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The primary structure of the iron-sulfur subunit of ubiquinol–cytochrome *c* reductase from *Neurospora*, determined by cDNA and gene sequencing

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The primary structure of the iron-sulfur subunit of ubiquinol–cytochrome *c* reductase from *Neurospora* mitochondria was determined by cDNA and genomic DNA sequencing. A first cDNA was identified from a cDNA bank cloned in *Escherichia coli* by hybridization selection of mRNA, cell-free protein synthesis and immunoadsorption. Further cDNA and geonomic DNA were identified by colony filter hybridization. The N-terminal sequence of the mature protein was determined by automated Edman degradation. From the sequence a molecular mass of 24749 Da results for the precursor protein and of 21556 Da for the mature protein. The presequence consists of 32 amino acids with four arginines as the only charged residues. The mature protein consists of 199 amino acids. It is characterized by a small N-terminal hydrophilic part of 29 residues, a hydrophobic stretch of 25 residues and a large C-terminal hydrophilic domain of 145 residues. The only four cysteines of the protein, which are assumed to bind the 2Fe-2S cluster, are located in a moderate hydrophobic region of this large domain. Cysteines 3 and 4 are unusually arranged in that they are separated by only one proline. From sequence data the arrangement of the subunit in the membrane is deduced.

Ubiquinol–cytochrome *c* reductase (cytochrome reductase) is a proton-translocating enzyme complex of the oxidative phosphorylation system in mitochondria. The enzyme isolated from *Neurospora* consists of the cytochromes *b* and c_1, an iron-sulfur subunit (Rieske 2Fe-2S protein, [1]) and six subunits without redox centers [2, 3].

The three-dimensional structure of cytochrome reductase, the arrangement of the structure in the mitochondrial inner membrane and the topography of most of the subunits within the structure have been studied by electron microscopy of two-dimensional crystals, neutron diffraction of enzyme/detergent preparations and biochemical characterization of isolated subunits [3–7]. With regard to the iron-sulfur subunit the studies showed that the subunit extends from the membrane with a large domain which carries the 2Fe-2S cluster into the intermembrane space of mitochondria and is anchored to the bilayer only by a small protein part.

The import of the cytoplasmically synthesized subunits of the *Neurospora* cytochrome reductase (all except cytochrome *b*, which is a mitochondrial coded protein) into mitochondria has been studied recently [8]. The subunits, except the 14000-Da subunit, are synthesized as larger precursors and proteolytically processed during or after their import into mitochondria. Similar results were obtained for the yeast enzyme [9–12].

In this article we report on the primary structure of the iron-sulfur subunit of *Neurospora* cytochrome reductase in its precursor and mature form. Until now no sequence of a Rieske-type iron-sulfur protein was published. We isolated a cDNA clone corresponding to a part of the mRNA from an ordered cDNA clone bank, identified further clones by colony/filter hybridization and used cDNA probes to isolate the cloned gene. From the nucleotide sequences the primary structure of the protein was derived. The N-terminal sequence of the mature protein was confirmed by Edman degradation. The primary structure is used for a more detailed prediction of the arrangement of the iron-sulfur subunit in the membrane.

MATERIALS AND METHODS

Strains, materials and methods for identification of cDNA and genomic DNA are detailed elsewhere [13–15]. cDNA was sequenced by the Maxam and Gilbert method [16] except for modifications at dA + dG, which were performed according to Burton [17]. Partial *Sau*3A fragments (6000–8000 bases) of genomic DNA [15] were cloned in the *Bam*HI site of plasmid pBR322. A cloned fragment of 7000 base pairs, containing the gene of the iron-sulfur subunit, was identified by colony filter hybridization. The *Sau*3A fragments of a *Bgl*II/*Eco*RI fragment were cloned in both directions in M13mp10 and sequenced by the Sanger method [18, 19].

Ubiquinol–cytochrome *c* reductase was prepared from *Neurospora* mitochondria as in [2]. The iron-sulfur protein was isolated from cytochrome reductase by preparative sodium dodecyl sulfate (SDS) gel electrophoresis, antibodies were raised as described [8]. For Edman degradation the protein was recovered from the SDS gel by electroelution for 16 h in 0.1 M phosphate pH 8, 0.1% SDS. The protein was concentrated to 30–50 μM by ultrafiltration using Amicon PM10 filters. An aliquot of 60 nmol was coupled to 70 mg

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Abbreviation. SDS, sodium dodecyl sulfate.

Enzyme. Ubiquinol–cytochrome *c* reductase (EC 1.10.2.2).

diisothiocyanate glass beads (23 nm) for 5 h at 37 °C and for another 1 h after addition of 100 µl ethanolamine [20]. The glass was washed with water, methanol and diethyl ether and dried. Sequencing was carried out with a solid-phase sequencer (model 12, Sequemat, Watertown, USA). The amino acid phenylthiohydantoins were identified by thin-layer chromatography [21].

RESULTS

Isolation of cloned cDNA and genomic DNA

A first clone was identified from an *E. coli* cDNA bank by hybridization selection, subsequent cell-free protein synthesis and immunoprecipitation (Fig. 1). The cDNA of this clone 24B8 contains 145 nucleotides coding for the iron-sulfur subunit. They correspond to nucleotides 604 – 748 of the coding sequence (Fig. 3). Further cDNA clones were identified by three rounds of colony filter hybridization. From 150000 clones the cDNA of only 24 hybridized with iron-sulfur cDNA probes. No full-length cDNA was found in these clones. Therefore, the overlapping cDNA fragments, shown in Fig. 2, were sequenced.

With the cDNA insert of clone 24B8, UH2 was identified. This clone allowed identification of UH1 and UH8, and UH8 was used to identify UH33. UH1 contains the poly(A) tail. None of these clones extends to the d(ATG) start codon.

The 5'end of the mRNA, not covered by cDNA clones, was deduced from the nucleotide sequence of the cloned gene. A comparison of cDNA and genomic DNA sequences revealed the presence of three introns after bases 274, 363 and 514 of the coding sequence (Fig. 3). The nucleotides after the intron boundaries correspond, with a few exceptions, to intron-exon junctions of yeast and other *Neurospora* genes [22].

The primary structure of the preprotein and the mature subunit

The derived nucleotide sequence of the mRNA contains an open-reading frame of 693 base pairs starting with the first d(ATG) codon after 223 base pairs of the 5' non-coding region. That presumed d(ATG) start codon is preceeded by a short sequence d(CACA) similar to the start codon of other *Neurospora crassa* mRNAs [13, 15, 22 – 25]. The start of the mRNA has not yet been determined. The mature iron-sulfur subunit was submitted to 24 cycles of automated Edman degradation. The established amino acid sequence coincides exactly with amino acids 33 – 56 of the open-reading frame. Accordingly, the preprotein of the iron-sulfur subunit consists of 231 amino acids and has a molecular mass of 24749 Da (Fig. 3). The presequence is 32 residues long, corresponding to a molecular mass of 3211 Da. The only four charged residues in the presequence are arginines. The sequence of the mature subunit contains 199 residues. The molecular mass, including the 2Fe-2S redox center, is 21728 Da. With regard to the distribution of hydrophobic residues the mature protein shows three characteristic parts (Fig. 4): (a) a small 29-residues-long hydrophilic part at the N terminus (amino acids 33 – 61 of the preprotein sequence), (b) a 25-residues-long stretch of predominantly hydrophobic amino acids (amino acids 62 – 86) and (c) a large hydrophilic domain (amino acids 87 – 231), which contains the only four cysteines of the sequence at positions 174, 179, 193 and 195 in a region of moderate hydrophobicity. Three of the four histidines of the subunit are also found in this region. It is conspicuous, that

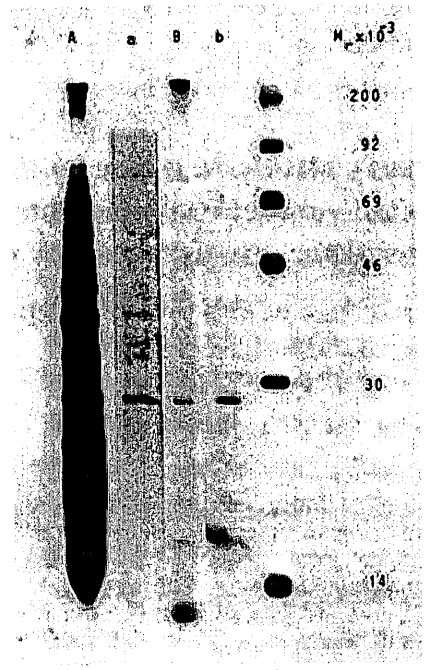

Fig. 1. *SDS gel electrophoresis of* ^{35}*S-labelled* in vitro *translation products.* cDNA plasmids were bound to diazobenzyloxymethyl-cellulose paper and hybridized with total poly(A) RNA. mRNAs, selected by hybridization with cDNA, were translated in a cell-free wheat germ system. An aliquot of each assay was incubated with iron-sulfur antibodies. Lanes A and B show total translation products, lanes a and b the immunoprecipitated products. Experiments A and a contained total polyadenylated RNA, and B and b mRNA selected by plasmid 24B8

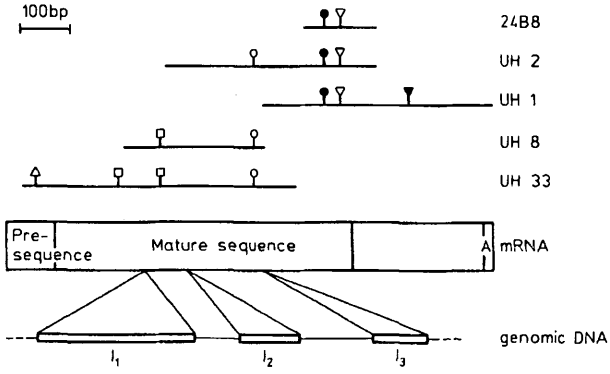

Fig. 2. *Schematic representation of mRNA and gene coding for the iron-sulfur subunit and of cDNA inserts used for sequencing.* Restriction sites are marked by the following symbols: (▼) *Aval*, (○) *BglI*, (▽) *Hin*dIII, (□) *Nco*I, (△) *Sma*I, (●) *XHo*I. The introns are indicated by I_1, I_2, and I_3

intron 1 of the gene occurs after the short hydrophobic protein segment and intron 3 just before the first cysteine.

By combining the sequence data with the results of Li et al. [6] and Karlsson et al. [3] we predict the arrangement of the iron-sulfur subunit in the membrane shown in Fig. 5. A larger catalytic domain of the subunit extends into the intermembrane space of mitochondria with the 2Fe-2S cluster facing the membrane. This domain is anchored to the membrane by a single α helix. A smaller hydrophilic domain extends into the matrix phase.

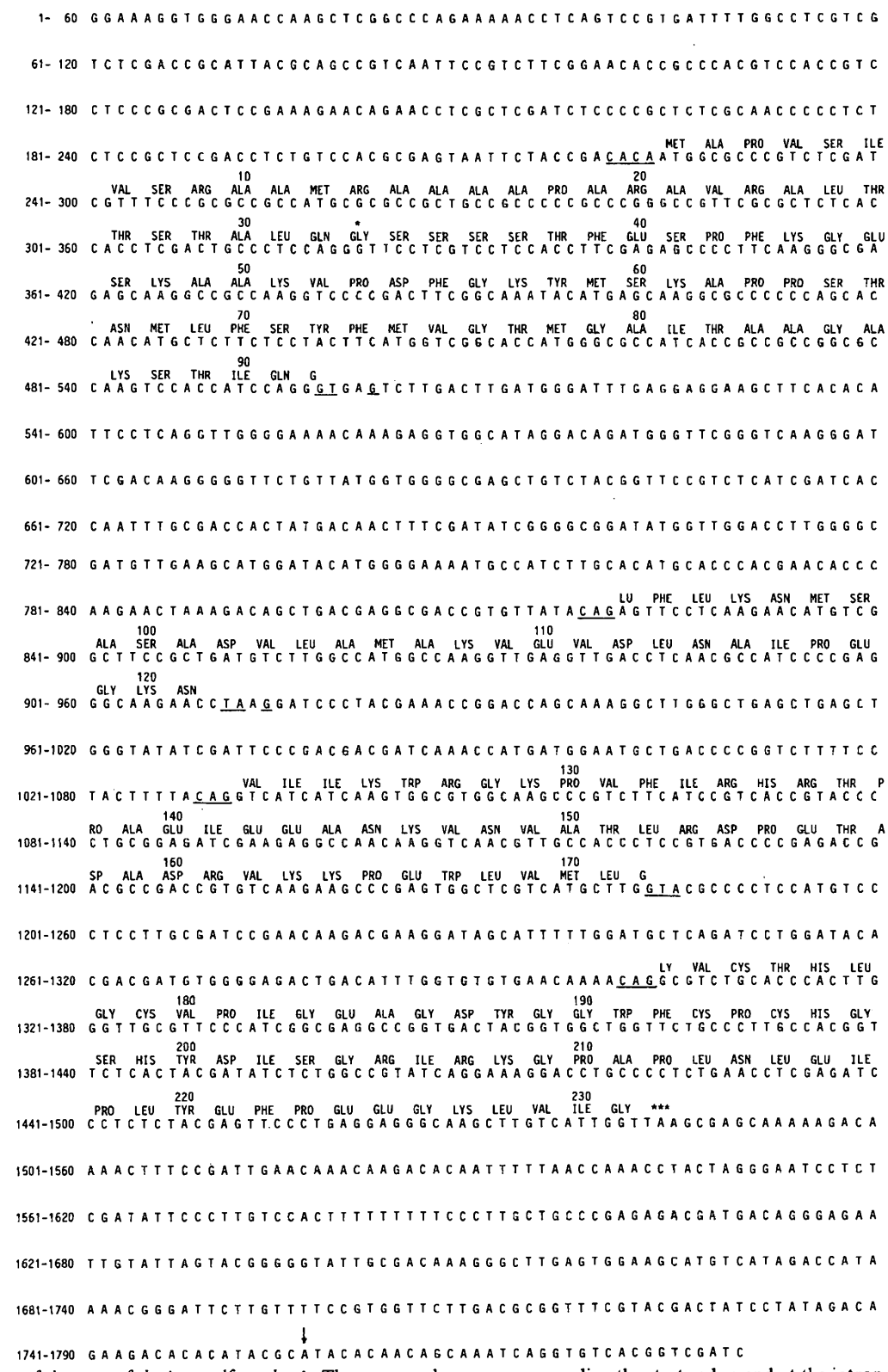

Fig. 3. *Sequence of the gene of the iron-sulfur subunit.* The conserved sequences, preceding the start codon and at the intron boundaries, are underlined. The polyadenylation site is marked by an arrow, the start of the mature subunit by an asterisk

DISCUSSION

The primary structure of precursor and mature form of the iron-sulfur subunit nicely explains previous results on the import of the subunit into mitochondria [8] and the arrangement in the membrane [6].

The precursor protein, synthesized in an *in vitro* system, was found to have a molecular mass upon SDS gel electro-

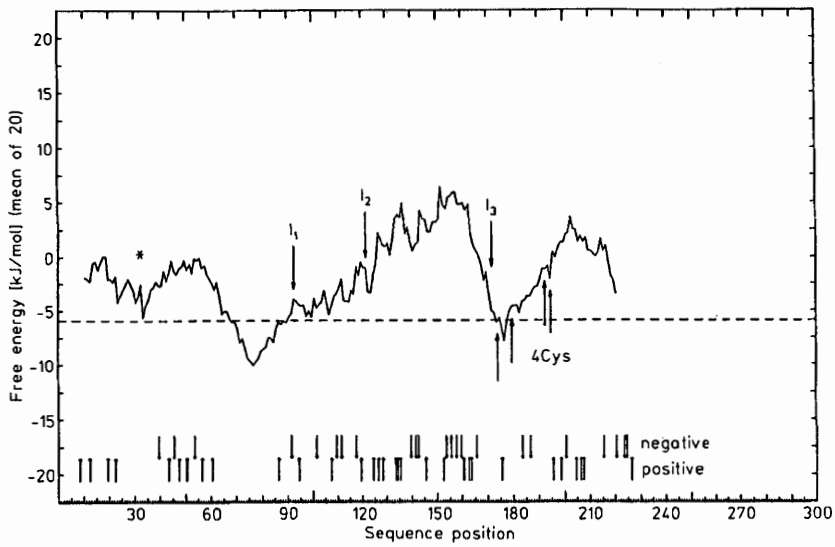

Fig. 4. *Polarity profile of the preprotein of the iron-sulfur subunit.* The gain of free energy during transition of a 20-residue segment from water in the membrane is calculated for all sequence positions according to von Heijne [38]. The area below the dotted line indicates an increased probability for the amino acid to be located in the membrane. The mature protein starts at position 33 as marked by the asterisk. The positions of the four cysteines and the introns are indicated by arrows. At the bottom positive and negative charges are indicated

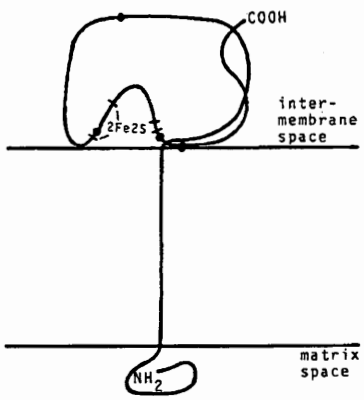

Fig. 5. *Predicted arrangement of the iron-sulfur subunit in the mitochondrial membrane.* The positions of the four histidines are indicated by spots

phoresis of 28000 Da, compared with 25000 Da of the processed subunit [8]. This molecular mass difference equals the molecular mass of 3211 Da of the presequence. The presequence, which is assumed to address the protein to the mitochondrial membrane, contains four arginines as the only positive charges but no negatively charged residue. An abundance of basic residues has also been found in presequences of other imported mitochondrial proteins [13, 26–29].

Of the 32 residues in the presequence of the iron-sulfur subunit 11 are alanines; some of them are clustered. This is reminiscent of but less pronounced than in the presequence of yeast cytochrome *c* peroxidase [28], an enzyme in the intermembrane space of mitochondria. Since the water-soluble domain of the iron-sulfur protein (see below) lies in the same mitochondrial compartment and both proteins are processed in the matrix space, one may speculate that they follow the same import pathway. In contrast to the precursors of the peroxidase and cytochrome c_1, the precursor of the iron-sulfur protein is not cleaved by the chelator-sensitive protease in the matrix space. Accordingly a putative recognition sequence for this protease [13, 26, 30] is not present at the cleavage site of the iron-sulfur protein precursor.

The isolated mature subunit, with the apparent molecular mass of 25000 Da in SDS, was found to be soluble only in the presence of detergent. Approximately 130 mol Triton X-100 is bound/mol protein. By chymotrypsin treatment of this subunit a water-soluble preparation with the apparent molecular mass of 16000 Da, which does not bind detergent, was obtained. This preparation shows the same electron paramagnetic resonance and light absorption spectra of the 2Fe-2S cluster as the whole subunit. It was concluded that part of the small fragment cleaved off by proteolysis is responsible for detergent binding and anchoring the subunit to the membrane [6]. The N terminus of the water-soluble preparation was not determined experimentally, but in the sequence a typical chymotrypsin cleavage site was found at Phe-93 and Leu-94. In the case of cleavage occuring behind Phe-93 a smaller part of 6403 Da and a larger one of 15171 Da would arise. The smaller part consists of a hydrophobic sequence, which is long enough to span the membrane [31], and a hydrophilic sequence at the N terminus. The larger part is predominantly hydrophilic and contains the only four cysteines. These cysteines, which are assumed to carry the 2Fe-2S cluster (for review see [32]), are located in a moderately hydrophobic region. Three of the total four histidines of the sequence are also found in this region.

In most models of the electron transfer through cytochrome reductase (for review see [33]) ubiquinol interacts with the iron-sulfur subunit as follows. In a first step, ubiquinol is deprotonated and bound to the subunit as anion. An electron is then transferred to the 2Fe-2S center. The semiquinone is deprotonated and bound as anion to the cytochrome *b* subunit. A hydrophobic protein pocket around the 2Fe-2S center with histidines as possible proton acceptors might be an appropriate environment for these reactions.

The four cysteines in the Rieske iron-sulfur protein are arranged as two pairs. One pair is separated by four amino acids including one histidine, the other pair only by one pro-

line. Such a short distance has not yet been found in other iron-sulfur proteins [32]. In bacterial and plant ferredoxins three cysteines are close together, separated by two to four amino acids, and the fourth cysteine is located about 30 amino acids away [34]. The rubredoxins contain two groups of two cysteines each separated by two amino acids [35]. In high-potential iron proteins only two cysteines are close together, the other two cysteines are not clustered [36]. Recently a Rieske-type of iron-sulfur protein, containing two 2Fe-2S centers, was isolated from *Thermus thermophilus* [37]. These centers are considered to be bound to the protein each by two cysteines and two other residues (His, Tyr, Glu). Whether this protein is related to the cytochrome reductase Rieske subunit cannot yet be decided.

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