TOPOGRAPHY OF THE IRON–SULPHUR SUBUNIT IN MITOCHONDRIAL UBIQUINOL:CYTOCHROME *c* REDUCTASE

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1. Introduction

The mitochondrial electron-transfer enzyme ubiquinol:cytochrome *c* reductase (EC 1.10.2.2) is a dimeric multiprotein complex with a 2-fold axis running perpendicular to the membrane. The monomeric unit has an elongated structure and extends ~15 nm through the membrane. The protein is unequally distributed; ~30% is located in the membrane, 50% in a section which extends 7 nm into the matrix space of mitochondria and 20% in a section which extends 3 nm into the intermembrane space [1].

We are studying the topography of the (most probably) 9 different subunits of cytochrome reductase within this structure by the following approach: the enzyme is dissociated under mild conditions into the subunits which are then isolated and characterised as hydrophilic, hydrophobic or amphiphilic, according to their solubility in aqueous buffers or detergent solutions. The hydrophilic subunits are water soluble without detergent and are assumed to lie outside the membrane. The hydrophobic subunits are soluble in detergent solution and are assumed to lie in the membrane. The amphiphilic subunits are soluble in monodisperse state only in detergent solution. These subunits, however, become water soluble without detergent when a hydrophobic protein stretch is cleaved off by proteolysis. Amphiphilic subunits are therefore assumed to extend mainly into the aqueous phase and to be anchored to the membrane by the hydrophobic protein stretch.

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Here we report that the iron–sulphur subunit [2] of cytochrome reductase, like the cytochrome c_1 subunit [3], is amphiphilic; the 25 000 M_r native subunit can be isolated in form of a protein–detergent complex. After limited proteolysis a 16 000 M_r preparation which is water soluble without detergent can be obtained. We will discuss this result with regard to a difference between the three-dimensional gross structures of total cytochrome reductase and a subunit complex from cytochrome reductase which lacks the iron–sulphur subunit [4].

2. Materials and methods

Cytochrome reductase was prepared and dissociated by salt treatment as in [4,5]. The cleavage procedure was modified by performing the gel filtration step at pH 6 instead of 7. The mixture of the two largest subunits and the iron–sulphur subunit [4] was concentrated to 10 mg protein/ml by ultrafiltration using an Amicon PM30 filter. Aliquots of 1.5 ml were layered on 12 ml sucrose density gradients which contained 5–20% sucrose in 50 mM Tris–acetate (pH 7.5) and 2 mM dithioerythritol (DTE). The gradients were centrifuged at 28 000 × *g* for 24 h. The complex of the 2 largest subunits sedimented halfway through the gradient. The iron–sulphur subunit and the Triton X-100 remained close to the top of the gradient. The fraction which contained the iron–sulphur subunit (crude preparation) was dialysed against 15 mM Tris–acetate (pH 7.5), 1 mM DTE and 0.05% Cemulsol LA90 detergent (Rhone-Poulenc, any other $C_{12}E_{8-10}$ alkyl polyoxyethylene detergent, for abbreviation see [6], could also be used). Protein (5 mg) was applied to a 1 × 10 cm DEAE-Sepharose CL6B

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(Pharmacia) column which was equilibrated with 15 mM Tris–acetate (pH 7.5) and 0.05% Cemulsol. The iron–sulphur subunit was eluted at 0.08 M NaCl using a 70 ml gradient from 0–0.2 M NaCl in the above buffer. The final yield was 20%. For proteolytic cleavage, 5 mg/ml of the crude iron–sulphur subunit preparation (final purification was unnecessary) and 1 mg chymotrypsin/ml in 15 mM Tris–acetate (pH 7.5), 1 mM DTE and 0.05% Cemulsol were incubated at 8°C for 30 min. The solution was then pumped onto a 0.7 × 4 cm DEAE-Sepharose CL6B column equilibrated with 15 mM Tris–acetate (pH 7.5). The iron–sulphur preparation was eluted at 0.12 M NaCl using a 30 ml 0–0.25 M NaCl gradient in the above buffer. The yield was 20%.

[^{14}C]Cemulsol binding was determined by absorbing the subunit on a small DEAE-Sepharose CL6B column, equilibrating the column with [^{14}C]Cemulsol (CEA, BP 2, Gif-sur-Yvette) and then eluting the subunit with 0.2 M NaCl in the [^{14}C]Cemulsol solution. The amount of bound detergent was determined from the ^{14}C radioactivity which coeluted with the subunit [6]. Acid-labile sulphur was determined as in [7]. EPR spectra were recorded and quantitated as in [8,9]. Protein was determined by quantitative amino acid analysis.

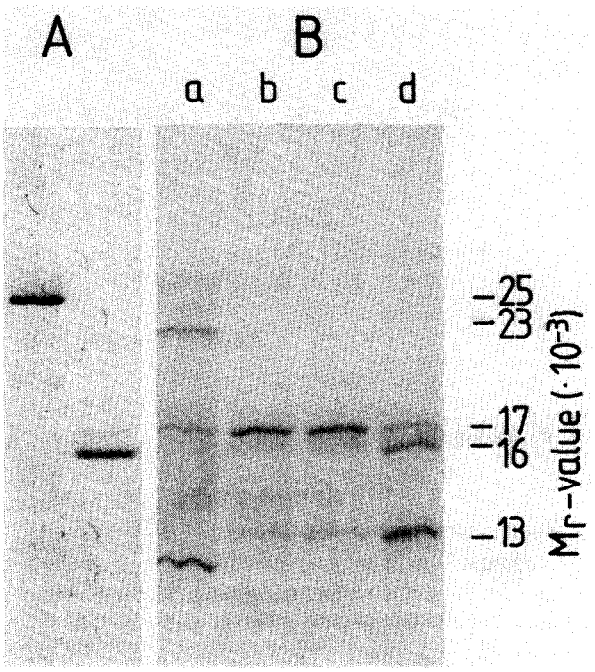

Fig.1. SDS gel electrophoresis of the native iron–sulphur subunit and the water-soluble domain of the subunit (A) and of polypeptides obtained by digesting the iron–sulphur subunit with an increasing amount of chymotrypsin (50 U/mg chymotrypsin) (B): 10 μg chymotrypsin/mg iron–sulphur subunit (a); 50 μg/mg (b); 100 μg/mg (c); and 500 μg/mg (d). (The 11 000 M_r band is a contamination present in the iron–sulphur subunit preparation used for digestion.)

3. Results and discussion

The native 25 000 M_r iron–sulphur subunit is isolated in monomeric state as protein–detergent complex (table 1). This protein–detergent complex is very sensitive to proteolysis. By treatment with increasing amount of chymotrypsin 4 intermediate polypeptides can be obtained with M_r-values 23 000, 17 000, 16 000, 13 000 (fig.1; the 11 000 M_r band is a contamination present in the iron–sulphur preparation used for proteolysis). The 16 000 M_r polypeptide was isolated. It is water soluble in a monomeric state without binding detergent (table 1).

The EPR spectrum of the 2 Fe–2 S cluster in this 16 000 M_r polypeptide is indistinguishable from the spectrum of the iron–sulphur subunit in cytochrome

Table 1
Properties of the iron–sulphur subunit preparations

Preparation	Cemulsol binding (g/mmol)	Stokes' radius (A)	M_r-Value of protein		Acid-labile sulfide-content (μmol/g protein)
			From gel filtration	From SDS gel electrophoresis	
Iron–sulphur subunit detergent complex	60–90	49–52		25 000	60–70
Water soluble iron–sulphur subunit	0	15.5	15 000	16 000	80–95

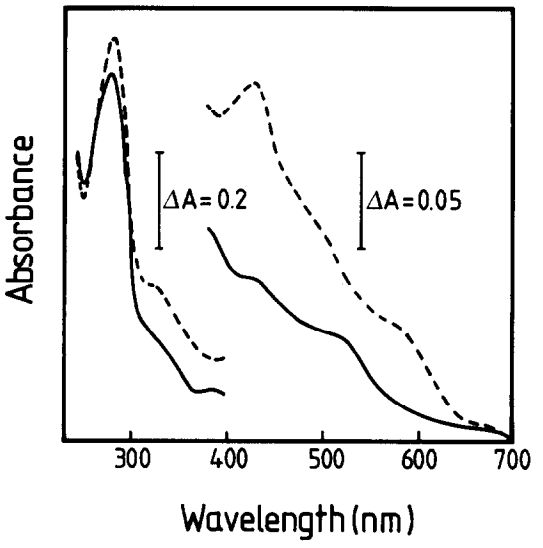

Fig.2. Light absorption spectra of the ferrycyanide-oxidized (---) and ascorbate reduced (——) 16 000 M_r iron–sulphur subunit preparation.

reductase, or in the isolated detergent-bound subunit (not shown). The g-values are the same as those reported for cytochrome reductase preparations from other organisms, namely $g_{z,\,y,\,x}$ = 2.03, 1.90, 1.75 at 36 K and 9.25 GHz (review [10]). This indicates that the protein environment of the 2 Fe–2 S cluster has not changed upon proteolytic cleavage. Also the light absorption spectra (review [11]) appear to be the same. The spectra of the oxidized and the reduced forms of the 16 000 M_r preparation are shown in fig.2. (Similar spectra were obtained for the isolated native subunit; the spectra, however, showed increasing absorbance towards the UV region due to the detergent present). The molar absorbance coefficient at 425 nm was estimated to be 8–9 $mM^{-1} . cm^{-1}$ for the oxidized form and 3.5–4.5 $mM^{-1} . cm^{-1}$ for the reduced form by quantitation of the EPR signals.

The amino acid composition of the 25 000 M_r and the 16 000 M_r preparations and the clipped 9000 M_r fragment, obtained by subtracting the composition of the 16 000 M_r preparation from that of the 25 000 M_r preparation, are shown in table 2. The polarity defined as mol% of the polar residues is highest in the 16 000 M_r preparation and lowest in the 9000 M_r fragment. Since the 9000 M_r fragment is responsible for detergent binding of the isolated subunit this fragment must also be responsible for anchoring the

Table 2
Amino acid composition of the iron–sulphur subunit preparations

Residue	25 000-M_r subunit	16 000-M_r preparation	9000-M_r 'fragment'
Asx	21	17	4
Thr	12	6	6
Ser	17	6	11
Glx	22	17	5
His	5	3	2
Lys	11	8	3
Arg	11	7	4
Gly	27	15	12
Ala	21	12	9
Val	15	10	5
Met	4	2	2
Ile	10	8	2
Leu	17	9	8
Tyr	9	4	5
Phe	7	4	3
Pro	16	12	4
Cys	n.d.	n.d.	n.d.
Trp	n.d.	n.d.	n.d.
Total no.	225	140	85
M_r-Value ($\times 10^{-3}$)	24	15	8.8
Polarity (%)	51	55	46

The number of residues was calculated from the percentage composition by assuming 17.0 leucine residues/molecule and an M_r of 25 000. Values for serine and threonine are calculated by extrapolation to zero time of hydrolysis; values for valine and isoleucine are the maximal ones for 72 h hydrolysis. The composition of the 9000 M_r 'fragment' was obtained by subtracting the composition of the 16 000 M_r preparation from that of the 25 000 M_r preparation

16 000 M_r part of the subunit to the membrane. If this fragment spans the membrane once in the form of an α-helix a strand of 21 hydrophobic amino acid residues would be required. Two strands of antiparallel β-structure would require 18 residues [12]. The fragment, however, contains 80–90 residues, and a considerable number of them are charged. Therefore only a part of this fragment can be located in the membrane.

As in [13], membrane crystals have been prepared for cytochrome reductase and for a subunit complex which contains the cytochromes b and c_1 and the 4 [14] small subunits but lacks the iron–sulphur subunit and the 2 large subunits [4]. The gross three-dimensional structure of these preparations has been

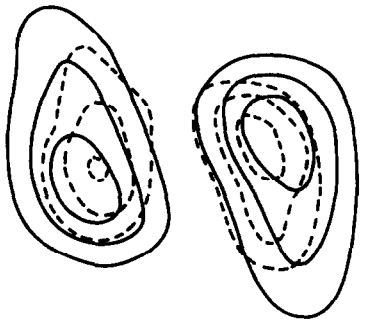

Fig.3. Projections perpendicular to the membrane plane of the small peripheral section of cytochrome reductase (——) and the peripheral section of the cytochrome bc_1 subunit complex (– – –). The density contours were obtained from tilted views of the negatively stained membrane crystals [1,15].

determined by electron microscopy [1,15]. As will be detailed elsewhere [15], the structure of the subunit complex as compared to the structure of the whole enzyme lacks the large peripheral section which extends 7 nm from the membrane. This section is assumed to be contributed by the 2 large (hydrophilic) subunits of cytochrome reductase [1]. Furthermore that section which extends 3 nm from the membrane is smaller in the subunit complex than in the whole enzyme and the distance between the mass centres of the 2 related sections of 1 dimer is shorter, namely 7.4 nm for the whole enzyme and 6.0 nm for the subunit complex (fig.3). This suggests that protein is missing from the outer part of each section (fig.4). We think that this loss of protein can be explained by the absence of the 16 000 M_r hydrophilic domain of the iron–sulphur subunit. If this assumption is correct, the domain of the iron–sulphur subunit which carries the 2 Fe–2 S cluster extends into the inter-

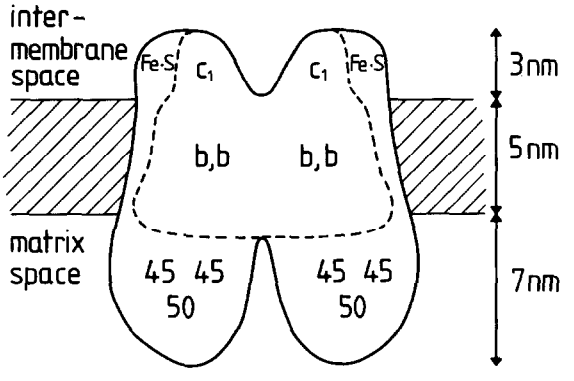

Fig.4. Schematic representation of the gross structure of the cytochrome reductase (——) and the cytochrome bc_1 subunit complex (– – –). The characters refer to the redox centres, the numbers to the M_r-values of the large subunits without redox centres.

membrane space of mitochondria and adjoins the heme-carrying domain of the cytochrome c_1 subunit [3]. The same pattern of subunit neighbours can be deduced from the pathway of electron transfer in cytochrome reductase as proposed in [16,17].

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