:cyt *c* reductase	*Rhodopseudomonas viridis*
FYSLHYLLP	H183	QH_2:cyt *c* reductase	*Rhodopseudomonas viridis*
AVYIHIFRG	H 96	QH_2:cyt *c* reductase	*Paracoccus denitrificans*
LVVVHIVAF	H197	QH_2:cyt *c* reductase	*Paracoccus denitrificans*
LRYLHANGA	H 82	QH_2:cyt *c* reductase	*Paracoccus denitrificans*
FFSLHYLLP	H183	QH_2:cyt *c* reductase	*Paracoccus denitrificans*
MRYIHANGA	H 82	QH_2:cyt *c* reductase	*Rhodobacter capsulatus*
AVYIHIFRG	H 96	QH_2:cyt *c* reductase	*Rhodobacter capsulatus*
LVAIHIWAF	H197	QH_2:cyt *c* reductase	*Rhodobacter capsulatus*

continued

Peptide	Histidine	Enzyme	Species
ASILHRVSG	H 30	succinate : Q reductase, C	*Escherichia coli*
ALAYHVVVG	H 84	succinate : Q reductase, C	*Escherichia coli*
SILIHAWIG	H 71	succinate : Q reductase, D	*Escherichia coli*
FRRLHSLLG	H 13	succinate : Q reductase, A	*Bacillus subtilis*
PLIYHAVYG	H 70	succinate : Q reductase, A	*Bacillus subtilis*
STIFHFSNG	H155	succinate : Q reductase, A	*Bacillus subtilis*
AALLHTKTW	H 83	QH_2 : fumarate reductase, C	*Escherichia coli*
FMIGHMFFV	H 44	QH_2 : fumarate reductase, *b*	*Wolinella succinogenes*
VFIAHAFLA	H 93	QH_2 : fumarate reductase, *b*	*Wolinella succinogenes*
LGSVHLYIM	H143	QH_2 : fumarate reductase, *b*	*Wolinella succinogenes*
AVELHGSVG	H182	QH_2 : fumarate reductase, *b*	*Wolinella succinogenes*
SNLFHIGIL	H 56	QH_2 : nitrate reductase, I	*Escherichia coli*
GIFVHFFGM	H 66	QH_2 : nitrate reductase, I	*Escherichia coli*
IFRLHLVLG	H187	QH_2 : nitrate reductase, I	*Escherichia coli*
SRLIHIWSV	H206	QH_2 : nitrate reductase, I	*Escherichia coli*
INMIHVSCG	H 47	*b*-561 dihaeam	*Escherichia coli*
TGLAHLGHL	H 86	*b*-561 dihaeam	*Escherichia coli*
LKSWHETLA	H139	*b*-561 dihaeam	*Escherichia coli*
VIGLHAAAA	H152	*b*-561 dihaeam	*Escherichia coli*
QITLHWAIA	H 14	succinate : Q reductase?	*Rhodobacter sphaeroides*
GHYLHVVVG	H 52	succinate : Q reductase?	*Rhodobacter sphaeroides*
SAGPHVLAA	H120	succinate : Q reductase?	*Rhodobacter sphaeroides*
LALVHAVSA	H134	succinate : Q reductase?	*Rhodobacter sphaeroides*
TAMYHFLFV	H 19	*bd* complex, *b*-595	*Escherichia coli*
VKFVHTVAS	H186	*bd* complex, *b*-558	*Escherichia coli*
MMILHVFRV	H 96	b_6f complex, b_6	*Nicotiana tabacum*
FMLMHFPMI	H197	b_6f complex, b_6	*Nicotiana tabacum*
MMILHVSRV	H 96	b_6f complex, b_6	*Spinacium oleracea*
FMLMHFLMI	H197	b_6f complex, b_6	*Spinacium oleracea*
IRSVHRWSA	H 82	b_6f complex, b_6	*Spinacium oleracea*
FYSLHTFVL	H182	b_6f complex, b_6	*Spinacium oleracea*
TKVLHGLLH	H109	*b*-561 chromaffin grana	*Bos taurus*
LYSLHSWCG	H143	*b*-561 chromaffin grana	*Bos taurus*
HGLLHVFAF	H113	*b*-561 chromaffin grana	*Bos taurus*
YRPQHVFFG	H182	*b*-561 chromaffin grana	*Bos taurus*
YWVIHSITI	H 23	Photosystem II, *b*-559 A	*Spinacium oleracea*
WLAIHGLAV	H 18	Photosystem II, *b*-559 B	*Spinacium oleracea*
WLAVHTLAV	H 23	Photosystem II, *b*-559 B	*Synechococcus*

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