Isolation and amino acid sequence of the 9.5 kDa protein of beef heart ubiquinol:cytochrome *c* reductase

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Received 14 February 1986

The 9.5 kDa protein of beef heart ubiquinol:cytochrome *c* reductase was isolated by a series of chromatographic steps involving dissociation of the complex by urea and guanidine. A clear distinction between the 9.5 kDa protein and the 9.2 kDa protein described earlier [(1982) J. Biochem. 91, 2077–2085] by SDS-PAGE was only achieved when the electrophoresis was performed according to Schägger et al. [(1985) FEBS Lett. 190, 89–94; (1986) Methods Enzymol. 126, 22] because in this gel system the apparent molecular mass of the 9.5 kDa protein is shifted to 11 kDa. The amino acid sequence was determined by solid-phase Edman degradation of the whole protein up to amino acid residue 80 and of the proteolytic cleavage fragments. The protein consists of 81 amino acid residues; its M_r was calculated to be 9507. Structure predictions have been made from average and sided hydropathy profiles. The 9.5 kDa protein is either bound to the core proteins within a 9.5 kDa-core protein subcomplex or else it aggregates easily with the core proteins during the isolation procedure.

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

1. INTRODUCTION

The ubiquinol:cytochrome *c* reductase (henceforth called the reductase) is a multiprotein complex consisting of 11 polypeptides. Three of these proteins carry the 4 redox centres while 8 'surplus' subunits do not contain redox centres. These surplus subunits are: the two core proteins with molecular masses of about 47 and 45 kDa; a 13.4 kDa protein denoted ubiquinone-binding protein of complex III (QP-C), since it has been labelled by NAPA-ubiquinone [7]; the 9.5 kDa protein described here, which is tightly linked to the two core proteins; the 9.2 and 7.2 kDa proteins closely associated with cytochrome c_1; the 9.2 kDa protein has also been named 'hinge' protein as it is asserted to be involved in cytochrome *c* binding [1]; an 8 kDa protein, which might be involved in the formation of the Q_0 centre; and finally the smallest subunit, the 6.4 kDa protein, which can be dissociated from the complex together with the iron-sulphur protein by 2 M urea.

The primary structures of the 6 small subunits have been established [1,2,4–6]: those of the two core proteins and that of the FeS protein of beef heart are still expected. A tentative folding pattern of the 9.5 kDa protein has been deduced from average and sided hydropathy profiles obtained by a new algorithm.

2. EXPERIMENTAL

2.1. *Isolation procedure*

All procedures are performed at 4°C. Mitochondria prepared according to Smith [8] 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. [9]. The reductase is prepared as described by Engel et al. [10] and Schägger et al. [3].

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00145793/86/$3.50

2.1.1. Step I – Cleavage of the reductase into 3 fractions: 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 1 (table 1). After washing the hydroxyapatite-bound reductase with 50 ml buffer 1, the 6.4 kDa and FeS proteins 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 [3].

2.1.2. Step II – Cleavage of the bc_1 subcomplex into a fraction comprising 6 proteins and a cytochrome c_1 subcomplex comprising 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 [4].

2.1.3. Step III – Separation of the fraction containing the two core proteins and the 9.5 kDa protein from the fraction containing cytochrome *b*, 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% of cytochrome *b* and almost 100% of the 13.4 kDa and 8 kDa proteins pass the column unbound, whereas the core proteins, 9.5 kDa protein and the remaining part of cytochrome *b* remain bound on the hydroxyapatite column. For separation of cytochrome *b*, 13.4 kDa and 8 kDa proteins, see [3].

2.1.4. Step IV – Separation of the 9.5 kDa protein from the two core proteins

The fraction still bound on the hydroxyapatite column and containing the 9.5 kDa protein and the two core proteins is eluted by adding buffer 8. After removing the guanidine by gel filtration on a 100 ml Sephadex G-25 column pre-equilibrated with buffer 9, the proteins are precipitated by an equal volume of acetone. The precipitate is washed with water and dissolved in a solution containing 4% SDS and 1% mercaptoethanol, to a final protein concentration of 10 mg/ml. Then the 9.5 kDa protein is separated from the two core proteins by

Table 1

Buffers for the preparation of the subunits of the bc_1 complex from beef heart mitochondria

	1	2	3	4	5	6	7	8	9
Triton X-100 (%)	0.05	1.0	0.05	0.5	0.5	0.5	0.5	0.5	0.1
NaCl (M)	0.05	0.4	0.4	0.2	0.25	–	0.2	–	0.2
Na phosphate (M)	0.035	0.025	0.05	0.025	0.085	0.35	0.01	–	–
K phosphate (M)	–	–	–	–	–	–	–	0.25	–
Mops (M)	–	–	–	–	–	–	–	–	0.01
Guanidine (M)	–	–	–	1.5	–	–	–	3.0	–
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 NaN_3

gel filtration at room temperature in 1% SDS on a Sephadex G-100 column of 300 ml. Core protein I is only partially separated from core protein II under these conditions.

2.1.5. Step V – Removal of SDS and minor impurities

The fractions containing the 9.5 kDa protein are again precipitated by addition of an equal volume of acetone and stored overnight at −20°C. The sediment is solubilised in 80% formic acid and chromatographed on a Sephacryl S-200 column in 80% formic acid.

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 to account for destruction and incomplete hydrolysis.

N-terminal solid-phase Edman degradation of the whole 9.5 kDa protein was performed with 20 nmol protein coupled to aminopropyl glass via its carboxyl groups.

Cleavage with endoproteinase Lys-C (Boehringer Mannheim) was performed in 0.1 M ammonium bicarbonate, pH 9.0, 0.1% SDS for 3 h at 37°C using a protease/substrate ratio of 1:50 (w/w). The resulting fragments were separated by gel chromatography on a Sephacryl S-200 column in 80% formic acid. Cleavage with *Staphylococcus aureus* V8 protease (Miles) was performed in 25 mM Tris-phosphate buffer, pH 7.5, 0.1% SDS for 4 h at 37°C (protease/substrate, 1:50, w/w). The fragments were separated by high-performance liquid chromatography (HPLC) on a Shandon WP 300 Hypersil octyl column using gradients of acetonitrile in 0.05% trifluoroacetic acid. Fragments obtained by digestion with chymotrypsin (Merck, 1:50, w/w) in 0.1 M ammonium bicarbonate, pH 8.0, 0.1% SDS (4 h at 37°C) were separated in the same HPLC system.

The C-terminal amino acid residues were determined by digestion of the whole protein with carboxypeptidase A and B (Boehringer Mannheim) in 0.1 M morpholine acetate buffer, pH 8.0, for different incubation times. The released amino acids were quantitated in the amino acid analyser.

All amino acid sequences were determined by automated solid-phase Edman degradation using a non-commercial sequencer with on-line detection of the released amino acid phenylthiohydantoin derivatives (PTHs) by HPLC [11]. The protein and the fragment peptides were immobilised on aminopropyl glass (77 Å) via their carboxyl groups using hydroxybenzotriazole-catalysed carbodiimide activation, or on diisothiocyanate-activated aminopropyl glass essentially as described in [11]. Cysteine was identified as cysteic acid PTH after performic acid oxidation.

3. RESULTS

3.1. *Amino acid sequence*

The protein consists of 81 amino acid residues. The amino acid composition obtained by hydrolysis agrees reasonably well with the composition derived from the amino acid sequence (table 2). The M_r is calculated to be 9507. The pro-

Table 2

Amino acid composition of the 9.5 kDa protein

	a		b
Asx	4.1	(4)	4
Thr	4.4	(4–5)	5
Ser	3.7	(4)	4
Glx	7.9	(8)	7
Pro	n.d.		6
Gly	4.3	(4)	4
Ala	5.7	(6)	6
Val	5.7	(6)	6
Cys*	0.9	(1)	2
Met*	0	(0)	0
Ile	2.9	(3)	3
Leu	5.2	(5)	5
Tyr	3.8	(4)	5
Phe	6.7	(7)	7
Lys	3.8	(4)	4
His	3.4	(3)	3
Arg	10.2	(10)	10
Trp	n.d.		0
Total			81

* Determined after performic acid oxidation

(a) Number of residues predicted from amino acid hydrolysis assuming a molecular mass of 10 kDa (nearest integer in brackets); (b) number of residues found by sequence analysis

tein contains neither tryptophan nor methionine, but 6 proline residues. The protein has a net charge of +9 (14 positively charged residues vs 5 negatively charged residues). The content of hydrophobic residues amounts to one third.

Automated solid-phase Edman degradation of the whole protein (20 nmol) resulted in a continuous sequence of 80 residues (fig.1). This sequence was confirmed by independent solid-phase degradations of a complete set of fragments obtained by enzymatic cleavage with endoproteinase Lys-C, *S. aureus* V8 protease, and chymotrypsin, and isolated by gel chromatography and/or reversed-phase HPLC (fig.2). A fragment overlapping residues 66–78 was found, resulting from incomplete cleavage by *S. aureus* V8 protease. The C-terminal end was confirmed by the release of a single arginine upon digestion with carboxypeptidase B, whereas no amino acid was found after incubation with carboxypeptidase A.

3.2. *Structure predictions from sided hydropathy profiles*

A tentative folding pattern of the 9.5 kDa protein (fig.3) 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 [13]. A strongly hydrophobic region (residues 43–63) was detected in the hydropathy plot according to Kyte and Doolittle [12], almost fulfilling their requirements for membrane-spanning α-helices (mean hydropathy 1.56). However, since this segment contains 2 of the 6 proline residues adjacent to each other, a

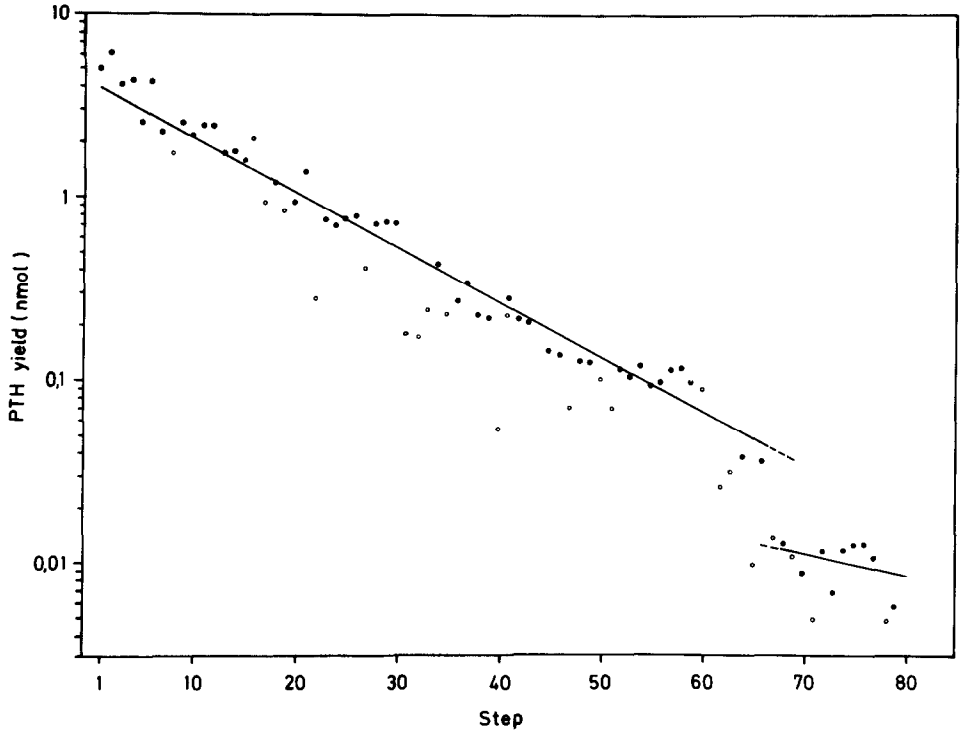

Fig.1. Yields of amino acid phenylthiohydantoin derivatives (PTH) obtained by automated solid-phase Edman degradation of the whole 9.5 kDa protein determined by high-performance liquid chromatography. Open circles represent yields which are decreased due to the instability of the amino acid to acid treatment (serine, threonine) or due to partial side-chain attachment to the support (glutamic and aspartic acid), enlarged by the overlap portion from the preceding identical residue, or yields of amino acids which give rise to more than one PTH derivative (proline, glycine). Prevalent side-chain attachment of Glu-65 to the support is the reason for the significant drop of PTH yields after this residue. The two separate regression lines calculated from the data represented by solid circles correspond to mean repetitive yields of 93.4 and 97.3%, respectively.

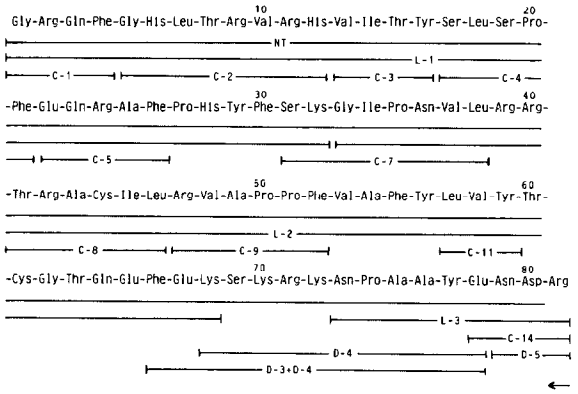

Fig.2. Amino acid sequence of the 9.5 kDa protein. Bars indicate the fragments used for sequence analysis: NT, sequence determined by automated solid-phase Edman degradation of the whole protein; this sequence was confirmed by complete sequencing of fragments obtained after cleavage with endoproteinase Lys-C (L-1–L-3), *S. aureus* V8 protease (D-3-4, D-4, D-5), and chymotrypsin (C-1–C-10). The arrow indicates the arginine residue released by carboxypeptidase B (←).

twist of the helix is to be expected. It could even result in a sharp bend of the helix so that the hydrophilic stretches on both sides of the hydrophobic helix may be located on the same side of the membrane.

The N-terminal region (residues 1–19) may form a membrane-penetrating, amphiphilic helix including 3 arginine residues. A third, strongly amphiphilic but short helix (residues 27–39) is proposed to be located at the surface of the membrane. The membrane-penetrating stretches contain solely arginine as polar residues while the C-terminal stretch is strongly hydrophilic but without a net charge (5 positively charged side chains vs 4 negatively charged side chains plus the terminal carboxy group). Therefore this protein shows an asymmetry in the distribution of charges on the two sides of the membrane, which has already been observed for some of the other small subunits of ubiquinol:cytochrome *c* reductase [13]. The arginine residues embedded in the hydrophobic

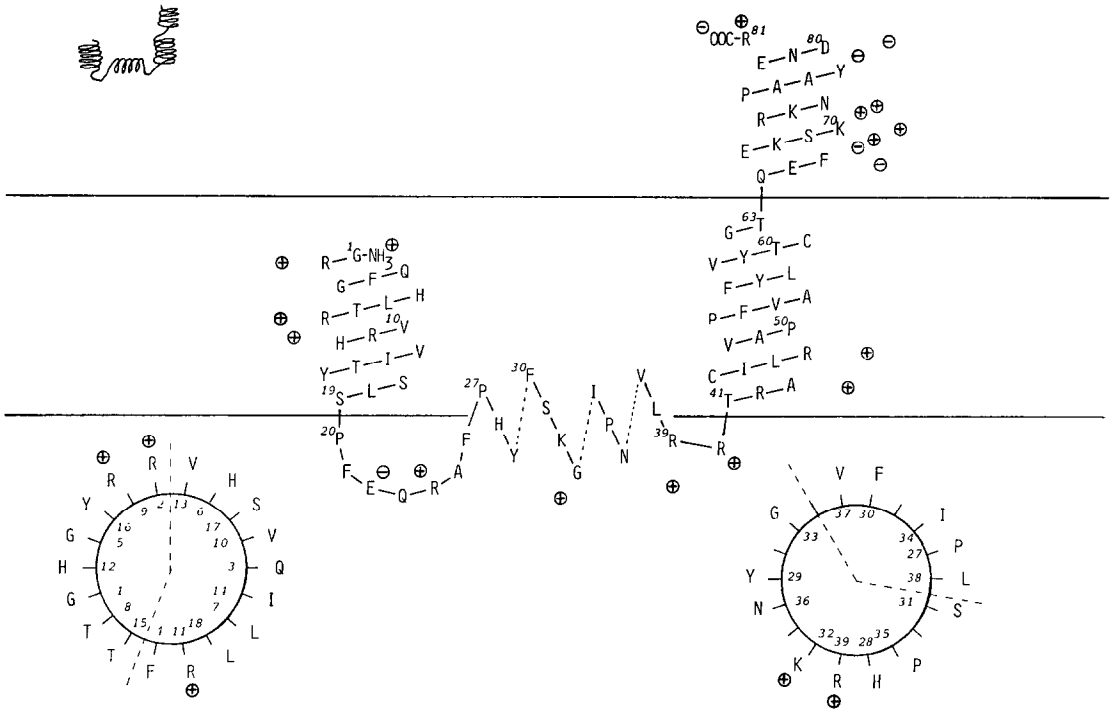

Fig.3. Tentative folding pattern of the 9.5 kDa protein. The folding pattern is proposed on the grounds of an analysis of hydropathy plots according to Kyte and Doolittle [12] and of sided hydropathy plots according to Link and Von Jagow [13]. The insert shows a helical wheel plot according to Schiffer and Edmundson [14] of two of the proposed helical regions (residues 1–18 and 26–39).

phase could be responsible for the tight binding between the 9.5 kDa subunit and the core proteins.

4. DISCUSSION

The function of the 9.5 kDa protein is not yet known. It seems to be strongly bound to the core proteins, as indicated by the following observations:

The 9.5 kDa-core protein fraction bound to hydroxyapatite can be partly eluted by buffers containing phosphate, e.g. buffer 5, without changing the ratio of 9.5 kDa protein to core protein. The fractions thus eluted proved to be a mixture of aggregates when chromatographed on Sepharose 6B-CL in the presence of Triton X-100. No significantly different ratio of the proteins was detected by SDS-PAGE when the first fractions, containing the strongly aggregated material, were compared with the last fractions, containing only slightly or not at all aggregated proteins.

However, there is no definite answer to the question whether the '9.5 kDa-core protein subcomplex', resistant even to treatment with 1.5 M guanidine, exists already in the native reductase or whether these proteins merely aggregate during isolation of the subunits. We, in fact, favour the existence of a native 9.5 kDa-core protein subcomplex. If unspecific aggregation of the core proteins did occur, the 9.5 kDa protein should not be the only protein to be trapped.

The apparent molecular mass of the 9.5 kDa protein is shifted to 11 kDa in the SDS-PAGE according to Schägger et al. [2,3]. This fact allows a clear distinction from the 9.2 kDa protein and the 13.4 kDa QP-C protein. In the SDS-PAGE systems using urea according to Merle and Kadenbach [15] and Swank and Munkres [16] the 9.2 kDa and 9.5 kDa proteins are very close together or not separated at all. Urea, although it generally improves the resolution power for small proteins, probably causes a deterioration of the resolution in this case, perhaps due to a more complete unfolding of the proteins. SDS gels according to Schägger et al. [2,3], using 3–8 M urea instead of glycerol, showed the same phenomenon.

ACKNOWLEDGEMENTS

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

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