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The structure of the dihaem cytochrome *b* of fumarate reductase in *Wolinella succinogenes*: circular dichroism and sequence analysis studies

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The fumarate reductase from *Wolinella succinogenes* contains two haem groups with markedly different midpoint potentials (−20 mV and −200 mV). The enzyme is made up of three subunits, the lipophilic one of which (cytochrome *b*) ligates the haems. Circular dichroism (CD) spectroscopy has been applied to the reductase in order to obtain information on the structure of the haems and of their environment. This approach is integrated with amino acid sequence comparison of the cytochrome *b* with other quinone-reacting membrane haemoproteins for predicting the axial ligands of the haems as well as their location relative to the membrane. The following results have been obtained: (1) the CD spectra in the Soret region show exciton coupling indicating haem–haem interaction, which is particularly evident in the reduced state and disappears upon denaturation of the enzyme; (2) The apoprotein of cytochrome *b* is predicted to consist of five hydrophobic helices (helices A–D and cd), four of which should span the membrane. Helices A, B, C and cd contain a histidine residue each which possibly forms one of the ligands of the haems. It is proposed that haem *b* (−20 mV) is ligated by H44 and H93, and haem *b* (−200 mV) by H143 and H182.

Introduction

The cytochrome *b* of the fumarate reductase of *Wolinella succinogenes* forms the anchor of the enzyme in the bacterial membrane and reacts with the donor of fumarate reduction, menaquinol [1–4]. In a preceding communication it was proposed that the cytochrome *b* consisted of a single polypeptide that would bind two haem groups [2]. In this respect the cytochrome *b* of fumarate reductase would resemble those of the various quinol : acceptor reductases, e.g., the bc_1 complex, that form two homologous families of proteins [5]. However, no obvious homology between the *Wolinella* cytochrome *b* and those of the bc_1 complexes was noticed from simple alignment of the amino acid sequences with standard procedures [2]. Furthermore, the redox potentials of both the haem groups in the dihaem *b* of bc_1 complexes are clearly more positive than one of the *b* haem in the *Wolinella* fumarate reductase ($E_m = -200$ mV), which is also much more electronegative than the donor of the enzyme, menaquinol ($E'_o = -75$ mV in ethanol). Isolation of the cytochrome *b* from the fumarate reductase resulted in a homogeneous protein which contained 0.3 mol of the high-potential *b* haem ($E_m = -20$ mV) per mol of protein, but no low-potential *b* haem [1]. This preparation confers quinone reactivity to the fumarate reductase depleted of cytochrome *b* [1]. Thus, the localization and function of the low-potential haem is not clear and requires further investigation.

To this end, we have here applied circular dichroism spectroscopy to the *Wolinella* fumarate reductase. Such a technique can discriminate between mono- and dihaem systems [5] from the spectral features in the Soret region [6,7] and also detect redox-linked conformational changes in the haem environment [5–7]. In addition, a detailed analysis of the amino acid sequence of the cytochrome *b* is undertaken with the aim of predicting the axial ligands of the haem groups and understand the structural determinants of their spectroscopic and redox properties.

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Methods

Fumarate reductase was prepared from *W. succinogenes* cultures as previously described [8]. The enzymatic activity with methylene blue and succinate [1,8] was 10 μmol/min per g of protein. The enzyme was stored in 20 mM Tris-HCl (pH 7.7), containing 0.1% Triton X-100, 70 mM NaCl and 1 mM malonate.

The cytochrome *b* content was measured optically from the reduced-minus-oxidized spectrum of the α band, using an extinction coefficient of 22.7 $mM^{-1} \cdot cm^{-1}$ at 562–575 nm [1].

CD spectra were measured at room temperature in a Jasco J-5 spectropolarimeter at a bandwidth of 1 nm under the conditions specified in Ref. 6. The reductase was either diluted to 6–8 μM cytochrome *b* in 25 mM Tris-HCl (pH 8.0) containing 0.1% purified cholate and 20% glycerol [6] or dissolved in the same buffer in which it was stored after preparation. The CD spectra were usually the average of 4–8 scans and corrected for background against the buffer [6].

Analysis of cytochrome *b* sequences was performed by using statistical hydropathy procedures [9,10] for determining the consensus prediction of the likely number of transmembrane segments [5,10]. The histidine-containing peptides were compared systematically for local sequence similarity by using various procedures [5], including the matrix of Levin and Garnier [11] and a data base of several peptides containing histidines known or predicted to bind membrane-buried haems [5]. A similar search of sequence similarity was also undertaken for the histidine-peptides that bind the quinone-reacting non-haem irons in photosynthetic reaction centres (cf. Ref. 7).

Results

CD properties of the fumarate reductase of W. succinogenes

Fig. 1A shows the wide-range CD spectra of the fumarate reductase of *W. succinogenes*, in which all its prosthetic groups manifest their dichroic signals. In the 430–480 nm region of the oxidized reductase, there is an overlapping of the dichroic absorption of the FAD group and of the three iron-sulphur clusters which are bound to the exstrinsic subunits FRDA and FRDB [1–4]. The broad negative band between 480 and 550 nm, however, is to be attributed almost exclusively to the iron-sulphur centres, since it closely resembles the features characteristic of other centres of this kind, such as those of xanthine oxidase [12]. In the 430–400 nm region, the CD absorption specific of the iron-sulphur clusters is obscured by the dominant positive band due to the Soret transitions of the *b* cytochrome (Fig. 1). In the oxidized state, the cytochrome shows a single positive band with λ_{max} at 421 nm, red-shifted from its optical maximum which is at 416 nm (Fig. 1B).

The optical activity of FAD disappears upon reduction [13]; therefore, the wide negative band with minimum at 470 nm that is seen in the reduced reductase (Fig. 1A, - - - - - -) is specifically due to the non-reducible irons of the iron-sulphur clusters [12,14]. The cyto-

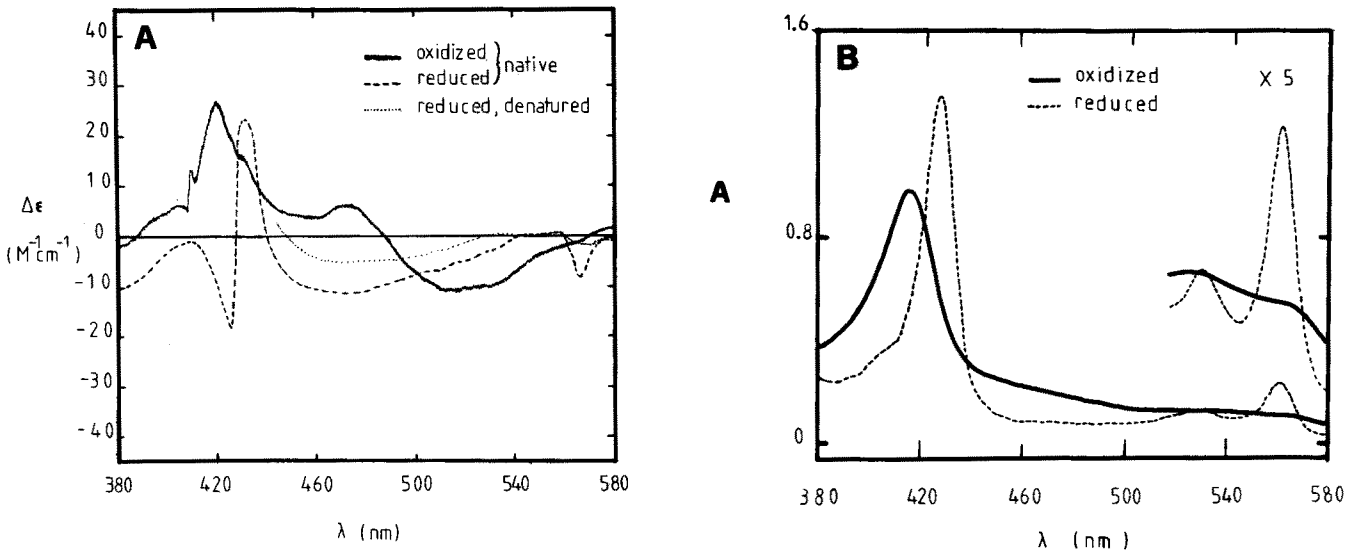

Fig. 1. CD and optical spectra of *W. succinogenes* fumarate reductase. (A) CD spectra of a preparation containing 20 μM cytochrome *b*, dissolved in the same buffer as that employed in the isolation procedure [8], which was oxidized by 50 μM potassium-ferricyanide and subsequently reduced by a few grains of sodium dithionite. The dotted spectrum is that obtained in the reduced reductase denatured after incubation at room temperature for 1 week. No change in the haem or non-haem iron content was detected upon denaturation of the reductase. (B) Optical spectra of a preparation containing 6.7 μM cytochrome *b*. In the insert, the α region is amplified 5-fold. Note that the optical spectrum is very similar to that of purified cytochrome *b* from yeast [6] except for the position of the maximum in the α band, which is at 562 instead of 561 nm.

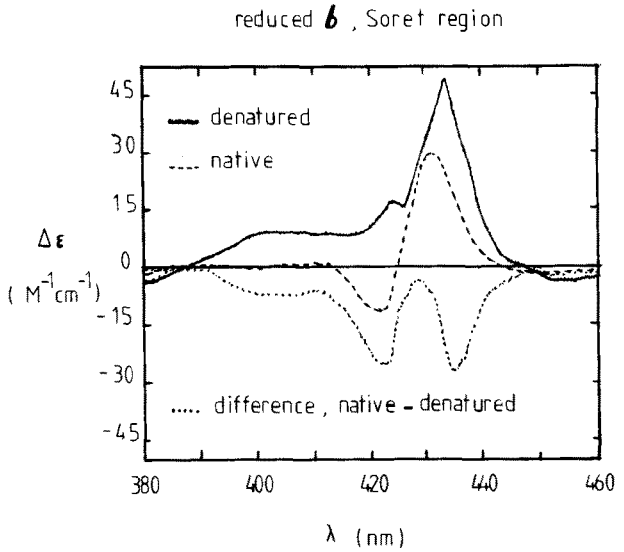

Fig. 2. Resolved CD spectra of the *b* cytochromes in the Soret region. The reductase was suspended at 6.6 μM cytochrome *b* in the Tris-glycerol-cholate buffer (6) and reduced with dithionite. The CD spectrum of reduced xanthine oxidase adjusted to the same signal at 470 nm was substrated from that of native reduced fumarate reductase. This was done to correct for the interfering spectrum of the 2Fe-2S cluster. The denatured enzyme was obtained as in Fig. 1 and its optical spectrum (not shown) was very similar to that of the native enzyme in the Soret region, and 1 nm blue-shifted in the α region.

chrome *b* shows, instead, a negative band at 567 nm in the α region and a bilobic signal in the Soret region (Fig. 1A).

The specific CD spectrum of the reduced *b* cytochrome of fumarate reductase can be resolved in the Soret region from the less intense signals of the 2Fe-2S iron-sulphur cluster, owing to the similarity of the latter with the previously published CD spectra of some iron-sulphur proteins [12–14]. The resolved spectrum of the cytochrome, deduced after subtraction of the estimated contribution of the iron-sulphur centre, is bisignate with a positive band at 430 nm and a negative band at 422 nm (Fig. 2). The zero-crossing that separates the two bands lies at 426.5 nm, which is close to the maximum of the optical band of the reduced cytochrome (428 nm, Fig. 1B). These CD features are typical of an exciton coupling signal originated by two interacting haems [6,15–18]. The sign of the exciton couplet in *W. succinogenes* is opposite to that usually seen in dihaem cytochromes – where the negative band is at longer wavelengths [6,7,15–18] – but similar to that estimated in haemoglobin [17].

Spectral changes of fumarate reductase upon denaturation

The fumarate reductase undergoes a complete inactivation after being incubated at room temperature for 1 week (results not shown). This process is likely to derive from a denaturation of the protein structures that entail function. The spectral changes occurring upon inactivation of the enzyme may pinpoint to some of these functionally relevant structural changes (Figs. 1A and 2). In particular, the Soret CD signal of the *b* cytochrome changes completely its shape in the reduced reductase (Fig. 2), whereas it remains quite unaffected in the oxidized reductase (results not shown). The optical spectra of the reduced cytochrome are just slightly blue-shifted by the inactivation process (results not shown). The CD changes occurring upon inactivation also include a weakening of the signals specific of the reduced iron-sulphur clusters and the almost complete disapperance of the negative band in the α region (Fig. 1A). Addition of external haemin to the inactive enzyme does not restore the CD features of the native cytochrome *b* (M. Degli Esposti, unpublished results), making it unlikely that loss of haem may be responsible for the spectral alterations.

Two concomitant phenomena could account for the variations in the CD spectra of reduced cytochrome *b*: (i) the loss of a symmetrical couplet centered around 426 nm which is due to haem-haem exciton coupling (Figs. 1A and 2); (ii) the change in sign of a strong negative band at 433 nm. CD changes similar to the latter occur frequently upon denaturation of haemoproteins, such as cytochrome *c* [19,20] and cytochrome *P*-450 [21], and derive from the modification of the orientation of an aromatic residue which is close to the haem [20]. On the other hand, the disappearance of the exciton signal may be due to even a slight reorientation of the two haems with respect to each other [17,18].

Local sequence similarity with other cytochromes

The magnetic circular dichroism (MCD) spectra of cytochrome *b* of the fumarate reductase in *W. succinogenes* are characteristic of low-spin haems in that they show a strong bisignate signal in the reduced α region (M. Degli Esposti, unpublished results). The MCD signals in the Soret also appear to be similar to those of the *b* cytochromes having bishistidine coordination that are reported in Refs. 22–28. Hence, although the visible MCD spectra can not completely exclude other types of haem ligand [26–28], it is highly probable that the axial ligands of both the haems of *Wolinella* cytochrome *b* are histidines, as generally seen in quinone-reacting membrane haemoproteins [5,25–28].

Table I shows the results of the application of three independent statistical procedures for predicting the most likely histidine ligands [5] in the apoprotein of the dihaem *b* of *W. succinogenes*. H254 and H255 have not been considered, since they are adjacent to the hydrophilic C-terminus [2]. Clearly, H114 and H120 can be excluded as potential haem ligands, whereas H93 (the only histidine that is surrounded by 4 specifically-enriched residues, cf. Ref. 5), H143 and H44 show the largest probability of binding the haems (Table I). Whereas H44 shows a substantial sequence similarity

TABLE I

Application of prediction procedures for the haem- and/or quinone-binding sites in the membrane-anchoring apocytochrome b of W. succinogenes fumarate reductase

histidine [a] peptide	(1) histidine [b] index	(2) SER [c]	(3) highest score of similarity [d]	(4) number of similarities [e]	Most similar *b*-haems [f]
FMIG H 44 MFFV	0.379	1	10 (9)	11	b_H, *T. brucei*; *b*-558, *B. subtilis* [g]
VFIA H 93 AFLA	0.810	4	8 (7)	7	b_H, *L. tarentolae*
TFKT H114 KDLM	0.444	2	– –	0	
DLMR H120 GDTT	0.322	1	– (7)	0	
LGSV H143 LYIM	0.422	2	10 (7)	14	b_H, man; rat; b_H, *D. yakuba*
AVEL H182 GSVG	0.720	3	8 (12)	3	*b*-556, SDHC *E. coli*

[a] Deduced from the gene sequence [2] from position −4 to position +4 around each histidine, except for H254 and H255 which are close to the C-terminal R256.

[b] Index calculated from position −3 to position +3 [5] that describes the cumulative probability of similarity with known ligand peptides.

[c] Specifically enriched residues [5] that are present from position −4 to position +4 around the histidine. When such residues are 4 or more, there is high probability that the protein stretch interacts with quinones and/or binds a haem in the membrane [5].

[d] Calculated from position −4 to position +4 against 110 peptides known or predicted to bind membrane-buried *b*-haems (QB data base [5]) by using the matrix of Levin and Garnier [11]. Only scores indicating a significant degree of structural similarity (i.e., 6 or more [5,11]) are considered. Note that the maximal score for two identical nonapeptides would be 18. The score shown in brackets refers to the maximal similarity that is seen in the comparison with 30 peptides known to bind the non-haem irons in bacterial and chloroplast reaction centres [30].

[e] Number of significant scores against 110 peptides of the QB data base. 0 means that no score exceeds the minimal value of 6.

[f] *b*-haems and species that show the highest score of similarity with the histidine-containing peptide examined. Only scores of 8 or more are shown.

[g] The peptide around H28 in cytochrome *b*-558 from the succinate:Q reductase of *B. subtilis* [42] that shows a score of 9, the highest among the haems that do not belong to bc_1 complexes.

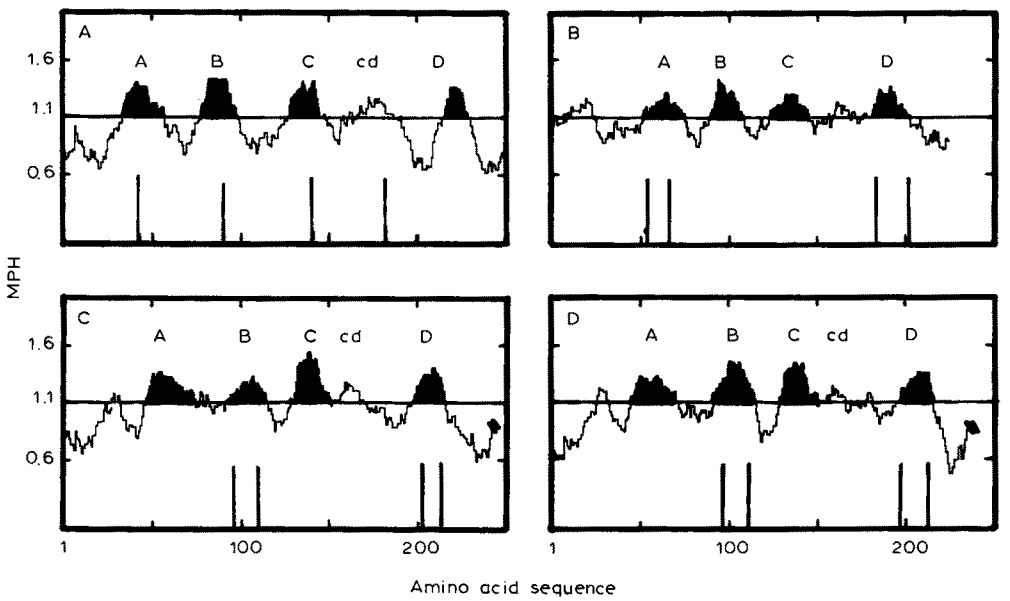

Fig. 3. Hydropathy plots of four bacterial dihaem cytochromes *b*. The statistical method of membrane propensity for haemoproteins (MPH [10,33]) was employed in all cases, with a scanning window of 13 residues (such a window enables the most correct prediction of the known ends of the transmembrane segments in bacterial reaction centres (40]). The nomenclature of the helices conforms to that proposed recently by Crofts et al. [36] for cytochrome *b* of bc_1 complexes, in which A, B, C, cd and D correspond to the former helices I, II, III, IV and V of the previous nomenclature [29,33]. This is extended also to *W. succinogenes* cytochrome *b* [2]. Segments that are predicted to span the membrane also by other statistical methods [9,10] are coloured in black. The vertical bars locate the histidine residues that are predicted to coordinate the haems' iron (Table I and Refs. 5,29). (A) The apocytochrome *b* of *W. succinogenes* [2]. (B) The protein codified by the *narI* gene of *E. coli* [37], which corresponds to the apoprotein of cytochrome *b*-556 of nitrate reductase [5,37]. (C) Apocytochrome *b* of the bc_1 complex of *P. denitrificans* (sequence taken from Ref. [29]). Only the N-terminal domain that binds the haems [29] is shown. (D) N-terminal domain of the protein from *B. japonicum* that binds both cytochrome *b* and c_1 and is homologous to mitochondrial cytochrome *b* [41].

with the putative histidine ligand H28 of cytochrome *b*-558 of *B. subtilis* (Table I, see also Ref. 2), the protein region around H93 shows even a clearer similarity with the ligand peptides located at the C-terminus of the second transmembrane helix in cytochrome *b* of bacterial bc_1 complexes (Ref. 2, cf. Ref. 29).

H182 may be the fourth haem ligand of the two cytochromes. Although it shows fewer sequence similarities with ligand peptides of other *b* cytochromes, this histidine displays some strong sequence similarities with the regions that are known to bind the non-haem iron in the bacterial reaction centres (Table I, cf. Ref. 30). Since all the above four residues appear to lie in hydrophobic regions of the apoproteins [2], it is indispensable to perform a thorough hydropathy analysis of the sequence in order to locate the ligands of each haem [5].

Hydropathy analysis of the apoprotein

The apoprotein of the cytochrome *b* subunit of *W. succinogenes* fumarate reductase is a rather hydrophobic polypeptide and shows five regions that might form transmembrane helices [2]. Such a conclusion was based on hydropathy plots [2] performed with the Kyte-Doolittle [31] algorithm. However, it is now increasingly clear that this method generally overestimates the true number of transmembrane segments in integral proteins with redox function [5,9,10,32,33]. A careful application of the statistical methods of Rao and Argos [9] and Degli Esposti et al. [33] avoids this error [10]. The consensus of the predictions obtained by these methods indicates that helix cd (former helix IV [2]) of *W. succinogenes* cytochrome *b* is unlikely to span the membrane (Fig. 3A).

The comparison with the hydropathy profile of cytochrome *b* of bacterial bc_1 complexes (Fig. 3C,D) strengthens this conclusion, in that also these proteins appear to have five hydrophobic regions in their haem-binding domain [29], the fourth of which is now regarded not to span the membrane [6,9,32–36]. A similar situation applies to the cytochrome *b*-556 of the nitrate reductase of *E. coli* (Fig. 3B) [5,37]. The amphiphilic helix cd of the *Wolinella* cytochrome *b* may be oriented parallel to the membrane plane and be either situated in the aqueous phase or partly embedded into one lipid layer of the membrane. As a consequence, the haem ligated by H182 should be situated in a relatively polar environment.

Discussion

This work describes the integration of the circular dichroic spectroscopy with the sequence analysis of a bacterial membrane haemoprotein, cytochrome *b* of the fumarate reductase from *W. succinogenes*. The exciton coupling signal in the CD spectra of the Soret region (Figs. 1A and 2) indicates that two interacting haems are present in this cytochrome, consistent with biochemical data [1]. The haem–haem interaction is particular evident in the reduced state and disappears upon inactivation of the reductase (Fig. 2). This suggests that conformational transitions are associated with the redox function of the cytochrome which influence the mutual orientation, and consequently the dipole coupling of the two haems. Despite the different type of chiral configuration, a similar conclusion has been previously reached in observing the redox-linked CD changes of mitochondrial cytochrome *b* [6,16,18]. Considering the above, we wish to rename the cytochrome subunit of the fumarate reductase as dihaem *b*-562 of *W. succinogenes*.

The localization of the irons' ligands is crucial for the elaboration of a structural model that can accommodate the two haems of cytochrome *b*-562. Four histidines in the sequence show several local similarities with histidines that bind membrane-bound cytochromes (Table I). They also appear to be located in two pairs at each side of the membrane (Fig. 3). H44 in transmembrane helix A (or I [2]) and H93 in transmembrane helix B (or II [2]) form the pair of possible ligands that are predicted to lie close to the negative side of the membrane (Fig. 3). It is suggested that these residues coordinate the high-potential form of *W. succinogenes* cytochrome *b* [1] because: (i) such histidines are embedded in protein regions that are more hydrophobic than those containing the other pair of histidines (especially H182, Fig. 3) – the redox potential of haemoproteins, in fact, increases by increasing the hydrophobicity of the haem environment [38] –; (ii) this haem is the primary electron acceptor from menaquinol and is in redox communication with the iron-sulphur clusters of the reductase [1] that are also located at the negative side of the membrane [1–4].

H143 in transmembrane helix C (or III [2]) and H182 in the amphiphilic helix cd (former helix IV [2], Fig. 4) may form the axial ligands of the low-potential *b* haem that is present in the *b* cytochrome of the reductase [1]. This second haem is predicted to be located at the positive side of the membrane, at an approximate distance of 24 Å from the other haem by assuming a homogeneous helical conformation of the transmembrane segments. The predicted location of this haem at the water/lipids interphase would imply that its environment is exposed to the aqueous medium, thereby explaining its very low midpoint potential [1] according to the theory of Kassner [38].

Semiquantitative calculations based on the exciton coupling theory [17,18,39] indicate that the interhaem distance in Wolinella cytochrome *b*-562 is longer than that existing in mitochondrial cytochrome *b* or chloroplast b_6 (approx. 20 Å [16,18,29,32,36]), which exhibit more intense CD signals (cf. Fig. 2 and Refs 6, 18). However, the deduced transmembrane folding (Fig. 3) and the sequence similarities around histidine residues

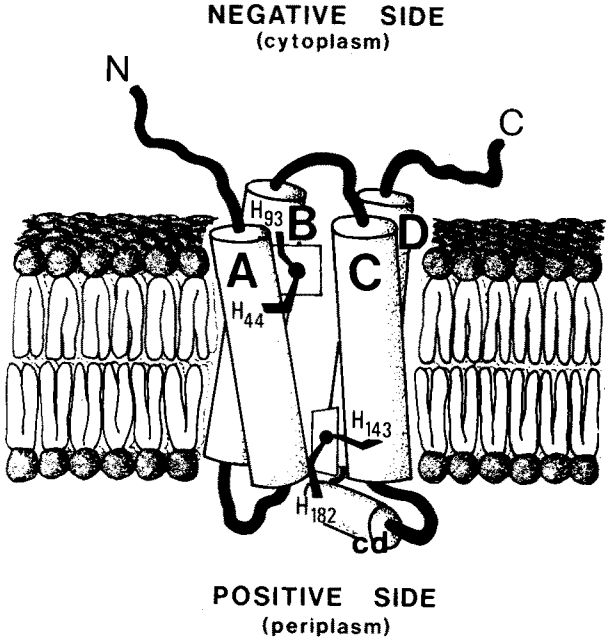

Fig. 4. A putative model for the folding of cytochrome *b* of *W. succinogenes*. The structure is postulated to conform to the general motif of four transmembrane helices packed in an antiparallel bundle [5,33], according to the hydropathy predictions (Fig. 3). The haem exposed at the negative side of the membrane is proposed to correspond to the menaquinol-reducible cytochrome *b* ($E_m = -20$ mV) [1] and should contact the other redox centres of the reductase that belong to the subunits protruding in the cytoplasm [1–4]. The other haem, that is usually not reducible by menaquinol [1], is predicted to be located at the water/lipid interphase of the positive side, due to the exclusion of the region around H182 from the membrane (Fig. 3, see also the text). The proposed ligands of the haems are assigned by considering the sequence similarities with other cytochromes (Table I). Note that the model is different from that of other dihaem cytochromes particularly in the arrangement of the haem at the positive side of the membrane (cf. Refs. 5, 7). The haems are shown in a chiral configuration to account for the observed exciton signal in the CD spectra (Fig. 2).

that may function as haem ligands (Table I and Ref. 2) suggest that the structure of *Wolinella* cytochrome *b*-562 could conform to the general motif of a four-helical transmembrane bundle with the two haems at its centre [5], as illustrated in the putative model of Fig. 4. According to this model, the structure of the high-potential haem of *W. succinogenes* cytochrome *b* would be rather similar to that proposed for the *b* haem exposed at the negative side of the membrane in the succinate : Q reductase of *B. subtilis* [5]. This is consistent with the apparent conservation of the N-terminal domain of several proteins that interact with quinones [5].

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