Analysis of the structures of the subunits of the cytochrome bc_1 complex from beef heart mitochondria

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The interaction of the protein subunits of the bc_1 complex from beef heart is analysed on the basis of protein chemical data and of secondary structure predictions suggesting a large number of amphipathic helices. Electrostatic interactions, i.e. helix-dipole interactions and ionic bonds, may play a major role in the stabilisation of the arrangement of the subunits within the multi-protein complex, formation of subcomplexes and maintenance of the steric strain of cytochrome *b*. A model of the heme-carrying 'core' of cytochrome *b*, i.e. of helices II–V, is presented consisting of a twisted '4-α-helical' bundle held together by helix-dipole interactions and stabilised by the interaction with other protein subunits of the bc_1 complex.

Ubiquinol:cytochrome c *reductase* bc_1 *complex* *Cytochrome* b *Structure prediction* *Membrane protein* *Helix interaction* *(Beef heart mitochrondria)*

1. INTRODUCTION

The *bc* complexes constitute the middle part of many electron transfer chains in mitochondria, chloroplasts, and bacteria. Although the *bc* complexes from different sources may utilise different electron donors and acceptors and may exhibit certain specificities in their inhibitor binding, they are strongly homologous and catalyse essentially the same reactions, i.e. the electron transfer from a quinone to the respective electron acceptor, a *c*-type cytochrome or plastocyanine, and a concomitant vectorial proton translocation.

Besides the strongly conserved subunits carrying redox centers, the energy-transducing complexes of the inner mitochondrial membrane contain several small proteins. Since the amino acid sequences of the small subunits of the beef heart bc_1 complex have been determined, an analysis of the arrangement of the subunits within the complex may help to elucidate the functional interrelation between catalytic and non-catalytic subunits. Therefore we have developed a new graphic method for the prediction of secondary structures of membrane proteins [1] by modifying the algorithm of Kyte and Doolittle [2]. The prediction of amphipathic membrane-spanning stretches is based on an analysis of the distribution of hydrophobic and hydrophilic residues on the surface of presumptive α-helices and β-sheets. The comparison of average hydropathy, α-helical sided hydropathy and β-sheet sided hydropathy patterns allowed the identification of amphipathic segments not detected by other methods. The work presented here deals with the interactions between these protein domains.

2. STRUCTURAL PREDICTION OF THE SUBUNITS OF THE bc_1 COMPLEX FROM BEEF HEART

The determination of the amino acid sequences of the 6 small subunits of beef heart mitochondrial bc_1 complex has been completed recently [3]. By application of the sided hydropathy plot, membrane-spanning or membrane-anchoring domains have been predicted for all these subunits (table 1) [1]. From these structural predictions we

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Table 1

Predicted membrane-anchoring domains of the subunits of the bc_1 complex from beef heart (FeS protein: *N. crassa*)

Band	Subunit	Segment	Type
III	cyt.*b*		
	I	29– 55	amphipathic
	II	76–103	hydrophobic
	III	113–140	hydrophobic
	IV	143–170	amphipathic
	V	175–202	amphipathic
	VI	226–252	hydrophobic
	VII	282–309	hydrophobic
	VIII	317–342	amphipathic
	IX	347–373	hydrophobic
IV	cyt.c_1	206–222	hydrophobic
V	FeS; (*N. crassa*)	33– 54	hydrophobic
VI	13.4 kDa ('QP-C')	1– 16	amphipathic
VII	9.5 kDa	1– 19	amphipathic
		41– 63	hydrophobic
VIII	9.2 kDa	13– 33	amphipathic
		58– 77	amphipathic
IX	8.0 kDa	10– 35	amphipathic
X	7.2 kDa	20– 43	hydrophobic
XI	6.4 kDa	12– 36	hydrophobic

Predictions according to Link and Von Jagow [1] (subunits IV–XI) and Saraste [8] (cytochrome *b*). Assignment of hydrophobic and amphipathic according to [1]

conclude that all the subunits may be integral membrane proteins or attached to the hydrophobic core of the complex. Deep integration of the small proteins within the membrane is suggested by the fact that most of the subunits bind rather high amounts of detergents, indicating the presence of large hydrophobic surfaces. Several membrane-spanning segments within the small proteins are also expected from the shape of the complex. The low-resolution three-dimensional structure of the bc_1 complex from *Neurospora crassa* indicates that approx. 30% of the protein mass is embedded in the membrane [4], corresponding to approx. 25 membrane-spanning helices of about 20 residues each. This value is compatible with the total number of possible membrane-spanning segments derived from the structure predictions, assuming that the core proteins have only few membrane-spanning segments since they extend largely into the mitochondrial matrix [4].

3. PROTEIN-PROTEIN INTERACTIONS OF THE SUBUNITS OF THE bc_1 COMPLEX AND THE FORMATION OF SUBCOMPLEXES

The structure predictions indicate a number of amphipathic helices for the subunits of the bc_1 complex from beef heart [1]. These helices contain several charged or ionisable amino acid side chains. The question arises as to whether these amphipathic helices span the membrane or belong to the extramembranous domains. Amphipathic helices may be exposed to the aqueous phase if their hydrophobic sides interact with other hydrophobic domains or they may traverse the membrane if their hydrophilic sides are compensated by complementary sides of other amphipathic helices. Charged residues within amphipathic membrane-spanning segments are expected to form ion pairs with other charged residues within the membrane since the occurrence of free charged residues in a hydrophobic environment is very unlikely for energetical reasons. These ion pairs may be essential for the stabilisation of the multi-protein complex.

The interactions between the protein subunits could be studied in detail during the stepwise dissociation of the complex. Different dissociating conditions led to the isolation of different subcomplexes which are assumed to represent functional entities.

Treatment of the bc_1 complex with 0.5% Triton X-100 resulted in the reversible dissociation of the iron-sulfur protein and of the 6.4 kDa protein. The re-association of the iron-sulfur protein can be described by a dissociation equilibrium assuming a K_d of approx. 10^{-6} M, depending on the experimental conditions [5].

Treatment of the bc_1 complex with Triton plus 2 M urea led to a complete dissociation of the iron-sulfur protein. The residual bc_1 subcomplex was destabilised and a subcomplex gradually released consisting of cytochrome *b*, the 13.4 kDa 'Q-

binding' protein and one of the two 'core' proteins. Within this subcomplex, cytochrome *b* maintained its spectral properties for some minutes before becoming denatured.

Treatment of the bc_1 complex with 1.5 M guanidine in the presence of Triton led to the isolation of a 'c_1 subcomplex'. This subcomplex consists of cytochrome c_1, the 9.2 kDa 'hinge' protein and the 7.2 kDa subunit and is the most stable functional unit of the complex. The other proteins did not form specific assemblies but were found either as separate proteins or in aggregated form.

The fact that chaotropic reagents like urea or even guanidine are required to obtain subcomplexes indicates that the interactions between the subcomplexes are not merely Van der Waals forces. In fact, the interactions within the subcomplexes are even stronger than those between different subcomplexes. These interactions are therefore assumed to be ionic. The two small subunits of the c_1 subcomplex, the 9.2 kDa and 7.2 kDa subunit, both contain a large number of charged residues within their amphipathic helices. The iron-sulfur protein and the 6.4 kDa protein, the only subunits that do not have charged residues within their membrane anchors and therefore are unable to bind to the rest of the complex through ion pairs, are the only subunits that are split off the complex without the use of chaotropic reagents.

4. THE STERIC STRAIN OF CYTOCHROME *b*

Cytochrome *b* the central component of the bc_1 complex, is a single polypeptide comprising two heme centers [6,7] in a transmembrane arrangement [8,9]. Cytochrome *b* is in a highly tensed state in the native bc_1 complex [10]. In the EPR spectrum, the g_z values of cytochrome *b* (3.4 and 3.78, respectively) indicate dihedral angles of almost 90° of the two pairs of histidines, each binding to one heme group; lower g_z values would indicate a more parallel alignment of the planes of the histidine pairs [11]. Isolation of cytochrome *b* led to a decrease of the g_z values and a concomitant decrease of the observed redox potential E_m (table 2; Von Jagow, G. and Albracht, S., unpublished). These observations are similar to data obtained from the investigation of the chloroplast cytochrome *b*-559 where the high-potential form occurring naturally (E_m = 400 mV) has higher g_z values than the low-potential form (E_m = 0 mV) observed after isolation of the protein [12]. However, in contrast to the data obtained from beef heart cytochrome *b*, T'sai and Palmer [13] found no change of the $g_z = 3.78$ signal in the yeast cytochrome *b* after modification with tetrahydrophthalic anhydride.

Table 2

Redox potentials and EPR g_z values of cytochrome *b* in different states of denaturation (Von Jagow, G. and Albracht, S., unpublished)

State	E_m (mV)	g_z
bc_1 complex (native)	+50, −50	3.78, 3.44
+ antimycin		3.78, 3.47
Isolated cyt.*b* (0.5% TX-100)	+10, −100	2.93, 2.42
Isolated cyt.*b* (SDS)	−140, −240	

The decrease of the g_z values and of the redox potentials of cytochrome *b* indicates that perturbation of the environment of cytochrome *b* leads to a release of the steric strain. Therefore it may be concluded that an interaction between cytochrome *b* and other subunits of the bc_1 complex is required for the stabilisation of the strained structure. These findings are consistent with the observation that cytochrome *b* maintained its spectral properties within a subcomplex formed together with the 13.4 kDa Q-binding protein and one of the core proteins (see above).

5. THE HEME-BINDING DOMAIN OF CYTOCHROME *b*

The structure predictions of the mitochondrial cytochromes *b* suggest the existence of 9 transmembrane helices [8,9]. The comparison of cytochrome *b* from mitochondria of 6 different species and of b_6 from spinach chloroplasts revealed 4 invariant histidines in a stretch comprising the first 5 membrane-spanning segments. Helix II and V each contain a pair of invariant histidines, separated by 13 residues in each case. Since the transmembrane segments are assumed to form α-helices, the histidines of each segment seem to be situated on one side of the same helix with a distance of 21 Å between them. The two hemes are

sandwiched between the membrane-spanning helices II and V. The Fe–Fe distance of 21 Å is consistent with distance measurements performed by EPR using dysprosium as a paramagnetic probe [14].

However, the picture of the heme-binding helices is not as simple as previously envisaged. Helix V contains a highly conserved proline residue situated between the two heme-binding histidines, 4 residues away from the N-terminal histidine (residue 182 in beef heart bc_1 complex). Therefore we examined the helix structure by means of model building studies applying CPK models. A proline residue can be incorporated into a helix without introducing a bend. The distortion of the helix is minimised if the proline is in the *cis* conformation which also prevents the side chain of the adjacent leucine residue from interfering with the δ-CH_2 group of the proline. This arrangement agrees with the fact that bulky L-amino acids situated at the N-terminus of a proline tend to force the proline into the *cis* form [15].

Incorporation of a proline into a helix in this way leads to a weakening of the helix and to a twist of the helix around the central axis. The heme-binding histidines are then not located exactly above each other on the same side of helix V but their α-carbon atoms form an angle of approx. 140° with the helix (fig.1). Therefore helices II and V cannot be arranged parallel to each other but form an angle of approx. 20° with each other.

The heme-binding helices II and V are connected by two membrane-spanning helices (III and IV) with very short intervening hydrophilic stretches [8]. Therefore helices II–V must be packed in a very tight structure. A '4-α-helical' structural motif has been observed in many proteins, including a number of heme-binding proteins [16,17]. This structural motif reveals 4 α-helices arranged in a symmetrical bundle with roughly 4-fold symmetry having a left-handed twist. Adjacent helices interact at distances of approx. 10 Å and at angles of approx. 18°. This assembly is stabilised by electrostatic helix-dipole interactions between the adjacent, antiparallel helices.

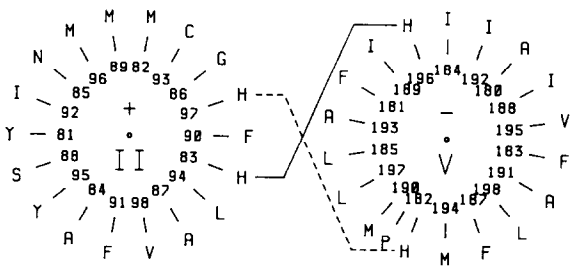


Fig.1. Helical wheel plot according to Schiffer and Edmundson [25] of helices II (residues 81–98) and V (residues 180–198) of cytochrome *b* from beef heart. The approximate position of the proline residue 186 is shown. + and − refer to the N- and C-termini of the helices, respectively. The two pairs of heme-binding histidines are indicated.

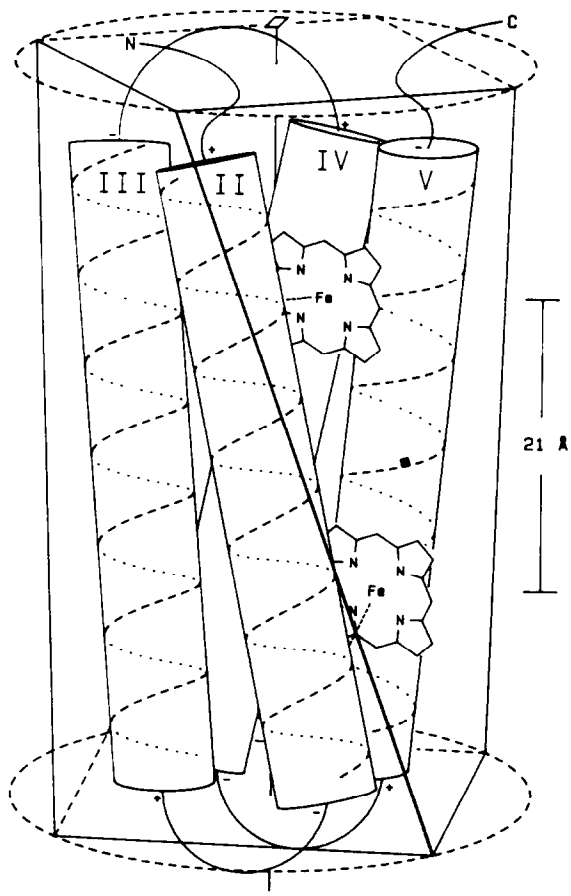
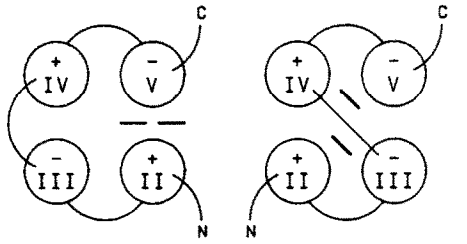


Fig.2. Proposed model of the 4-α-helical bundle formed by helices II–V of cytochrome *b*. The position of the proline residues 186 in helix V is indicated by a black square. The net dipole charges of the helices are indicated by + and −, respectively. The grid surrounding the bundle shows the distortion of the helices around the central axis of (approximately) 4-fold symmetry.

We therefore propose a model for the heme-binding core of cytochrome *b* consisting of the 4 helices II–V arranged in an antiparallel bundle (fig.2). The diameter of the bundle is approx. 30 Å. The porphyrins are mainly buried within the core. The propionyl groups of the hemes interact with basic amino acid residues within helices II and V (arginines 80, 100 and 177 in beef heart). The reaction cleft of the porphyrins is assumed to be formed by the groove between helices II and V.

The remaining 5 helices of cytochrome *b* are connected to this core by comparatively long hydrophilic stretches and are therefore expected to form a somewhat looser arrangement. Nevertheless, they may contribute to the stabilisation of the native structure of cytochrome *b* together with membrane-spanning domains of other subunits.



Helices	Antiparallel bundle		Partially parallel bundle	
		energy		energy
II - III	adjacent	-10	adjacent	-10
II - IV	diagonal	+ 6	adjacent	+10
II - V	adjacent	-10	diagonal	- 6
III - IV	adjacent	-10	diagonal	- 6
III - V	diagonal	+ 6	adjacent	+10
IV - V	adjacent	-10	adjacent	-10
Total		-28		-12

Fig.3. Comparison of two possible arrangements of 4 adjacent transmembrane helices: (A) antiparallel bundle, (B) partially parallel bundle. The position of the porphyrins is indicated by the heavy lines. The interaction energies (kJ/mol) are estimates taken from Sheridan et al. [17].

6. DISCUSSION

The twisted 4-α-helical arrangement that we suggest produces the most favourable interactions between helices II–V (fig.3). An α-helix dipole is comparable to a line dipole with charges of + and − 0.5 electrons lying at either end of the helix axis, the amino terminus being positive [18]. Therefore the all-antiparallel arrangement of helices II–V is energetically favourable compared to an arrangement where adjacent helices are partially arranged parallel to each other. The model of bacteriorhodopsin exhibits an antiparallel arrangement of groups of 4 helices each, interacting at angles of approx. 20°, which is similar to the structure we propose for cytochrome *b* [19].

Our model also gives an explanation for the occurrence of the highly conserved proline residue in helix V since the twist within helix V induced by the *cis*-proline is a prerequisite for the formation of a twisted bundle incorporating the two heme groups and thus for the steric strain of cytochrome *b*. Proline residues occur rather frequently within proposed membrane-spanning stretches. Although proline residues are strong helix breakers in globular proteins [20], the situation may be quite different in membrane proteins where a β-turn within the hydrophobic center of the membrane would introduce a large number of highly unfavourable interactions between the amide units and the hydrocarbon chains of the phospholipids. Within the membrane-spanning segments of bacteriorhodopsin, 3 dipeptides similar to the dipeptide Leu-Pro occurring in helix V of cytochrome *b* are found (Val-Pro, Thr-Pro, and Tyr-Pro) [21].

The structure predictions of the small subunits suggest several amphipathic helices within the subunits of the bc_1 complex from beef heart and a number of charged residues buried within the membrane. Amphipathic helices are frequently observed in the membrane-spanning segments of energy-transducing proteins; bacteriorhodopsin, for instance, has 9 charged residues buried within the membrane [21]. Four out of the 9 proposed transmembrane stretches of cytochrome *b* are amphipathic.

Different types of bonds contribute to the stabilisation of the three-dimensional arrangement of the multi-protein complex: (i) Van der Waals

forces; (ii) electrostatic interactions, i.e. dipole-dipole interactions, ionic bonds, and hydrogen bonds; and (iii) covalent bonds, i.e. cystine bridges. Electrostatic interactions between sided α-helices and the formation of ion pairs between different subunits may constitute an important factor in the stabilisation of the tertiary structure and in the arrangement of the protein subunits [22]. Since the magnitude of the dipole-dipole interactions is determined by Coulomb's law ($\Delta E = e_1e_2/\epsilon d$; e_1, e_2, charges; ϵ, effective dielectric constant; d, distance), helix-dipole interactions could be even more important for membrane proteins (ϵ_{membr} = 4) than for globular proteins (ϵ_{water} = 78.5) [23].

The formation of subcomplexes within the bc_1 complex seems to depend on the formation of ionic bonds between the protein subunits. Stabilisation of helix-helix interactions through ion pairs seems to occur more frequently than stabilisation by cystine bridges; the only protein of the bc_1 complex that has hitherto been shown to form intramolecular cystine bridges is the 9.2 kDa hinge protein [24]. Helix-dipole interactions may be particularly important in the formation of the tertiary structure of cytochrome *b* since this protein has no charged residues within the membrane that could participate in the formation of intramolecular ion pairs. The maintenance of the high strain of cytochrome *b* seems to depend on helix-dipole interactions within cytochrome *b* inducing the twisted 4-α-helical structure and on the stabilisation of this structure by the binding of additional, small subunits.

The structural analysis of the mammalian bc_1 complexes is impeded by the fact that these complexes contain a large number of non-catalytic subunits not present in the bacterial bc_1 complexes or in the chloroplast b_6f complex. The structure prediction suggests that the surface-oriented subunits, i.e. the 8.0 kDa protein and the 13.4 kDa Q-binding protein, may contribute to the formation of the Q_o or Q_i reaction centers and probably also to the process of proton transduction. The binding of other subunits to cytochrome *b* may be essential for the maintenance of the steric strain; this would explain why dissociation of the complex induces severe structural changes in cytochrome *b* as monitored by EPR, light absorption measurements, and redox potentiometry.

For the remaining subunits a regulatory function may be considered, although no regulation has as yet been demonstrated. Furthermore, some subunits may serve a specific function during the assembling process of the complex from its mitochondrially (cytochrome *b*) and nuclear coded subunit precursors.

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REFERENCES

[1] Link, T.A. and Von Jagow, G. (1986) submitted.
[2] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[3] Borchart, U., Machleidt, W., Schägger, H., Link, T.A. and Von Jagow, G. (1986) FEBS Lett. 200, 81–86.
[4] Karlsson, B., Hovmöller, S., Weiss, H. and Leonard, K. (1983) J. Mol. Biol. 165, 287–302.
[5] Engel, W.D., Michalski, C. and Von Jagow, G. (1983) Eur. J. Biochem. 132, 395–402.
[6] Schägger, H. (1980) Thesis, Universität München.
[7] Von Jagow, G., Engel, W.D., Schägger, H., Machleidt, W. and Machleidt, I. (1981) in: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F. et al. eds) pp.149–161, Elsevier, Amsterdam, New York.
[8] Saraste, M. (1984) FEBS Lett. 166, 367–372.
[9] Widger, W.R., Cramer, W.A., Herrmann, R. and Trebst, A. (1984) Proc. Natl. Acad. Sci. USA 81, 674–678.
[10] Carter, K.R., T'sai, A.-I. and Palmer, G. (1981) FEBS Lett. 132, 243–246.
[11] Palmer, G. (1985) Biochem. Soc. Trans., 548–560.
[12] Babcock, G.T., Widger, W.R., Cramer, W.A., Oertling, W.A. and Metz, J.G. (1985) Biochemistry 24, 3645–3650.
[13] T'sai, A.-I. and Palmer, G. (1982) Biochim. Biophys. Acta 681, 484–495.
[14] Ohnishi, T. and Von Jagow, G. (1985) Biophys. J. 47, 241a.
[15] Hetzel, R. and Wütherich, K. (1979) Biopolymers 18, 2589–2606.

[16] Weber, P.C. and Salemme, F.R. (1980) Nature 287, 82–84.
[17] Sheridan, R.P., Levy, R.M. and Salemme, F.R. (1982) Proc. Natl. Acad. Sci. USA 79, 4545–4549.
[18] Hol, W.G.J., Van Duijnen, P.T. and Berendsen, H.J.C. (1978) Nature 273, 443–446.
[19] Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–32.
[20] Chou, P.Y. and Fasman, G.D. (1974) Biochemistry 13, 222–245.
[21] Engelman, D.M., Henderson, R., McLachlan, A.D. and Wallace, B.A. (1980) Proc. Natl. Acad. Sci. USA 77, 2023–2027.
[22] Engelman, D.M. (1982) Biophys. J. 37, 187–188.
[23] Moore, G.R. and Rogers, N.K. (1985) J. Inorg. Biochem. 23, 219–226.
[24] Mukai, K., Miyazaki, T., Wakabayashi, S., Kuramitsu, S. and Matsubara, H. (1985) J. Biochem. 98, 1417–1425.
[25] Schiffer, M. and Edmundson, A.B. (1967) Biophys. J. 7, 121–135.