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Isolation and amino acid sequence of the 'Rieske' iron sulfur protein of beef heart ubiquinol:cytochrome *c* reductase

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The sequence of the 'Rieske' iron sulfur protein from the bc_1 complex of beef heart mitochondria has been determined by solid phase Edman degradation of the whole protein and of various proteolytic fragments. The protein consists of 196 amino acid residues. The molecular mass of the apoprotein was calculated to be 21 536 Da, that of the holo-protein including the Fe_2S_2 cluster as 21 708 Da. The protein is mainly hydrophilic with a polarity index of 42.9% and 25% of charged residues. It contains a hydrophobic membrane anchor which is predicted to form a 'hairpin' structure. The iron sulfur cluster is bound near the C-terminus of the protein between a hydrophobic and a more amphipathic domain. This reflects the fact that the cluster is located near the outer surface of the inner mitochondrial membrane. A folding pattern describing all known features of the protein is proposed.

Ubiquinol:cytochrome *c* reductase; bc_1 complex; Iron sulfur protein; Primary structure; Membrane protein; (Beef heart mitochondria)

1. INTRODUCTION

The *bc* complexes are the ubiquitous middle parts of respiratory and photosynthetic electron transfer chains in mitochondria, chloroplasts, and many bacteria. They are multiprotein complexes consisting of three to eleven different subunits, three of which are present in all species. These conserved subunits are: (i) a two-heme cytochrome *b*, the only mitochondrially coded subunit; (ii) cytochrome c_1; (iii) the 'Rieske' iron sulfur protein (ISP) containing a 2-Fe-2-S cluster with an unusually high redox potential (+280 mV). The ISP constitutes part of one of the quinone reaction sites of the bc_1 complex and is the primary acceptor of one electron on oxidation of a hydroquinone. While the sequences of ISPs from various *bc* complexes have already been established, the sequence of the ISP from the beef heart mitochondrial bc_1 complex presented here is the first mammalian 'Rieske' sequence and so far the only one that has been determined completely by amino acid sequencing. The sequences of nine out of the eleven subunits of the beef heart bc_1 complex have now been determined, with only the sequences of the two 'core' proteins, large subunits without redox centres, lacking.

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2. EXPERIMENTAL

2.1. *Isolation of the iron sulfur protein*

All procedures were performed at 4°C unless otherwise indicated. Uniquinol:cytochrome *c*

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reductase was prepared as described by Engel et al. [1] and Schägger et al. [2].

2.1.1. Hydroxyapatite chromatography

Freshly prepared reductase (100 mg) in 0.05% Triton X-100, 100 mM NaCl, 10 mM Mops was applied onto a 100 ml hydroxyapatite column after addition of 2 nmol of antimycin per nmol cytochrome c_1 and after addition of Na-phosphate buffer, pH 7.2, to a final concentration of 35 mM. The hydroxyapatite column had to be equilibrated in advance with 0.05% Triton X-100, 50 mM NaCl, 35 mM NaP_i, 1 mM NaN_3, pH 7.2. The bound complex was washed with 100 ml of a buffer containing 0.05% Triton, 250 mM NaCl, 50 mM NaP_i, 1 mM NaN_3 before the 6.4 kDa protein and the ISP were split off by a buffer containing 0.5% Triton, 250 mM NaCl, 85 mM NaP_i, 1 mM NaN_3, 0.2 mM PMSF. This buffer eluted both proteins, but the 6.4 kDa protein eluted almost quantitatively in the first fractions (in parallel with the rising Triton concentration), whereas the elution of the iron sulfur protein began with a delay of some fractions. For a complete elution of the ISP, two column volumes of buffer were necessary, because the cleavage conditions are so mild that the dissociation–association equilibrium of the ISP was maintained. This preparation method differs from the method described [3] in two main aspects. Antimycin was used for stabilisation of the bc_1 subcomplex and urea was omitted, so that very little contamination of the ISP by other subunits of the reductase was observed. After taking samples for protein determination and SDS-PAGE, all fractions were stored at −20°C.

2.1.2. Removal of salt and detergent from the iron sulfur protein

The dilute ISP fractions were thawed and concentrated by pressure filtration on an Amicon PM 10 membrane after addition of 1 mM EDTA and 0.2 mM PMSF. The ISP was then precipitated by addition of the same volume of acetone and stored overnight at −20°C to complete the precipitation. Warming up to 40°C helped to resolubilise the phosphate that coprecipitated. After centrifugation, the sediment was washed with water and was then used directly for cleavage reactions for example by CNBr or 80% formic acid. If necessary, the protein was further purified by preparative SDS-PAGE.

2.1.3. Preparative SDS-PAGE for separation of protein fragments

A new SDS-PAGE method for separation of small proteins without urea was used [2,4]. Without prior staining of the protein bands, the gels were cut into slices by a stack of 15 cm long razor blades, mounted at a distance of 2 mm from each other. The slices were then transferred to plastic tubes filled with 5–10 ml of a 0.1% SDS solution. After shaking overnight at room temperature, the extract was decanted through a net. The extraction was repeated one more time. The extracts were lyophilised, resolubilised in a minimal volume of 80% formic acid and chromatographed at room temperature on a 130 ml glass column filled with Sephacryl S-200 in 80% formic acid. Proteins with acid labile Asp-Pro bonds, like the Rieske ISP, had to be desalted before they were chromatographed at 4°C.

2.2. *Amino acid sequence determination*

For amino acid analysis, the protein samples were hydrolysed in 5.7 M HCl at 105°C for 24 h. Cyanogen bromide cleavage was performed in 80% formic acid for 12 h at room temperature using a 300-fold molar excess of cyanogen bromide (Serva) over methionine. For acidolytic cleavage, the protein was incubated in 80% formic acid for 16 h at 40°C. Digestion with *Staphylococcus aureus* protease V8 (Miles) was done in 0.05 M ammonium bicarbonate buffer, pH 7.8, for 20 h at 37°C. For digestion with endoproteinase Lys-C (Boehringer Mannheim), the protein was incubated for 12 h at 37°C in 0.1 M ammonium bicarbonate buffer, pH 9.0 (protease/substrate, 1:50, w/w). The peptides resulting from cleavages were isolated by gel chromatography on a Sephacryl S-200 column in 80% formic acid and/or by preparative gel electrophoresis as described above. C-terminal residues were obtained after carboxypeptidase C (Röhm) digestion of the whole protein. The protein was incubated in 0.1 M morpholine acetate buffer at pH 6.0 for varying 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 amino acid phenylthiohydantoin derivatives (PTH) by HPLC [5]. The protein and its fragments were covalently attached to porous glass supports as described [5,6].

3. RESULTS

3.1. *Amino acid sequence*

The complete amino acid sequence of the Rieske iron sulfur protein is given in fig.1. The protein consists of 196 amino acid residues. The molecular mass of the apoprotein was calculated to be 21536 Da. The molecular mass of the holoprotein with the Fe_2S_2 center is calculated to be 21708 Da. The amino acid composition is shown in table 1. The protein does not contain post-translationally modified residues.

The first 57 residues were sequenced by N-terminal degradation of the whole protein (N-T). Acidolytic cleavage of a single Asp-Pro bond resulted in two fragments (Ac 1 and Ac 2, residues 1–119 and 120–196) which were overlapped by the cyanogen bromide peptide CB 4. Cyanogen bromide cleavage of the whole protein resulted in the formation of several main fragments (CB 1, residues 1–62; CB 1–2, res. 1–71; CB 1–3, res. 1–87; CB 4, res. 88–192; CB 4–5, res. 88–196; CB 5, res. 193–196) and of minor amounts of the fragments CB 2–4, res. 63–192; CB 2–5, res. 63–196; CB 3–4, res. 72–192 and CB 3–5, res. 72–196. The gap between the sequenced residues

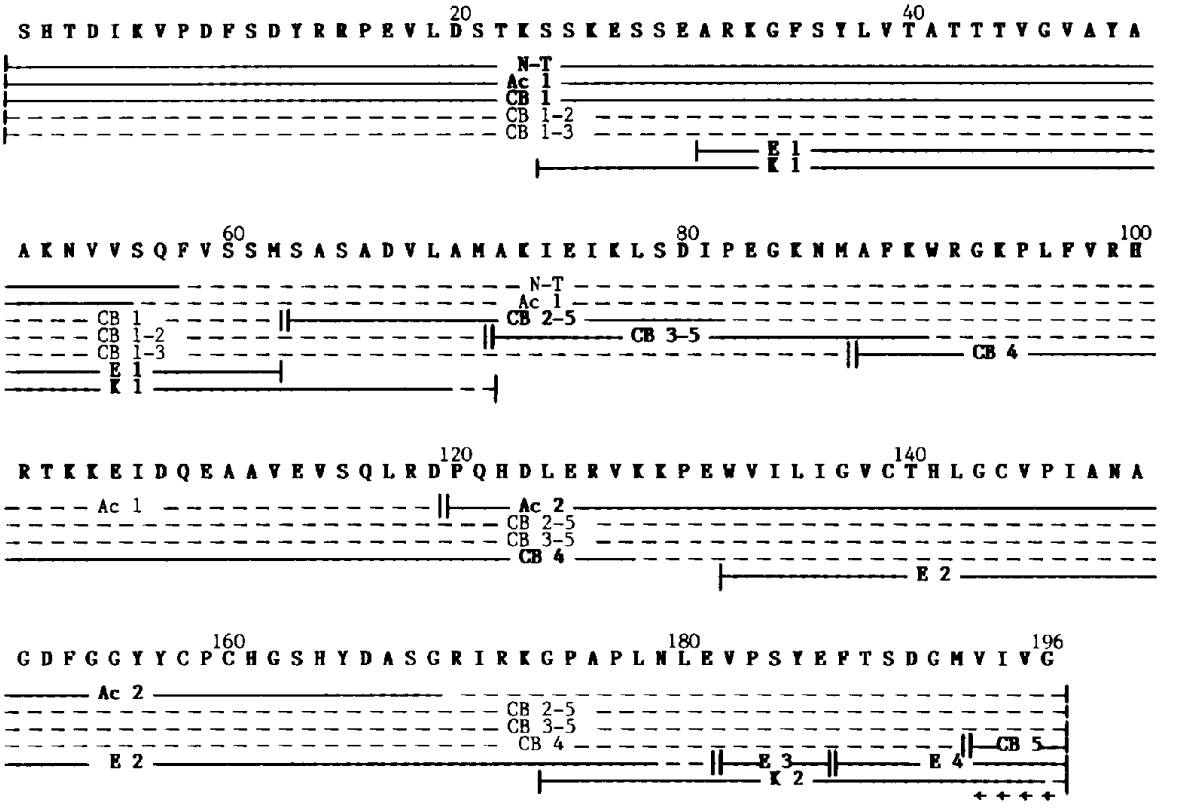

Fig.1. Amino acid sequences of the Rieske iron sulfur protein and of the fragments used for sequence determination. Sequenced parts of the fragments are indicated by solid lines. Broken lines, standing for the parts that were not sequenced, help to indicate the total length of the fragments used. Arrows indicate the amino acid residues obtained by cleavage with carboxypeptidase C. N-T, N-terminal sequence; Ac, acidolytic fragments; CB, cyanogen bromide fragments; E, fragments from digestion with *Staphylococcus aureus* protease V8; K, fragments from digestion with endoproteinase Lys-C.

Table 1

Amino acid composition of the Rieske iron sulfur protein

	Calculated	24 h hydrolysate
Aspartic acid	12	16.5
Asparagine	4	
Threonine	9	8.7
Serine	19	17.8
Glutamic acid	12	16.4
Glutamine	4	
Proline	11	n.d.
Glycine	14	12.7
Alanine	16	15.9
Valine	19	16.5
Cysteine	4	n.d.
Methionine	4	1.9
Isoleucine	10	9.5
Leucine	11	16.0
Tyrosine	7	7.0
Phenylalanine	7	8.2
Lysine	15	13.7
Histidine	6	6.7
Arginine	10	11.1
Tryptophan	2	n.d.
Total	196	

of CB 1 and CB 2–5 was filled by the sequence of fragment E 1, obtained by cleavage of CB 1 with *Staphylococcus aureus* protease V8 (specific for glutamyl bonds). The overlapping sequence K 1 to fragment CB 2–5 was obtained by endoproteinase Lys-C cleavage (specific for lysyl bonds) of the cyanogen bromide fragment CB 1–2. The C-terminal acidolytic fragment, Ac 2, was sequenced by degradation of the whole peptide and of its endoproteinase V8 subfragments E 2, E 3, and E 4. Overlapping of these subfragments and confirmation of the C-terminal end of the molecule was achieved using a typical solid-phase strategy: the whole Ac 2 peptide was digested with endoproteinase Lys-C and the unseparated fragments were coupled to *p*-phenylene diisothiocyanate activated aminopropyl glass via their amino groups. The C-terminal fragment containing no lysine, K 2, was selectively released by treatment with trifluoroacetic acid, coupled via its carboxyl groups and sequenced. The C-terminal residues were confirmed by analysis of the amino acid residues released by carboxypeptidase C. For the identification of cysteines the C-terminal acidolytic fragment was oxidised with performic acid before Edman degradation.

3.2. *Hydrophobicity analysis*

The beef heart ISP contains 25% charged residues with a single positive net charge. The polarity index according to Vanderkooi and Capaldi [7] is 42.9%, indicating large hydrophilic domains. These can be seen also in the hydrophobicity profile (fig.2). This observation is in agreement with the facts that (i) the ISP extends largely into the intermembrane space; (ii) the ISP is in a dissociation–association equilibrium in the detergent-solubilised complex; (iii) the redox centre is located near the accessible surface of the complex (Ohnishi, T., personal communication). In addition, two hydrophobic domains are found at positions 34–72 and 132–151. While the second hydrophobic stretch constitutes part of the iron sulfur cluster binding domain, the first hydrophobic domain may form a membrane an-

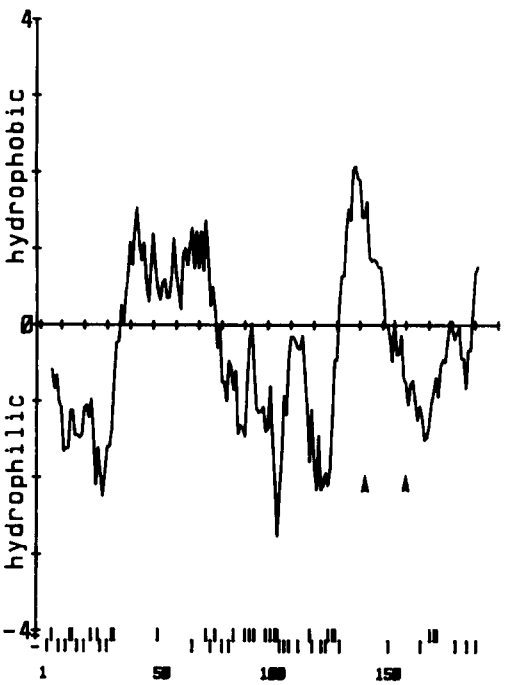

Fig.2. Hydropathy profile of the Rieske iron sulfur protein from beef heart bc_1 complex according to Kyte and Doolittle [8] using a span setting of 11. The arrows indicate the approximate positions of the two pairs of cysteine residues.

chor. Since the length of this domain allows for two membrane spanning α-helices, we suggest that it forms a 'hairpin' structure with two polar residues (Lys-52 and Asn-53) introducing a turn (cf. fig.5). The N-terminus is therefore predicted to be on the outside of the inner mitochondrial membrane, in agreement with protease digestion experiments (Neupert, W., personal communication).

4. DISCUSSION

4.1. *Sequence analysis and comparison with other ISP sequences*

Including the beef heart sequence described here, the sequences of six Rieske ISPs have been determined by amino acid sequencing (beef heart and spinach b_6f (partially) [9]) or deduced from the DNA sequence (spinach b_6f [10], *Neurospora crassa* [11], *Saccharomyces carlsbergensis* [12], *Rhodobacter capsulatus* [13], and *Paracoccus denitrificans* [14]). An alignment of these sequences is given in fig.3. The C-terminal parts of all six sequences are strongly conserved while the N-terminal parts show only few conserved residues. However, the hydropathy patterns of all sequences are very similar (fig.4). All six sequences have two hydrophobic stretches, one near the C-terminus comprising two conserved cysteines and

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                                                           :  :              *               *
Spinach:               ATSIPADNVPDMQKRETLNLLLLGALSLPTGYMLLPYASFFVPPGGGAGTGGTIAKD         ALGND
                                                                                            67
Beef:         SHTDIKVPDFSDY   RRPEVLDSTKSSKESSEA        RKGF SYLVTATTTVGVAYA AKNVVSQFVSSMSASAD

N. crassa:  GSSSSTFESPFKGESKAAKVPDFGKYMSKAPPSTNM          LF SYFMVGTMGAITAAG AKSTIQEFLKNMSASAD
S. carlsb.:            KSTY   RTPNFDDVLKENNDADKG         RSYA YFMVGAMGLLSSAG AKSTVETFISSMTATAD
P. denitr.:     MSHADEHAGDHGATRR                          DFL YYATAGAGTVAAGA A   AWTLVNQMNPSAD
Rb. caps.:      MSHAEDNAG    TRR                          DFL YHATAATGVVVTGA A   VWPLINQMNASAD
                              +                            :  *              *          *   **


            * *                      * * :                                    -  +
Spinach:    VIAA   EWLKTHAPGDRTLTQGLKGDPTYL                                 VVESDKTLATF GIN
                                                                                           130
Beef:       VLAMAKIEIKLSDIPEGKNMAFKWRGKPLFVRHRTKKEIDQEAAVEVSQLRDPQH           DLERVKKP       E

N. crassa:  VLAMAKVEVDLNAIPEGKNVIIKWRGKPVFIRHRTPAEIEEANKVNVATLRDPET           DADRVKKP       E
S. carlsb.: VLAMAKVEVNLAAIPLGKNVVVKWQGKPVFIRHRTPHEIQEANSVDMSALKDPQT           DADRVKDP       Q
P. denitr.: VQALASIQVDVSGVETGTQLTVKWLGKPVFIRRRTEDEIQAGREVDLGQLIDRSAQNSNKPDAPATDENRTMDEA     GE
Rb. caps.:  VKAMSSIFVDVSAVEVGTQLTVKWRGKPVFIRRRDEKDIELARSVPLGALRDTSAENANKPGAEATDENRSLAAFDGTNTGE
            * *             *     ** *** * * *   -*     *    * *              *  *


                   *******       -   : ****** *   **  +****  *
Spinach:         AVCTHLGCVVPFN AAE NKFICPCHGSQYNNQGRVVRGPAPLSLALAHCD VDDGKVVFVPWTETDFRTGEAPWWSA
                                                                           196
Beef:       WVILIGVCTHLGCVPIAN AGDFGGYYCPCHGSHYDASGRIRKGPAPLNLEVPSYEFTSDGMVIVG

N. crassa:  WLVMLGVCTHLGCVPIGE AGDYGGWFCPCHGSHYDISGRIRKGPAPLNLEIPLYEFPEEGKLVIG
S. carlsb.: WLIMLGICTHLGCVPIGE AGDFGGWFCPCHGSHYDISGRIRKGPAPLNLEIPAYEF DGDKVIVG
P. denitr.: WLVMIGVCTHLGCVPIGDGAGDFGGWFCPCHGSHYDTSGRIRRGPAPQNLHIPVAEFLDDTTIKLG
Rb. caps.:  WLVMLGVCTHLGCVPMGDKSGDFGGWFCPCHGSHYDSAGRIRKGPAPRNLDIPVAAFVDETTIKLG
            *    * ********     **:**::*********  ****+**** **  *   *        *
                   ▲ ▲  ▲              ▲ ▲▲        ▲
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Fig.3. Alignment of the sequences of Rieske iron sulfur proteins from spinach b_6f [9,10], beef heart (this paper), *N. crassa* [11], *S. carlsbergensis* [12], *Rb. capsulatus* [13], and *P. denitrificans* [14]. The top line above the spinach sequence shows residues conserved in all six sequences while the bottom line below the Rb. caps. sequence shows residues conserved in the mitochondrial and bacterial sequences (excluding the spinach). (*) Conserved residues; (+ and −) conserved charges; and (:) an exchange of one aromatic residue against another.

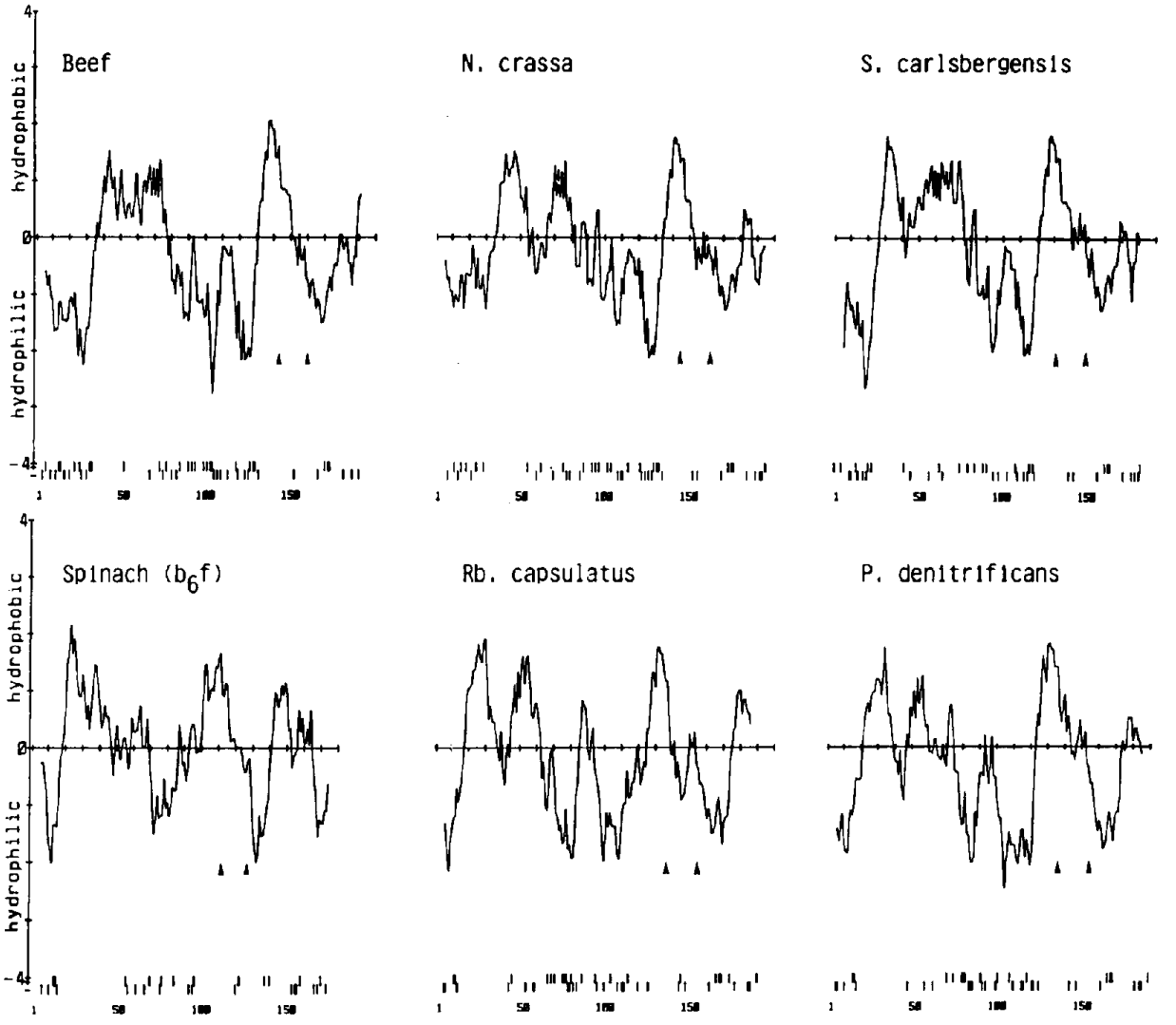

Fig.4. Comparison of the hydropathy profiles of the six Rieske iron sulfur proteins. The hydropathy values of Kyte and Doolittle [8] were used, applying a span setting of 11.

one conserved histidine, and one near the N-terminus allowing for two membrane spanning helices, except in the case of the spinach b_6f ISP, which appears to have only one membrane spanning stretch. In the five mitochondrial and bacterial ISPs sequenced so far, the occurrence of two hydrophobic membrane anchors is well conserved, but without strong amino acid homology between these sequences. Considering the orientation of the chloroplast b_6f complex, i.e. the iron sulfur cluster facing to the inside while it is directed outwards in the mitochondrial bc_1 complex, the absence of a second membrane spanning helix in the chloroplast ISP would bring the N-termini of both the mitochondrial and chloroplast ISPs to the cytosolic side of the respective membranes, which may be relevant considering the import pathways of these nuclear coded proteins [15].

The strongly conserved sequence near the C-terminus (fig.3) seems to be responsible for coordinating the iron sulfur cluster. It comprises four conserved cysteines that have been proposed to bind the redox centre, in analogy to the situation found in plant ferredoxins where the Fe_2S_2 cluster

is also coordinated by four cysteine residues. However, there is no homology between the iron sulfur cluster binding site in the Rieske ISP and the plant ferredoxins and the redox potentials differ by about 700 mV (Rieske: +280 mV; spinach ferredoxin: −420 mV). Moreover, the Rieske type ISP from *Thermus thermophilus* contains two apparently identical Fe_2S_2 clusters but only four cysteines, so that several ligands have to be non-sulfur atoms [16]. Recent measurements in yeast mitochondrial bc_1 complex have confirmed that the iron sulfur cluster is coordinated by at least one, and probably two nitrogen ligands [17], one of which is an imidazole ring nitrogen of a histidine side chain [18].

Further inspection of the sequence alignment reveals that, besides the four cysteine residues (beef heart positions 139, 144, 158, and 160), two histidines (beef heart 141 and 161) and one arginine (beef heart 170) are fully conserved, but no lysine. Therefore, four out of these seven residues may coordinate the iron sulfur cluster. These residues are found in two groups, one in a hydrophobic stretch, the other in a more amphipathic environment. It is therefore tempting to speculate that the redox centre is bound between two protein domains, one lying within the membrane and the other shielding the iron sulfur cluster from the outside.

The predicted folding pattern (fig.5) summarises the known features of the protein: (i) the amino- and carboxy-termini both lie on the outside of the inner mitochondrial membrane; (ii) the main part of the protein is hydrophilic and extends into the intermembrane space; (iii) the iron sulfur cluster is located near the membrane surface; (iv) the N-terminal part forms a membrane anchor with a hairpin-like structure; (v) the iron sulfur cluster is bound near the C-terminus in a strongly conserved region between a hydrophobic and an amphipathic region; (vi) besides two or three of the four conserved cysteine residues, two conserved histidines or one conserved arginine (not shown) may coordinate the redox centre.

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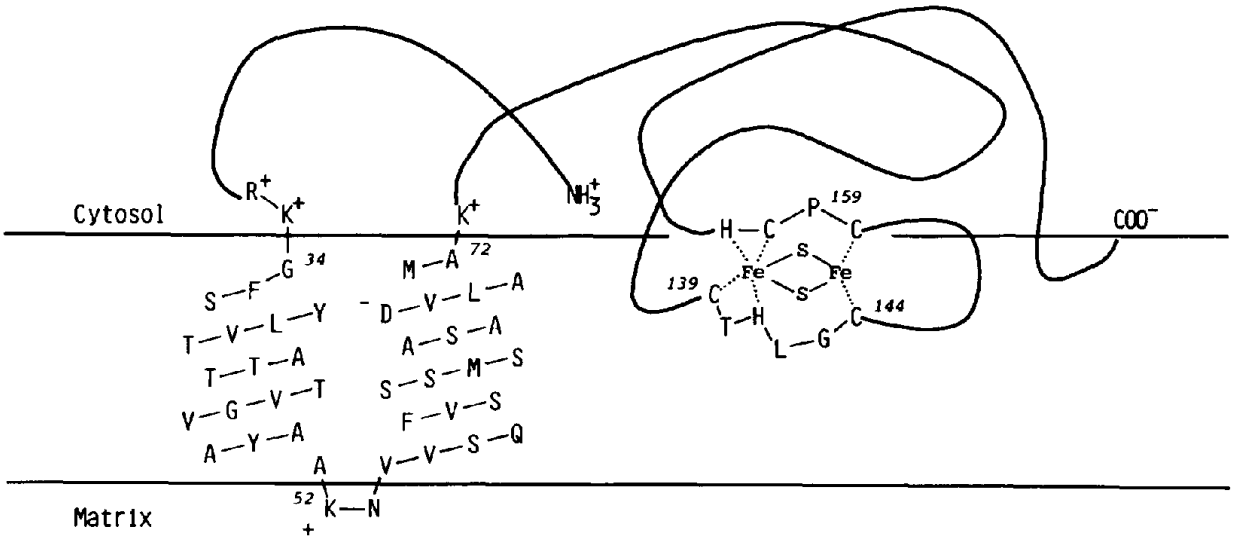

Fig.5. Predicted folding pattern of the beef heart iron sulfur protein in the inner mitochondrial membrane. Since the coordination of the iron sulfur cluster has not been firmly established, merely possible ligands are indicated, two of which may bind to each iron atom.

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