Location of haem-binding sites in the mitochondrial cytochrome *b*

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Amino acid sequences of 6 mitochondrial cytochromes *b* are analysed to locate the binding sites of the two protohaems. Four invariant histidines are found in two protein segments which span the membrane. In each, two histidines are separated by 13 residues. This would place them on the same sides of α-helices, and the protohaems could be sandwiched as bis-imidazole complexes between the two transmembrane segments. In this model the haems are located in different halves of the bilayer; the Fe–Fe distance is about 20 Å.

Membrane protein *Mitochondria* *Cytochrome* b *Amino acid sequence* *Haem-binding site*

1. INTRODUCTION

Ubiquinol:cytochrome *c* oxidoreductase (EC 1.10.2.2) is a part of the electron transport chain of mitochondria, chloroplasts and some bacteria. It is a multisubunit complex (called cytochrome bc_1 and b_6f in mitochondria and chloroplasts, respectively) which contains different cytochromes and an iron-sulphur centre [1,2]. Two spectroscopically distinct cytochrome *b* components (*b*-562 and *b*-566) with different midpoint potentials are found in the monomeric (or protomeric [1]) unit. In addition, a detailed spectroscopic and kinetic study [3] has proposed that all 4 cytochrome *b* haems of the dimeric complex [4] have distinct properties. Isolation of cytochrome *b* from the complex abolishes spectral differences between the haems [5,6]. However, in one case it has been shown that redox titration of the purified cytochrome *b* is still biphasic [6].

A single gene in the mitochondrial or chloroplast DNA codes for apocytochrome *b* [7,8]. Several nucleotide sequences of the mitochondrial genes have been recently published [9–14]. The predicted proteins have M_r values around 45 000, i.e., considerably higher than the previous estimates around 30 000. There is no indication that apocytochrome *b* would be synthesised as a precursor (see [1,5,6,15]). This leads to corrections of previously calculated stoicheiometries of the cytochrome *b* subunit in the complex, as well as in the haem/protein molar ratio. It now seems that there is only one cytochrome *b* subunit in the monomeric complex and that it binds both protohaems [1,15,16]. Using this model, the amino acid sequences are analysed here to find the most probable haem-binding sites. This study is based on the evolutionary invariance and probable folding of the polypeptide in the membrane. A preliminary account of this work has been published [17].

2. MATERIALS AND METHODS

Amino acid sequences of human [9], bovine [10], mouse [11], *Saccharomyces* [12], *Aspergillus* [13] and maize [14] cytochrome *b* are those predicted from the nucleotide sequences. Amino acid compositions of the isolated proteins [5,6] agree well with the predicted ones. Hydropathic profiles of the polypeptides were calculated using the index for amino acid side chains described in [18].

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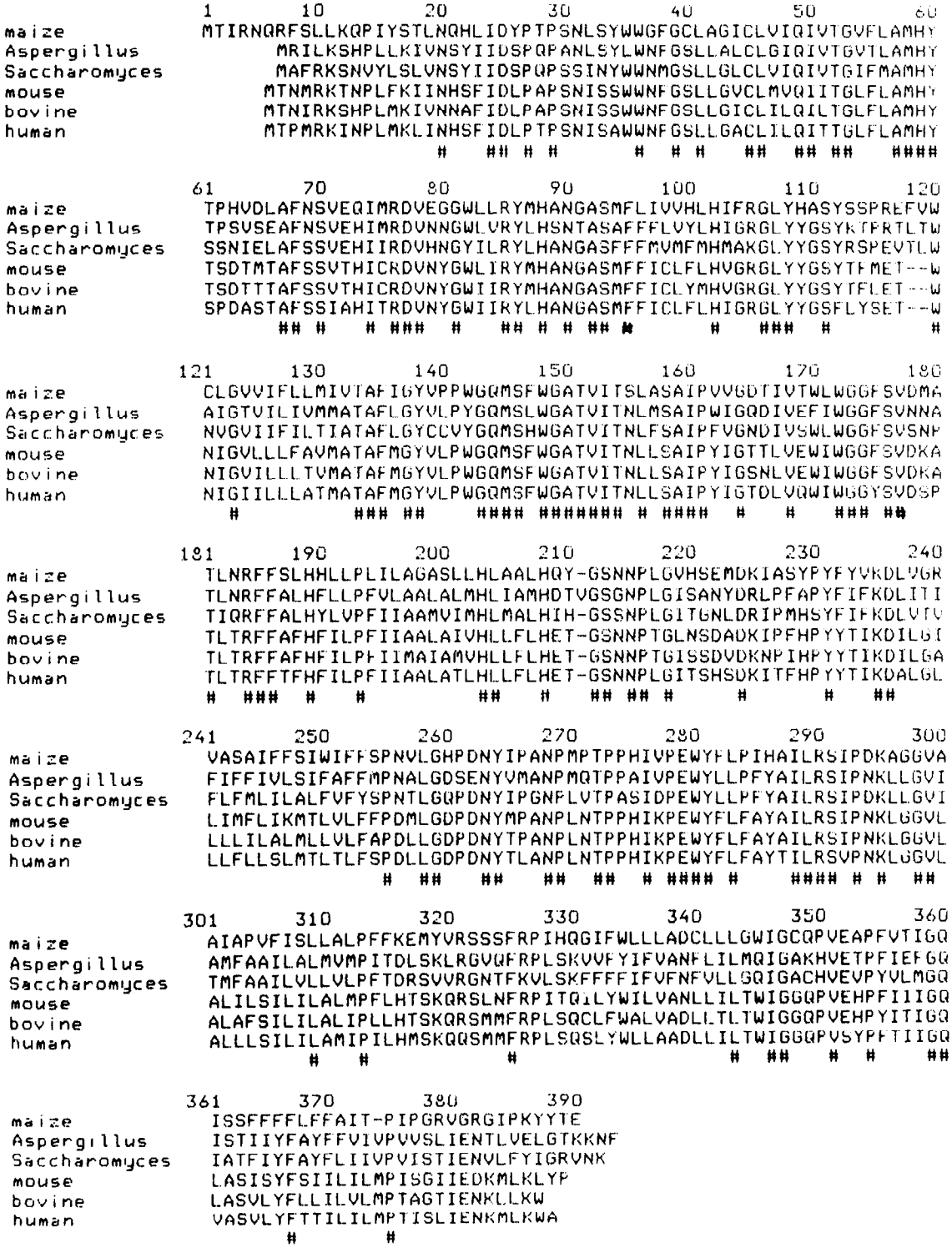

Fig. 1. Alignment of amino acid sequences of 6 cytochromes *b*. Identical residues are underlined with #, and deletions indicated with —. Residue number 240 in the maize protein is probably W and not R. It is coded by CGG which seems to stand for tryptophan in maize mtDNA [14].

3. RESULTS AND DISCUSSION

Six amino acid sequences of apocytochrome *b* are aligned in fig. 1. 30% of the residues are identical in all of them. Conservation is more pronounced in the middle part of the protein and weaker close to the N- and C-termini. All sequences have very similar hydropathic profiles (fig.2).

3.1. *Folding of the polypeptide in the membrane*

Surface labeling studies have shown that cytochrome *b* spans the membrane [19]. Hydrophatic profiles show 9 long hydrophobic segments interrupted by more hydrophilic sequences (fig.2). The hydrophobic sequences are long enough, that is approx. 25 residues, to span the bilayer in α-helical conformation. In this respect the profiles are similar to many others calculated for membrane-embedded proteins [17,18,20,21].

The path of the cytochrome *b* polypeptide in the membrane is schematically shown in fig. 3. Most of the protein is buried in the membrane; only 4 longer stretches are predicted to be on membrane surfaces – residues 1–35 at the N-terminus, residues 61–80, 210–233 and 260–290. The last sequence is very rich in prolines and also highly conserved; in *Saccharomyces* [12] two introns are located within this sequence.

The pattern of conserved residues observed in cytochrome *b* is consistent with assumed α-helical conformations of the trans-membrane segments. In several cases the conserved residues (circled in fig.3) would cluster to one half of a helical surface (segments I, III, V, VII and IX) or form invariant ridges along the helices (segments I, IV and IX). The latter point is interesting because one of the stabile helix/helix contacts employs such ridges [22]. Such conserved faces of the helices must be important either in the packing of the cytochrome *b* molecule itself or in its interactions with other subunits of the complex. Among the 121 identical residues there are 17 glycines and 12 prolines. These amino acids often occur at the ends of the trans-membrane segments and might assist in formation of bends in the polypeptide chain on the membrane surfaces.

3.2. *Haem-binding sites*

The axial ligands of the haems in cytochrome *b* are not definitely known. EPR measurements [23–25] show unusually high g_z values, 3.4–3.8. This does not readily fit with a bis-imidazole coordination but suggests that axial ligands may be one histidine and one amino group [26]. However, authors in [27] have shown that such EPR properties can be generated with bis-imidazole model compounds. They concluded that in cytochrome *b* axial histidine ligands might be subjected to steric strain.

Six invariant histidines and two lysines are found from the sequence alignment (fig.1). Four histidines are embedded in the membrane and two are predicted to reside on one membrane surface (fig.3). The membrane-buried histidines in segments II and V are separated in each case by 13 residues. Thus, on a helical surface, they are located almost exactly above each other on the same side of the helix. Consequently two bis-imidazole protohaems could be sandwiched between segments II and V as shown in fig. 4. Two invariant basic amino acids, arginine in position 85 and arginine or lysine in 105 (fig.1), are located in such a way that they could form salt bridges to propionate side chains of the protohaems (fig.4).

This model places the haems in different halves of the bilayer. The Fe–Fe distance would be approx. 20 Å and each Fe atom would be about 10 Å inside the membrane. The haem planes are known to be perpendicularily oriented with respect to the membrane plane [28]. Sandwiching the haems between α-helices which run roughly perpendicular to the membrane, gives a simple explanation for this unique orientation.

Alternatively, if it should turn out that the axial ligands are a histidine and an amino group, two invariant lysines (235 and 295, fig.1) might be involved in haem binding (see below). They are found at the edges of segments VI and VII close to the different membrane surfaces (fig.3). Also in this alternative model the haems would be located close to the surfaces of the bilayer. Therefore both models imply that electron transfer between two cytochrome *b* haems is electrogenic as proposed in [29,30].

The bis-imidazole model of fig. 4 is preferred for two reasons. The pattern of the invariant histidines in the hydrophobic segments is by itself very strik-

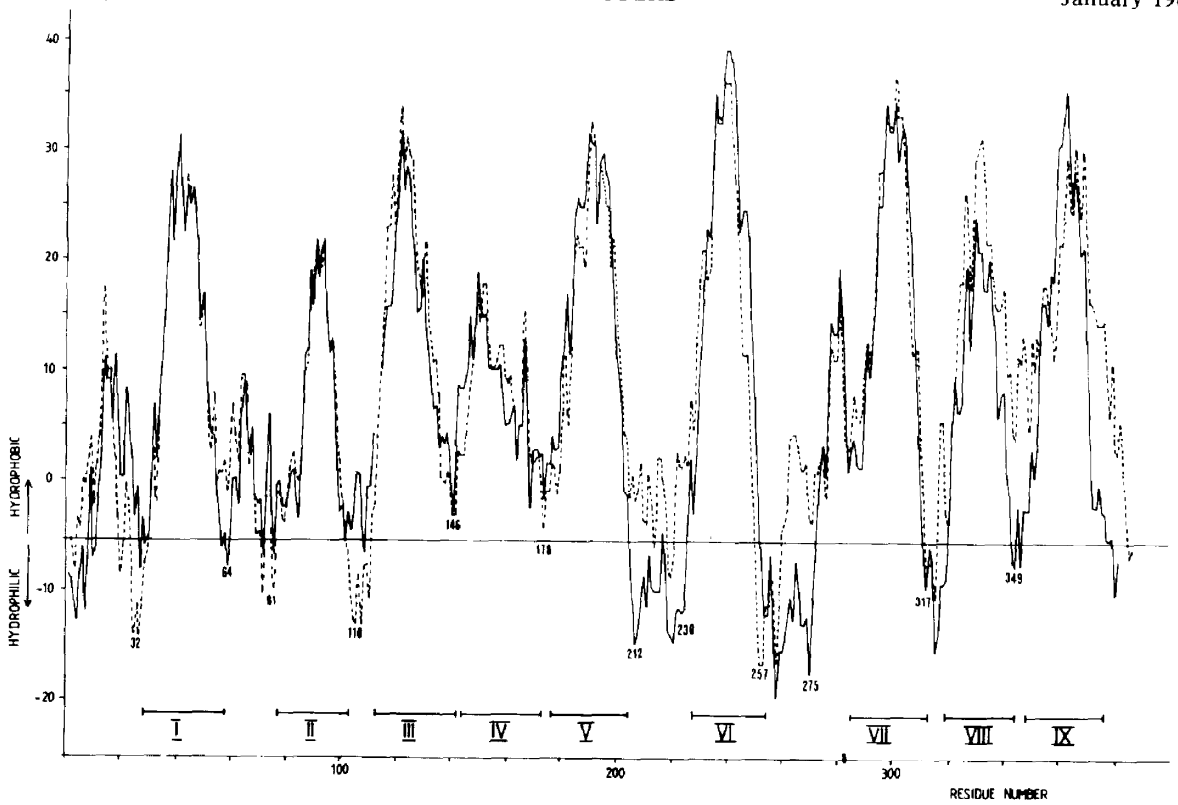

Fig. 2. Hydrophatic profile of the cytochrome *b* polypeptide. The profiles were calculated using the index given in [18] and a sliding window of 11 amino acids. Hydrophobicity is increased upwards, hydrophilicity downwards; the vertical line indicates the mean hydropathy for a peptide of 11 amino acids. Residue numbers on the abscissa refer to the *Saccharomyces* sequence, and numbers inside the figure to the sequence alignment (fig.1). (——) bovine cytochrome *b*; (---) *Saccharomyces* cytochrome *b*. Roman numerals indicate the proposed membrane-penetrating segments.

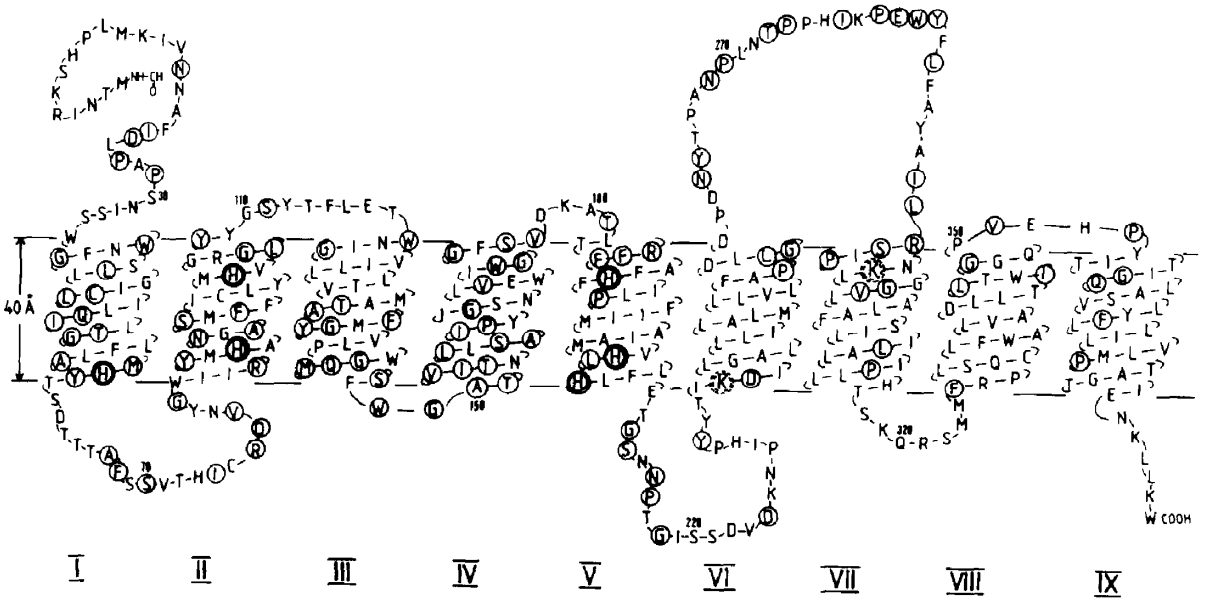

Fig. 3. Schematic model of the cytochrome *b* folding in the membrane. Circled residues are identical in all sequences (fig.1). Six invariant histidines (double circles) and two lysines (double dotted circles) are indicated. The sequence shown is bovine cytochrome *b*, but the residue numbers refer to the alignment of fig. 1.

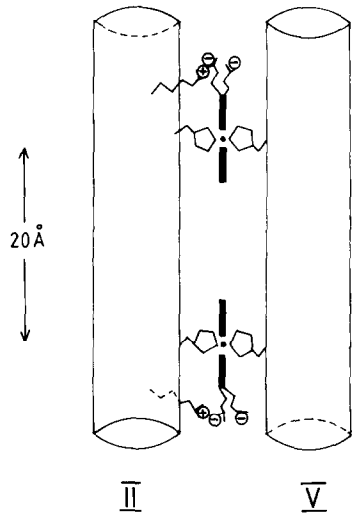

Fig. 4. Probable haem binding sites in cytochrome *b*. Two haems are bound between trans-membrane segments II (histidines 88 and 102) and V (histidines 189 and 203). The invariant basic amino acids (arginine – 85 and arginine/lysine – 105) at the edges of segment II are included in the figure.

ing and readily gives the structural design. Furthermore, the model explains nicely the orientation of the haem planes in the membrane [28]. Secondly, the chloroplast cytochrome b_6 which is related to the mitochondrial protein, is much smaller with M_r 23 000 [8]. It appears to correspond to the N-terminal part of the mitochondrial cytochrome *b* while a small 17 000 subunit of the b_6f complex [8] is homologous to the C-terminal part; the proposed haem-binding histidines are present in cytochrome b_6 [31]. Thus it seems that cytochrome b_6 lacks the part of the mitochondrial protein where the two invariant lysines are found.

Although two protohaems are apparently symmetrical in the model, their spectroscopic and redox properties can be distinguished by the different protein surroundings in the ends of α-helices II and V (fig.3), and by the interaction of cytochrome *b* with other subunits of the complex.

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