Isolation and amino acid sequence of the smallest subunit of beef heart bc_1 complex

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The 11 subunits of beef heart bc_1 complex can be separated either by a new SDS-PAGE system or by a series of chromatographic steps involving dissociation of the complex by salt treatment. The amino acid sequence of the smallest subunit was determined by complete solid-phase Edman degradation and was confirmed by sequencing the N-terminal part and the C-terminal tryptic fragment by liquid-phase Edman degradation. The protein consists of 56 amino acid residues; the M_r was calculated to be 6363. The protein ('ISP binding factor') might be entangled in the reassembly of the iron-sulfur protein with the bc_1 subcomplex.

Cytochrome bc_1 *complex* *SDS-PAGE system* *Amino acid sequence* *Smallest subunit* *Membrane protein* *Hydroxyapatite chromatography*

1. INTRODUCTION

The bc_1 complex is not only the central part of the respiratory chain in mitochondria but it is also homologous to the b_6f complex of the photosynthetic electron transport system and to the *bc* complex of photobacteria. Therefore it can be regarded as an ubiquitous enzyme system. Elucidation of the primary structure of all subunits of the bc_1 complex is an important prerequisite for the understanding of electron and proton transport in general.

The bc_1 complex from beef heart was previously believed to contain 8–10 subunits [1–3]. Using a new slab gel system, the existence of 11 subunits was revealed [4]. All 11 subunits can now be isolated and purified [4]. The isolation and the amino acid sequence of the smallest subunit, a 6.4 kDa protein, are described here.

2. EXPERIMENTAL

2.1. *SDS-polyacrylamide gel electrophoresis*

The SDS-PAGE method was developed because none of the systems described in the literature allowed the complete resolution of all the protein subunits of the bc_1 complex. A modified staining procedure was necessary to detect the eleventh subunit, the 8 kDa protein, which does not appear on application of the methanolic staining solution. The resolving power of this SDS-PAGE method increases inversely to the molecular mass of the proteins. Thus a very good resolution in the range of 3–25 kDa is combined with an overall separation range of 2–100 kDa. Hence only one gel is required for the resolution of all 11 subunits of the bc_1 complex. In a semilogarithmic plot a linear correlation between molecular mass and migration distance is observed over the whole separation range.

2.2.1 Gel preparation

The stock solutions prepared for gel electrophoresis are given in table 1. They are kept at room temperature with the exception of the acrylamide-bisacrylamide mixture which is stored at 4°C. The separating gel (table 2) is polymerized within 15 min by addition of 100 μl ammonium persulfate (10%) and 10 μl TEMED. To ensure the formation of a smooth surface between separating gel and

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Table 1

Stock solutions for SDS-PAGE

Buffer	Tris (M)	Tricine (M)	HCl (M)	SDS (%)
Anode buffer	0.2	–	–*	–
Cathode buffer	0.1	0.1	–	0.1
Gel buffer	3	–	1	0.3
Overlay solution	1	–	0.33	0.1

Acrylamide-bisacrylamide mixture: 45% acrylamide (w/v), 1.5% bisacrylamide (w/v)

* Adjusted to pH 8.9 with HCl

Table 2

Composition of separating and sample gels

	16% separating gel	4% sample gel
Acrylamide-bisacrylamide mixture	10 ml	0.5 ml
Gel buffer	10 ml	1.5 ml
Glycerol	6 g	–
Water	ad 30 ml	ad 6 ml

sample gel, the separating gel is covered with the overlay solution before polymerization. Some minutes after termination of the polymerization the overlay solution is decanted and replaced by the sample gel mixture (table 2) which is polymerized by addition of 50 μl ammonium persulfate (10%) and 5 μl TEMED. The lower end of the inserted comb should be at least 1 cm away from the separating gel.

2.1.2. Sample preparation

The protein samples are incubated in a buffer with the following final concentrations: 5% SDS, 15% glycerol, 50 mM Tris, 2% mercaptoethanol, 0.003% bromphenol blue, pH 6.8, adjusted with HCl.

2.1.3. Electrophoresis conditions

Slab gels (14 × 14 × 0.15 cm) are used in a vertical apparatus. The electrophoresis at room temperature is run at 30 V for 2 h and continued at 90 V constant for 16 h.

2.1.4. Staining

After fixing the protein for 2 h in a solution consisting of 65% methanol and 10% acetic acid, the gel is stained for at least 3 h in a solution containing 10% acetic acid and 0.1% Coomassie brilliant blue G-250.

2.2. *Isolation procedure*

All procedures are performed at 4°C. Mitochondria, prepared according to Smith [5], are concentrated by a 15 min centrifugation step at 27 000 × g to reduce the amount of sucrose. The hydroxyapatite used is prepared according to Tiselius et al. [6]. This material has a better flow rate than the commercially available material. The bc_1 complex is prepared as described in [1,2].

100 mg of freshly prepared bc_1 complex in 100 mM NaCl, 10 mM Mops, 0.05% Triton X-100 are applied onto a 100 ml hydroxyapatite column after addition of Sodium phosphate buffer, pH 7.2, to a final concentration of 35 mM. The hydroxyapatite column has to be equilibrated in advance with buffer 1 (table 3). After washing the hydroxyapatite-bound bc_1 complex with 50 ml buffer 1, the 6.4 kDa protein and the 'Rieske' iron-sulfur protein are split off by application of 30 ml of buffer 2. After consecutive application of 50 ml of buffer 3 and 50 ml of buffer 1, the 6.4 kDa protein elutes before the iron-sulfur protein. The 6.4 kDa protein is found in the colourless fractions containing the high Triton concentrations as detected by the absorbance at 280 nm. The fractions of crude iron-sulfur protein, eluting just after the 6.4 kDa protein, are slightly coloured. The collected protein fractions are passed through Sephadex G-25 col-

Table 3

Buffers for the isolation of the 6.4 kDa protein

		1	2	3	4	5
Triton X-100	(%)	0.05	1	0.05	0.05	0.1
Na-chloride	(M)	0.05	0.4	0.4	0.2	–
Na-phosphate	(M)	0.035	0.025	0.05	–	0.15
Mops	(M)	–	–	–	0.01	–
Na-dithionite	(M)	–	0.002	0.002	–	–
PMSF	(M)	–	0.0002	0.0002	0.0002	–
Urea	(M)	–	2.0	2.0	–	–

All buffers are adjusted to pH 7.2 and contain 1 mM Na-azide

umns pre-equilibrated with buffer 4 and are then stored at −20°C.

For further purification the 6.4 kDa protein is precipitated by adding an equal volume of acetone and leaving overnight at −20°C. After centrifugation the sediment is resolubilized in 4% SDS and 1% mercaptoethanol and chromatographed at room temperature on a Sephadex G-50 superfine column, equilibrated with 1% SDS. The hydroxyapatite-bound bc_1 subcomplex can be eluted by buffer 5 and stored for a few weeks at −20°C in 50% glycerol (w/w). The yield of 6.4 kDa protein is 65%, that of the iron-sulfur protein and the bc_1 subcomplex 60% and 90%, respectively.

2.3. *Amino acid sequence determination*

2.3.1. Amino acid analysis

Samples were hydrolyzed in 5.6 M HCl at 105°C for 24 h and for 24 h after performic acid oxidation.

2.3.2. Coupling of the protein

The 6.4 kDa protein (50 nmol) was precipitated in 50% acetone, stored overnight at −20°C, washed, dried, and preincubated in anhydrous trifluoroacetic acid. The dried protein was dissolved in dimethylformamide and was attached to aminopropyl glass (79 Å pore size) by the hydroxybenzotriazole-catalyzed carbodiimide procedure [7].

2.3.3. Tryptic cleavage of the protein

Cleavage by trypsin was performed for 3 h at 37°C in 0.1 M ammonium bicarbonate solution. The fragments were separated by high performance liquid chromatography (HPLC) on a reversed-phase RP-18 column in 50 mM ammonium acetate, pH 4.0, with an acetonitrile gradient of 0–50%.

2.3.4. Sequence analysis

The amino-acid sequence was determined by automated solid-phase Edman degradation [8] and by automated liquid-phase Edman degradation in non-commercial sequencers. Identification of amino acid phenylthiohydantoins by HPLC and computerized analysis of data were performed as described [9].

3. RESULTS

3.1. *Amino acid analysis and primary structure*

The solid phase degradation gave the complete sequence depicted in fig.1. Parts of the sequence were confirmed by liquid phase degradation of the N-terminus of the protein and of the tryptic fragment T6, as shown in fig.1.

The protein consists of 56 amino acid residues. The amino acid analysis (table 4) is almost identical to that derived from the amino acid sequence, indicating the high purity of the isolated material. The sequence was found to contain 4 acidic residues (4 Asp) and 8 basic residues (3 Lys, 5 Arg) resulting in a net positive charge of 4. The protein contains no histidine and no cysteine. The molecular mass was calculated to be 6363 Da.

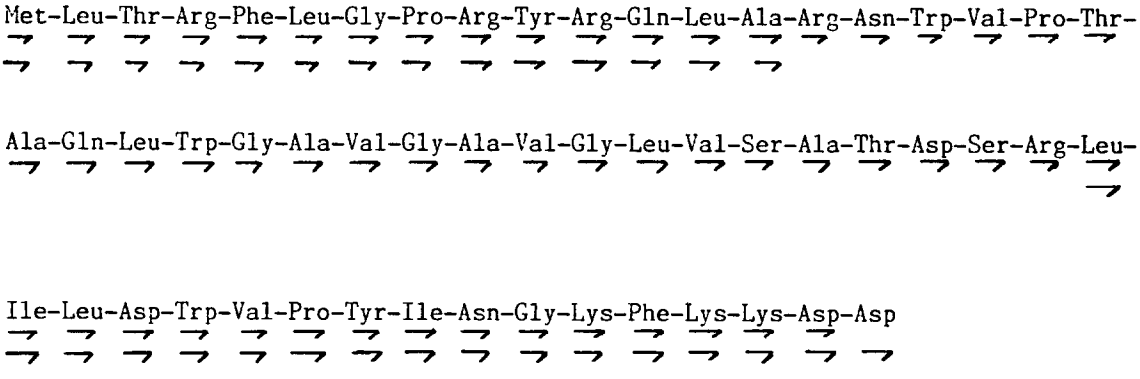


Fig.1. Complete amino acid sequence of the 6.4 kDa protein of bc_1 complex from beef heart mitochondria. Arrows indicate residues established by solid phase degradation and liquid phase degradation, respectively.

Table 4

Amino acid composition of the 6.4 kDa protein and of the tryptic fragment T6

	6.4 kDa Protein		T6 Fragment	
	Sequence	24 h hydrolysate	Sequence	24 h hydrolysate
Aspartic acid	4	5.5	3	4.0
Asparagine	2		1	
Threonine	3	3.1	0	0.3
Serine	2	2.1	0	0.4
Glutamic acid	0	2.0	0	0.5
Glutamine	2		0	
Proline	3	>0	1	>0
Glycine	5	5.8	1	1.8
Alanine	5	5.7	0	0.6
Cysteine[a]	0	0	0	n.d.
Valine	5	4.3	1	0.6
Methionine[a]	1	0.8	0	n.d
Isoleucine	2	2.1	2	2.0
Leucine	7	6.7	2	2.0
Tyrosine	2	1.5	1	0.5
Phenylalanine	2	2.2	1	1.2
Lysine	3	2.7	3	3.0
Histidine	0	n.d.	0	n.d.
Arginine	5	4.7	0	0
Tryptophan	3	n.d.	1	n.d.
Total	56		17	

[a]Obtained by analysis of the hydrolysate after performic acid oxidation

3.2. *Secondary structure*

The content of aliphatic hydrophobic residues exceeds one third; this already indicates that it is a membrane protein. This view is further supported by the plot of the hydropathy profile (fig.2). It displays a hydrophobic region from residues 16–36 with hydrophilic stretches extending to the N- and the C-terminus. The C-terminal stretch has no net charge, while the N-terminal stretch contains 4 positive arginine residues. The length of the hydrophobic region would be sufficient to form an α-helix through the membrane. The comparison of the amino acid sequence with those of other mitochondrial proteins gave no detectable homology.

4. DISCUSSION

The new SDS gel method described in this paper reveals that beef heart bc_1 complex consists of 11 intrinsic protein subunits (fig.3). A satisfying resolution of the small proteins in the previously used SDS gel electrophoresis systems had not been possible; furthermore, the 8 kDa protein could not be traced since it was dissolved again during the routine staining procedure. It only became visible when methanol was omitted from the staining solution.

The primary structures of cytochrome *b* [11], cytochrome c_1 [12], and the iron-sulfur protein [13] are available so far, although that of the iron-sulfur protein has been established only in *Neurospora crassa*. Out of the 6 small proteins 4 subunits have been analyzed. The primary structures of the core proteins, the 8 and 11 kDa proteins are still to be established. The protein structures of the 9 and 7 kDa proteins, belonging to the c_1 subcomplex, have been established by Wakabayashi et al. [14]

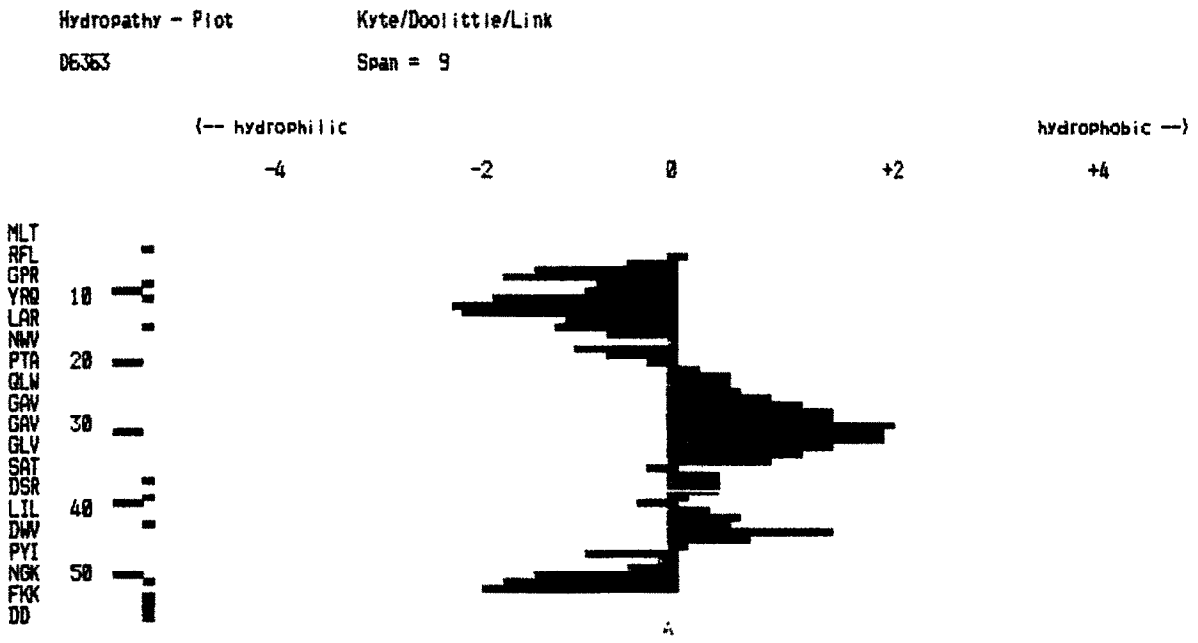
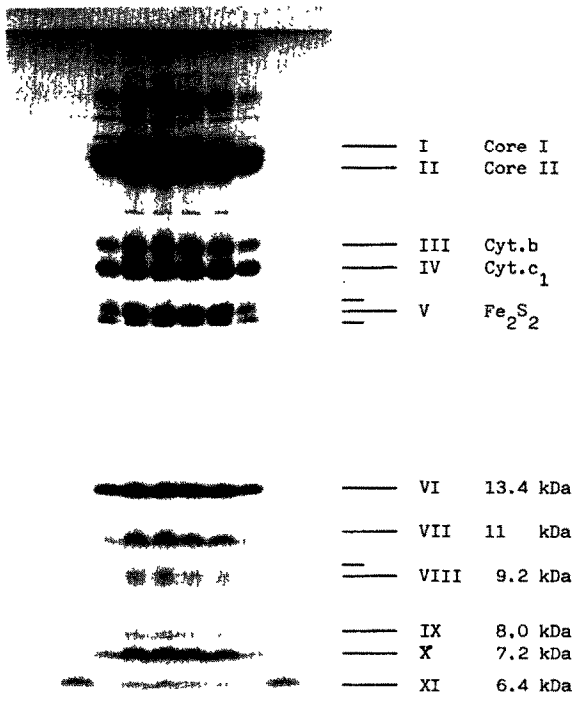


Fig.2. Hydropathy plot of the 6.4 kDa protein according to Kyte and Doolitte [10].



and Schägger et al. [15], respectively, and just recently Wakabayashi et al. [16] have also published the amino acid sequence of the protein revealing an apparent molecular mass of 12 kDa in our gel; they found it to possess a molecular mass of 13389 Da. It has been asserted to be a Q-binding protein (QP-C), indicating that this protein is a structural part of a ubiquinone reaction center.

The question arises to which domain of the bc_1 complex the 6.4 kDa protein, the smallest subunit,

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Fig.3. SDS-PAGE of beef heart bc_1 complex and its 6.4 kDa protein subunit. Outer lanes, isolated 6.4 kDa protein; inner lanes, different preparations of bc_1 complex, prepared according to [1,2]. The iron-sulfur protein appears as 3 bands in this gel. This effect is observed from time to time and can probably be attributed to an incomplete breakdown of intramolecular disulfide linkages by mercaptoethanol. The same effect is observed for the 9.2 kDa protein under the incubation conditions used (SDS/mercaptoethanol, 1 h). The 9.2 kDa protein appears as one single band if mercaptoethanol is omitted from the incubation mixture.

can be assigned and what the function of this protein is. In this respect it should be noted that during isolation it is cleaved off the complex together with the iron-sulfur protein, probably as a result of its close linkage to this protein [17]. In retrospect it is clear that the reconstitution experiments performed by us previously, reconstituting the iron-sulfur protein with the Fe_2S_2 depleted Triton bc_1 subcomplex, were in fact a reassembly of a mixture of the iron-sulfur protein and this 6.4 kDa protein with the bc_1 subcomplex. New reconstitution experiments with the bc_1 subcomplex and the separated iron-sulfur and this 6.4 kDa protein will have to show, whether the 6.4 kDa protein can be looked upon as an 'ISP binding factor', i.e. whether it is essential for a complete reassembly of the ubiquinol oxidation center, and therefore for full ubiquinol:cytochrome *c* reductase activity.

The amino acid sequences of the 8 and 11 kDa proteins are under study.

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