Isolation and amino acid sequence of the 8 kDa DCCD-binding protein of beef heart ubiquinol:cytochrome *c* reductase

U. Borchart, W. Machleidt, H. Schägger, T.A. Link and G. von Jagow

Institut für Physiologische Chemie und Physikalische Biochemie, Goethestrasse 33, 8000 München 2, FRG

Received 22 July 1985

The 8 kDa protein of beef heart ubiquinol:cytochrome *c* reductase was detected by means of a new SDS-PAGE [(1985) FEBS Lett. 190, 89–94] system and was isolated by a series of chromatographic steps involving dissociation of the complex by salt treatment. The amino acid sequence was determined by solid-phase Edman degradation of both the N-terminal part of the whole protein and proteolytic cleavage fragments of the protein. The protein consists of 78 amino acid residues: its M_r was calculated to be 7998. Structure predictions have been made from average and sided hydropathy profiles. The suggested structure encompasses an α-helix and a β-strand, the latter comprising a glutamic acid residue situated in a relatively hydrophobic neighbourhood. This residue may be responsible for the fact that the 8 kDa protein is the first subunit of the whole reductase (consisting of 11 subunits) to be labelled by DCCD when the reductase is in free form or inlaid in phospholipid vesicles.

Ubiquinol:cytochrome c reductase *Primary structure* *Hydropathy profile* *Structure prediction* *DCCD-binding protein* *Membrane protein* *(Beef heart mitochondria)*

1. INTRODUCTION

The ubiquinol:cytochrome *c* reductase (henceforth called the reductase) consists of 11 subunits. Cytocrome *b*, cytochrome c_1 and the iron-sulfur protein are the only subunits carrying redox centers. The 8 'surplus' subunits lacking redox centers are a 6.4 kDa protein described recently [1], a 7.2 kDa protein [2], the 8 kDa protein described here, a 9.2 kDa protein [3], an 11 kDa protein, a 13.4 kDa protein (QPC,4) and the 2 'core' proteins. Thus the primary structures of 5 out of the 6 smallest surplus subunits have been elucidated; those of the 2 core proteins, the FeS protein and 11 kDa protein have yet to be determined. The primary structure of the 8 kDa protein was used to derive average and sided hydropathy profiles. From these data a tentative folding pattern of the 8 kDa protein has been deduced.

2. EXPERIMENTAL

2.1. *Isolation procedure*

All procedures are performed at 4°C. Mitochondria prepared according to Smith [5] are concentrated by a 15 min centrifugation step at 27000 × *g* to reduce the amount of sucrose. The hydroxyapatite used is prepared according to Tiselius et al. [6]. The reductase is prepared as described by Engel et al. [7] and Schägger et al. [8].

2.1.1. Step I

Cleavage of the reductase into 3 fractions – the 6.4 kDa protein, FeS protein and 'bc_1 subcomplex': 100 mg freshly prepared reductase in 100 mM NaCl, 10 mM Mops, 0.05% Triton X-100 are applied onto a 100 ml hydroxyapatite column after addition of NaP_i buffer, pH 7.2, to a final concentration of 35 mM. The hydroxyapatite column has to be equilibrated in advance with buffer

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

Table 1

Buffers for the preparation of the 8 kDa protein of beef heart mitochondrial ubiquinol:cytochrome *c* reductase

		1	2	3	4	5	6	7	8
Triton X-100	(%)	0.05	1.0	0.05	0.5	0.5	0.5	0.5	0.05
Na-chloride	(M)	0.05	0.4	0.4	0.2	0.25	–	0.2	0.1
Na-phosphate	(M)	0.035	0.025	0.05	0.025	0.085	0.35	0.01	–
Mops	(M)	–	–	–	–	–	–	–	0.01
Guanidine	(M)	–	–	–	1.5	–	–	–	–
Na-dithionite	(M)	–	0.002	0.002	–	–	–	–	–
PMSF	(M)	–	0.0002	0.0002	–	–	–	0.0002	0.0002
Urea	(M)	–	2.0	2.0	–	–	–	–	–

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

1 (table 1). After washing the hydroxyapatite-bound reductase with 50 ml buffer 1, the 6.4 kDa protein and FeS protein are split off by application of 30 ml buffer 2. After consecutive application of 50 ml buffer 3 and 50 ml buffer 1, the 6.4 kDa protein elutes in front of the FeS protein. For further processing of these fractions see [8].

2.1.2. Step II

Cleavage of the bc_1 subcomplex into a fraction comprising 6 proteins and a cytochrome c_1 subcomplex composed of 3 proteins: The bc_1 subcomplex still bound to hydroxyapatite is cleaved further by application of one column volume of buffer 4, leading to the elution of a fraction comprising 6 proteins. The cytochrome c_1 subcomplex remains bound on the column. It is washed with 100 ml buffer 5 at 4°C and eluted with buffer 6 at room temperature. For separation of the 3 proteins of the cytochrome c_1 subcomplex see [2].

2.1.3. Step III

Separation of the fraction containing the 2 core proteins and 11 kDa protein from the fraction containing cytochrome *b*, and 13.4 kDa and 8 kDa proteins. The fraction obtained in step II, containing 6 of the 11 proteins of the reductase, is separated from guanidine by passing it through a 250 ml Sephadex G-25 column, pre-equilibrated with buffer 7. The eluate is then applied on a 30 ml hydroxyapatite column, pre-equilibrated with buffer 7, and subsequently washed with 30 ml of the same buffer. More than 60% cytochrome *b* and almost 100% of the 13.4 kDa and 8 kDa proteins pass through the column unbound, whereas the core proteins, 11 kDa protein and the remaining part of cytochrome *b* remain bound on the hydroxyapatite column.

2.1.4. Step IV

Separation of cytochrome *b*, and 13.4 kDa and 8 kDa proteins from each other: The fraction containing cytochrome *b*, and 13.4 kDa and 8 kDa proteins is concentrated by a factor of 10 by pressure filtration on an Amicon YM 5 membrane. PMSF is present in the buffer to prevent protease degradation of the 8 kDa protein. The Triton concentration should not exceed 5%. The material is then applied onto a Sephadex G-50 sf (superfine) column of 600 ml volume, pre-equilibrated with buffer 8. The gel filtration leads to the separation of the 8 kDa protein, only slightly contaminated by the 13.4 kDa protein. Further purification is achieved by gel filtration on Sephacryl S-200 in 80% formic acid after lyophilization of the eluate fractions. For separation of the 11 kDa protein from the 2 core proteins and for separation of cytochrome *b* from the 13.4 kDa protein see [8].

2.2. *Amino acid sequence determination*

For amino acid analysis, protein samples were hydrolysed in 5.7 M HCl at 105°C for 24, 48, 72 and 120 h, and for 24 h after performic acid oxidation. The data from the amino acid analyser (Kontron Liquimat II) were corrected for destruction and incomplete hydrolysis. The composition of fragment peptides was obtained from a 24 h hydrolysis without further correction.

80 nmol protein in 1 ml of 80% (v/v) formic acid were cleaved with cyanogen bromide (Serva) for 24 h at ambient temperature. The fragments were separated by gel chromatography on a Sephacryl S-200 column.

Cleavage by trypsin was performed for 3 h at 37°C in 0.05% Triton X-100 at pH 8. The fragments were separated by high-performance liquid chromatography (HPLC) on a reversed-phase RP-18 column in 0.05% trifluoroacetic acid, pH 2, with a gradient from 0 to 35% acetonitrile/2-propanol (1:1, v/v). Acidolytic fragments were prepared by incubation of the protein in 80% formic acid for 4 days at 50°C and separated by gel chromatography on a Sephacryl S-200 column.

N-terminal amino acids of all fragments were identified by dansylation [9] and by the 4-*N,N*-dimethylaminoazobenzene-4′-isothiocyanate/phenylisothiocyanate (DABTIC/PITC) method according to [10]. The N-terminal acetylmethionine of the protein which was obtained after cleavage of the N-terminal tryptic peptide with pronase (Boehringer Mannheim) was isolated by reversed-phase HPLC and identified by comparison of its elution time with that of a synthetic standard (Bachem, Switzerland) followed by amino acid analysis before and after hydrolysis. The C-terminal amino acids of the protein were determined by digestion with 1% (w/w) of carboxypeptidase A and B (Boehringer Mannheim) in 50 mM triethylamine/HCl at pH 7.5 for different incubation times. After stopping the reaction with formic acid, the released amino acids were quantitated in the amino acid analyser.

All amino acid sequences were determined by automated Edman degradation [11]. The peptides were attached to aminopropyl glass (77 Å) via their carboxyl groups using hydroxybenzotriazole-catalysed carbodiimide activation. The coupling reactions were performed in dimethylformamide after pre-incubation of the peptides in anhydrous trifluoroacetic acid. The immobilised peptides were degraded in a non-commercial solid-phase sequencer with an on-line identification of the released phenylthiohydantoin derivatives of amino acids by HPLC [11].

3. RESULTS

3.1. *Amino acid sequence*

The complete amino acid sequence of the 8 kDa protein is given in fig.1; the amino acid composi-

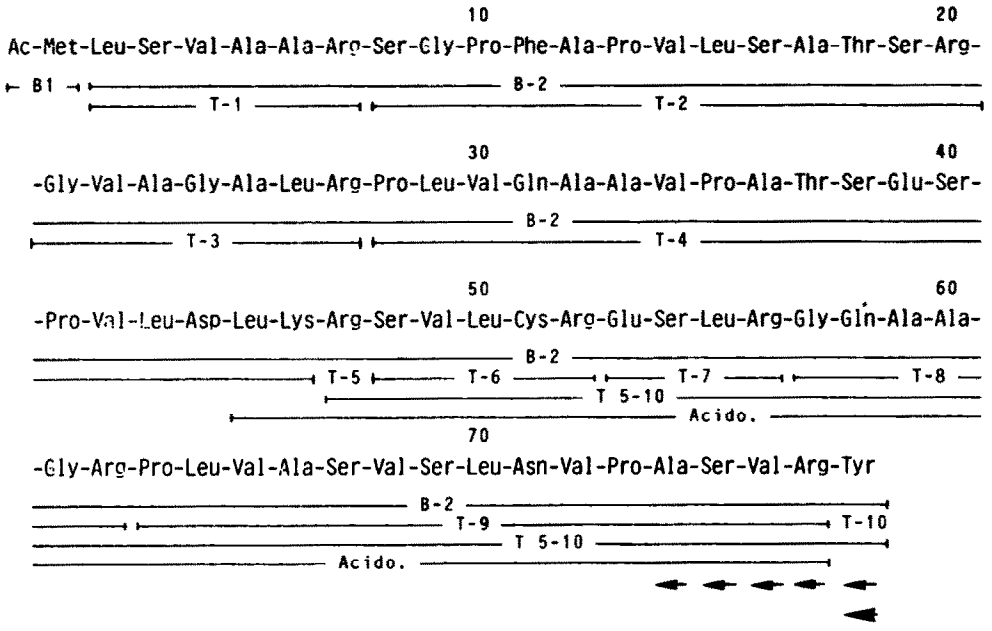

Fig.1. Amino acid sequence of the 8 kDa protein. Bars indicate the fragments used for sequence analysis: B-1, B-2, cyanogen bromide fragments; T-1–T-10, tryptic fragments; T 5-10, overlapping fragment resulting from limited cleavage with trypsin; Acido., acidolytic fragment. Arrows indicate residues released by carboxypeptidase A (◄) and B (◄).

tion is shown in table 2. The protein consists of 78 amino acid residues; its M_r was calculated to be 7998. The protein contains no tryptophan, isoleucine or histidine and only a single lysine, phenylalanine, tyrosine, methionine and cysteine residue.

Sequencer experiments performed with the native protein indicated that it is N-terminally blocked and that the blocking group was not removed under deformylating conditions. Cyanogen bromide cleavage of the protein followed by gel chromatography resulted in a fragment of virtually the same size as the original protein which was devoid of methionine. This fragment was sequenced over 50 steps (fig.1). The rest of its sequence was determined from subfragments obtained by cleavage with trypsin which were isolated by reversed-phase HPLC. The alignment of subfragments was confirmed by partial sequencing of an acidolytic fragment of the whole protein.

The amino acid composition of the tryptic subfragment T-1 suggested that the blocked N-terminal residue was methionine. It was released by pronase digestion of the peptide and was identified as *N*-acetylmethionine according to its elution time on a calibrated reversed-phase column. On this column the *N*-acetylmethionine was eluted separately from *N*-formylmethionine and from free methionine. The collected sample was not detected in the amino acid analyser but yielded methionine after acidic hydrolysis.

Table 2
Amino acid composition of the 8 kDa protein

	A		B
Asx	2.44	(2)	2
Thr	1.85	(2)	2
Ser	9.09	(9)	11
Glx	4.71	(5)	4
Pro	n.d.		7
Gly	4.75	(5)	5
Ala	12.39	(12)	13
Val	10.85	(11)	11
Cys[a]	0.32	(0–1)	1
Met[a]	1.16	(1)	1
Ile	0	(0)	0
Leu	10.15	(10)	10
Tyr	1.02	(1)	1
Phe	0.94	(1)	1
Lys	1.07	(1)	1
His	0	(0)	0
Arg	8.15	(8)	8
Trp	n.d.		0

[a] Determined after performic acid oxidation

(A) Number of residues predicted from hydrolysis assuming an M_r of 8000 (nearest integer in parentheses); (B) number of residues found by sequence analysis

The C-terminal sequence of the protein was identified by carboxypeptidase digestions. The amino acid composition calculated from the sequence agrees well with the integer values predicted from hydrolysis (table 2). The single cysteine was localised by sequence analysis of the tryptic peptide T-6 after performic acid oxidation. Both free arginine (T-5) and free tyrosine (T-10) were isolated from the tryptic digest.

3.2. *Structure predictions from sided hydropathy profiles*

A tentative folding pattern of the 8 kDa protein (fig.2) was derived from average hydropathy profiles according to Kyte and Doolittle [12] and from sided hydropathy profiles (α-helix, β-strand) according to Link and Von Jagow (in preparation). No distinct hydrophobic stretch can be observed when calculating with the average hydropathy algorithm, but the existence of a sided helix and of a sided β-strand becomes obvious when using the sided hydropathy program. A short polar stretch (8 residues) at the N-terminus of the sequence is followed by a membrane-spanning α-helix consisting of presumably 26 residues, beginning and ending with a proline. The helix seems to be distorted, since it includes a third proline. The 2 arginines included in the membrane lie on the same side of the helix as can be seen in the helical wheel plot [13].

A sided β-strand follows, which is connected to the helix by a short intervening polar stretch of about 8 residues. The C-terminus of the β-strand is linked to a second, short α-helical domain (10 residues) which may reach back into the membrane to a depth of about 15 Å.

The sided β-strand begins with leucine and extends over 22 residues. One side is strongly hydrophilic comprising 5 charged residues and is probably directed towards the aqueous bulk phase whereas the other side of the β-sheet seems to face the lipid polar head group region. This side contains merely 1 arginyl and 1 glutamic acid residue.

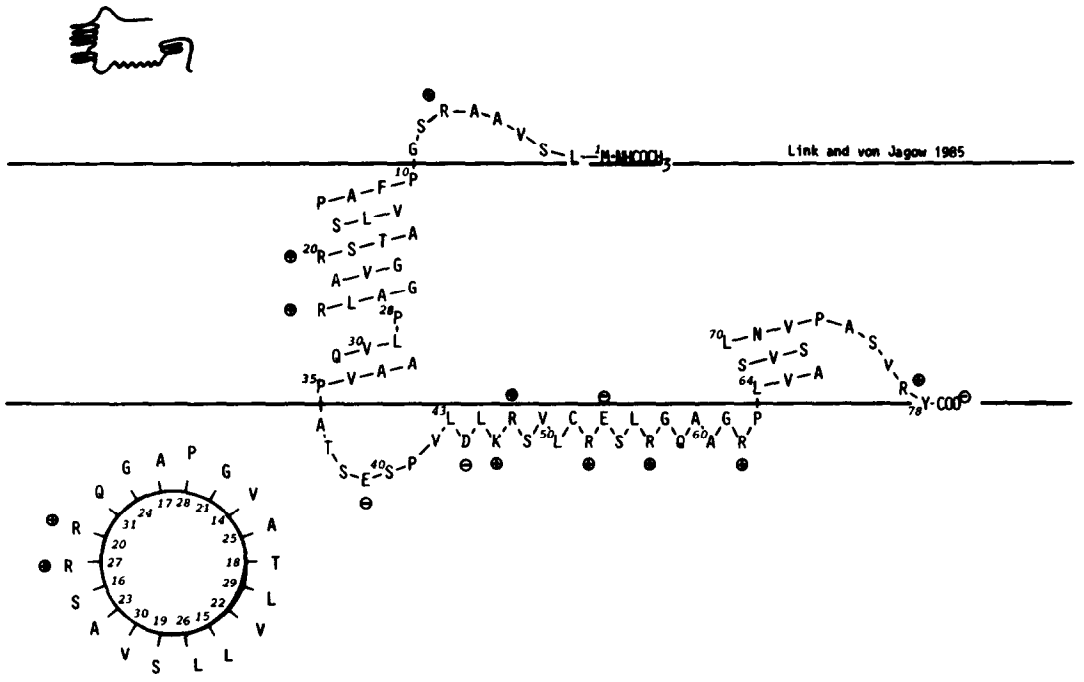

Fig.2. Tentative folding pattern of the 8 kDa protein. The folding pattern has been proposed from the analysis of hydropathy plots according to Kyte and Doolittle [12] and of sided hydropathy plots according to Link and Von Jagow (in preparation). The inset shows a helical wheel plot according to Schiffer and Edmundson [13] of the proposed helical region (residues 14–31).

4. DISCUSSION

The 8 kDa protein was not detected previously, because the resolution power of the available gels was insufficient in this molecular mass region and because the protein was poorly stained when methanol was present in the staining solution. The protein seems to be in close proximity to cytochrome *b*, since it is co-isolated with it. At present it is still unknown whether it contributes to the formation of either the Q_o or Q_i center. It is the protein which is preferentially labelled by DCCD. The glutamic acid residue (position 53) sitting in a relatively apolar region may be the preferential site of reaction with DCCD.

ACKNOWLEDGEMENTS

We gratefully acknowledge the valuable technical assistance of Miss U. Linner, Miss C. Michalski and Miss K. Wiedenmann and the skillful engineering of Mr H. Hofner. This work was supported by a grant to G.v.J. from the Deutsche Forschungsgemeinschaft.

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