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Primary structure, cell-free synthesis and mitochondrial targeting of the 8.2 kDa protein of cytochrome *c* reductase from potato

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Abstract

Cytochrome *c* reductase from potato comprises ten subunits with apparent molecular sizes between 55 and < 10 kDa. The subunit with the highest electrophoretic mobility on SDS–polyacrylamide gels was isolated and analysed by cyclic Edman degradation. Mixtures of degenerative oligonuleotides were derived from the obtained sequence data and used for the isolation of corresponding cDNA clones. The clones encode a protein of 72 amino acids which exhibits significant sequence identity with a 9.5 kDa subunit of cytochrome *c* reductase from bovine and a 11 kDa subunit of the enzyme complex from yeast. Comparison between the deduced amino acid sequence of the open reading frame and the sequence of the mature protein reveals that only the initiator methionine is absent in the functional subunit. Hence the protein has a calculated molecular mass of 8.2 kDa. Transcripts of the potato 8.2 kDa protein were not translated in reticulocyte lysates but in vitro translation worked efficiently with wheat germ lysate. Import of the radiolabelled protein into isolated mitochondria from potato seems to depend on a potential across the inner membrane and confirms the absence of a cleavable mitochondrial presequence.

Keywords: Cytochrome *c* reductase; Cytochrome bc_1 complex; Respiratory chain; Protein transport; Mitochondrion; (*S. tuberosum*)

1. Introduction

The mitochondrial cytochrome *c* reductase complexes from bovine and yeast comprise ten subunits: the three respiratory proteins cytochrome *b*, cytochrome c_1 and the 'Rieske' iron sulfur protein, two 'core' proteins and five small proteins with a molecular mass below 20 kDa [1,2]. In addition the presequence of the iron sulfur protein is retained as an eleventh subunit in the enzyme complex from bovine [3,4]. One of the small proteins is the 11 kDa subunit from yeast, that resembles a 9.5 kDa subunit of the enzyme complex from bovine. The primary structure of this nuclear encoded protein is only known for these two organisms [5,6] and its function is object of recent investigations [7–9].

Upon cleavage of the isolated cytochrome *c* reductase from bovine with guanidine the 9.5 kDa protein cofractionates with the two 'core' proteins, which are localized on the matrix side of the enzyme complex [1,5]. On the other hand, the results of immunological and crosslinking experiments also indicate an exposure of the 9.5 kDa protein towards the mitochondrial intermembrane space [7,10]. The subunit is therefore assumed to span the inner membrane. Cytochrome *b* and the 9.5 kDa protein were shown to be the only components of the respiratory protein complex from bovine that can be photo-affinity labelled with a quinone derivative [7]. Hence the 9.5 kDa subunit is called the small ubiquinol-binding protein of cytochrome *c* reductase (QPc) and most likely forms part of the ubiquinol reduction or oxidation site.

The 11 kDa subunit of cytochrome *c* reductase from yeast was immunologically localized on the intermembrane space side of the enzyme complex [11]. There is genetic evidence that the 11 kDa subunit interacts with cytochrome *b* [12–14]. Disruption of the gene encoding

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the 11 kDa subunit results in a loss of ubiquinol cytochrome *c* oxidoreductase activity. The mutant cells display decreased levels of cytochrome *b*, the Rieske iron sulfur protein and the 14 kDa subunit indicating a role of the 11 kDa protein in the assembly of the enzyme complex [6,15,16]. Experiments with truncated versions of the 11 kDa protein from yeast reveal that the C-terminal 28 amino acids are dispensable for the enzymatic activity of the protein complex [14]. Recently a highly aromatic stretch of amino acids was reported to be important for the assembly and function of cytochrome *c* reductase [8,9]. As mutational alterations in this region affect the binding of the inhibitor myxothiazol to the enzyme complex the 11 kDa subunit seems to contribute to the structure of the ubiquinol oxidation site (center P).

The cytochrome *c* reductase complex from potato also consists of ten subunits, including at least four proteins with a molecular mass below 20 kDa [17–19]. Initial characterization of the primary structure of these proteins allowed a preliminary identification of similarities to corresponding subunits of cytochrome *c* reductase from bovine and yeast [20]. Here we report on the isolation of cDNA clones for a 8.2 kDa protein from potato, that resembles the 11 kDa subunit from yeast and the 9.5 kDa 'core-linked' subunit from bovine. Data are presented on the cell-free synthesis of the protein and on its import pathway into mitochondria, which is shown to be independent of a cleavable mitochondrial targeting sequence.

2. Materials and methods

2.1. Isolation of potato mitochondria and preparation of cytochrome c reductase

Mitochondria were isolated from potato tubers (*Solanum tuberosum* L. var. Bintje) as described in Braun et al. [21]. Cytochrome *c* reductase was prepared from about 150 mg mitochondrial membrane protein. The protein complex was solubilized with 3.3% Triton X-100 and purified by affinity chromatography and gel filtration as outlined in Braun and Schmitz [18]. The purified protein complex was stored at −70°C.

2.2. Analysis of proteins by SDS-PAGE and gas phase sequencing

The protein concentrations of mitochondrial fractions were determined with the method of Bradford [22] using a kit supplied by BioRad. Polypeptides were separated in SDS–polyacrylamide slab gels [23]. For protein sequencing, the subunits of cytochrome *c* reductase were fractionated in 12% polyacrylamide gels and blotted onto Immobilon membranes. Protein bands were stained with Ponceau S (Sigma), cut out and subjected to Edman degradation in an Applied Biosystems pulsed-liquid phase sequencer.

2.3. Screening of cDNA libraries

A cDNA library from potato tubers was screened with radiolabelled oligonucleotide mixtures as previously described [24]. Two sets of oligonucleotides with the lowest degeneracy were derived from peptide sequences which had been obtained from the 8.2 kDa polypeptide. The oligonucleotide mixtures contained the full complement of sequences that could potentially encode the octapeptide Lys-Gln-Pro-Val-Lys-Leu-Lys-Ala or the nonapeptide Pro-Phe-Gln-Gln-Lys-Ile-Met-Pro-Gly. The oligonucleotides were end labelled with T4 polynucleotide kinase and [γ-^{32}P]dATP (Amersham) and used for isolation of positively reacting phages.

2.4. Cloning, sequencing and computer analysis of DNA and protein sequences

DNA inserts from phages hybridizing to the labelled oligonucleotides were cloned into Bluescript vectors (Stratagene) and sequenced on both strands following the didesoxynucleotide method [25]. Overlapping subclones were produced with the exonuclease III deletion strategy [26]. DNA and protein sequences were analysed with programs of the Staden package on a VAX computer.

2.5. Synthesis of radiolabelled precursor proteins and in vitro import into isolated mitochondria

The cDNA encoding the 8.2 kDa protein was transcribed under the control of the T7 promoter using RNA polymerase as recommended by the supplier (BRL). To dissolve potential secondary structures the generated RNA was heat treated at 67°C for 10 min and subsequently kept on ice for 5 min prior to starting the translation assay. Translation was performed with different cell-free lysates from reticulocytes (Amersham, Promega) and wheat germ (Promega) in the presence of [^{35}S]methionine and of [^{3}H]leucine following the instructions of the suppliers. For in vitro import reactions mitochondria were purified on Percoll step gradients as previously described [21]. Mitochondrial protein was adjusted to 10 mg/ml in a buffer containing 0.4 M mannitol, 10 mM KH_2PO_4, pH 7.2 (KOH) and 0.1% BSA. In vitro import reactions were performed in a reaction volume of 200 μl (Braun and Schmitz, unpublished data). After completion of the import the mitochondria were layered on a sucrose cushion (25% sucrose, 10 mM KH_2PO_4, pH 7.2) and reisolated by centrifugation for 15 min at 13000 × *g*.

The pellet was resuspended in Laemmli buffer and analysed by SDS-PAGE [28]. The gels were incubated for 30 min in Amplify (Amersham) and radiolabelled proteins were visualized by exposure to X-ray film at −70°C.

3. Results

3.1. Primary structure of the 8.2 kDa protein of cytochrome c reductase from potato

The subunits of the cytochrome *c* reductase complex from potato were resolved by SDS gel electrophoresis, blotted onto membranes and the band corresponding to the smallest subunit of the enzyme complex was analysed by Edman degradation. Internal sequence data were obtained after proteolytic fragmentation of the protein, HPLC separation and N-terminal sequencing of the generated peptides [20]. Two stretches of amino acids were suitable to derive oligonucleotide mixtures with a low degeneracy: a 23-mer with 1536 and a 26-mer with 718 combinations. Screening of a cDNA library of potato tuber with the radiolabelled 26-mer yielded several positively reacting clones. Clones which also reacted with the 23-mer were isolated and further analysed. Sequencing of a clone termed pCR10–1 revealed an insert of 509 bp that includes an open reading frame (orf) of 216 bp encoding a protein of 72 amino acids (Fig. 1). The 5′ non-coding region includes the stop codon TGA 9 bp upstream of the start codon ATG, indicating that the orf is complete. The 3′ non-coding region of 271 bp does not terminate with a poly A-tail and also lacks a sequence motif that resembles a polyadenylation site. Therefore the 3′ noncoding region of the corresponding mRNA may be further extended.

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                        M   G   K   Q   P   V   K   L   K   A    10
GAATTCGCAGTGAACGAAG ATG GGG AAG CAA CCG GTG AAG CTG AAG GCT    49

 V   V   Y   A   I   S   P   F   Q   Q   K   I   M   P   G    25
GTT GTA TAC GCT ATA TCT CCA TTT CAA CAG AAA ATT ATG CCT GGT    94

 L   W   K   D   L   P   G   K   I   H   H   K   V   S   E    40
TTA TGG AAG GAT CTT CCT GGT AAG ATC CAT CAC AAA GTG TCT GAA   139

 N   W   I   S   A   T   L   L   L   G   P   L   V   G   T    55
AAT TGG ATC AGC GCT ACT CTC TTG CTT GGT CCA CTC GTC GGA ACC   184

 Y   S   Y   V   Q   H   F   L   E   K   E   K   L   E   H    70
TAC TCG TAT GTG CAG CAC TTT CTG GAA AAG GAG AAG TTA GAA CAC   229

 R   Y   *                                                   72
AGA TAC TAA AGATGTGTTCTTCATCTGTCTGTTGAAGATCTCTATTCCATTTTGCA  285

GTTTTCAACAGGAAATAAGCTACTATTGTTGCATTTTTGGAATTTTTCATTTTGAAGAT  344

ATTTACTTGTATGGCCCTTATAATAGCAATTAAATCTCTGTTAGGGTCTTTTGAAAGTC  403

TTGTGCATCCTAAATCTTATTCAGAAATAGTCTGATAAAATAATTTGTAACACATTTCA  462

TTGACTTGCTGTTAAAAGATCCCATTGTGATTTCACAATGAACAGTT              509
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the insert of clone pCR10–1. The sequence data have been submitted to the EMBL sequence data banks and are available under the accession number X79275.

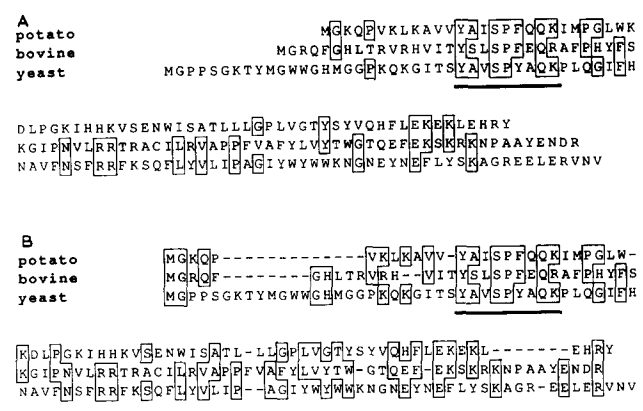

Fig. 2. Comparison between the amino acid sequence of the 8.2 kDa protein of cytochrome *c* reductase from potato and the sequences of the 9.5/11 kDa proteins of the enzyme complex from bovine/yeast [5,6,8,34]. (A) Alignment of the sequences without interruptions. (B) Improved alignment with 2–5 gaps per sequence. Identical residues are boxed. The bar marks the only stretch of amino acids beeing highly conserved.

The sequences of the peptides of the smallest subunit of cytochrome *c* reductase are identical to segments of the amino acid sequence deduced from clone pCR10–1. Comparison of the predicted primary structure with the protein sequences in the SwissProt data banks did not reveal homology to any known protein. Therefore the deduced sequence was directly compared to sequences of the small subunits of cytochrome *c* reductase from yeast and bovine. The 9.5 kDa subunit from bovine and the 11 kDa subunit from yeast turned out to exhibit similar sequence motifs. However, based on the alignment in Fig. 2A the overall identity between the protein from potato and the 9.5/11.0 kDa subunits is only 15%/14%. Most conserved is a stretch of 9 amino acids close to the N-terminus, which includes four of the five residues that are identical in all three organisms (indicated by a bar in Fig. 2). On the other hand the yeast and the bovine proteins also only comprise 17% identity and were discussed to be homologous components [6]. An alignment of the three sequences allowing 2–5 breaks per sequence results in an significant increase of sequence identity (Fig. 2B; potato/bovine: 32%; potato/yeast: 21%; bovine/yeast: 28%). Like the subunits from bovine and yeast the potato protein has a surplus of basic amino acids (14 basic residues versus 5 acidic residues). The polarity profile of all three proteins is very similar (Fig. 2).

3.2. Characterization of the mature 8.2 kDa protein

The smallest subunit of cytochrome *c* reductase from potato was subjected to direct cyclic Edman degradation in order to obtain the N-terminal sequence of the mature protein. The first 27 residues are

GKQPVKLKAVVYAISPFQQKIMPGLWK. The sequence corresponds to amino acids 2–28 of the open reading frame, indicating that only the N-terminal methionine is removed. Hence the protein lacks a cleavable mitochondrial presequence. The calculated molecular mass of the mature subunit is 8.2 kDa. Different values varying between 5 and 10 kDa were reported for the apparent molecular mass of the protein upon analysis by SDS-PAGE using the glycine buffer system of Laemmli [17,18]. Reinvestigation of the apparent molecular mass by the tricine-SDS-PAGE system of Schägger and von Jagow [28], which was reported to have a high resolution capacity for small proteins, revealed a size of 7 kDa (not shown). Consequently there is a discrepancy of 1.2 kDa between the calculated and the apparent molecular mass, which might be due to the excess of positively charged residues. In fact the 8.2 kDa protein has a slightly higher electrophoretic mobility than the 8.0 subunit and the 7.8 kDa 'Hinge' subunit of cytochrome *c* reductase from potato (Ref. [29] and unpublished data). Based on the calculated molecular size the potato 8.2 kDa protein is therefore not the smallest, but the third smallest subunit of the respiratory enzyme complex.

3.3. Cell-free synthesis of the 8.2 kDa protein and targeting into isolated mitochondria from potato

To investigate the import pathway of the 8.2 kDa subunit of cytochrome *c* reductase into isolated mitochondria the insert of clone pCR10–1 was transcribed and the generated mRNA used for cell-free synthesis of the protein. Translation of the transcripts with reticulocyte lysate in the presence of of [^{35}S]methionine did not result in the production of radiolabelled protein. The addition of [^{3}H]leucine to the translation mixture to compensate for the low number of methionines of the potato 8.2 kDa protein did not improve this result. Therefore wheat germ lysate was tested for translation and led to efficient synthesis of a radiolabelled protein of the correct size (Fig. 3). As a control, transcripts for the 8.0 kDa subunit of cytochrome *c* reductase [27] were also translated with reticulocyte and wheat germ lysates. For this protein both systems worked equally efficiently. The reason for the impossibility to synthesize the 8.2 kDa protein with reticulocyte lysate is not understood.

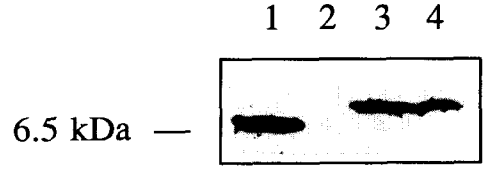

Fig. 3. Cell-free synthesis of the 8.0 and 8.2 kDa proteins of cytochrome *c* reductase from potato. The radiolabelled proteins were analysed by SDS-PAGE and fluorography. Translation was performed in the presence of wheat germ lysate (lanes 1 and 3) or reticulocyte lysate (lanes 2 and 4). The 8.0 kDa protein (lanes 3 and 4) is efficiently synthesized in both lysates, while the 8.2 kDa protein (lanes 1 and 2) is translated only by wheat germ lysate. The 8.0 kDa protein has a lower electrophoretic mobility than the 8.2 kDa protein; the size of a 6.5 kDa standard protein is given on the left.

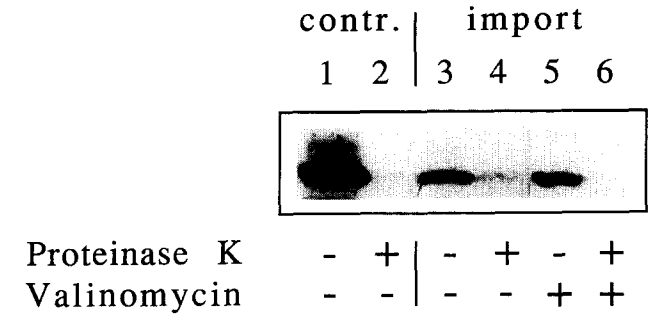

Fig. 4. Import of the 8.2 kDa protein into isolated mitochondria from potato. Radiolabelled protein was analysed by SDS-PAGE and fluorography. Susceptibility of the translation product (lane 1) towards proteinase K is demonstrated in lane 2. Import experiments (lanes 3–6) were performed as described in Section 2. The presence or absence of proteinase K and valimomycin is indicated by plus and minus.

Prior to import experiments, the sensitivity of the radiolabelled 8.2 kDa protein towards proteinase K was proven (Fig. 4, lane 2). Upon incubation with isolated mitochondria the protein cofractionates with the reisolated mitochondria (lane 3). Part of the protein becomes protected against proteinase K (lane 4), indicating translocation into the organelles. However, for unknown reasons, the import efficiency was low. Therefore the targeting experiment was repeated in the presence of valinomycin, which is an ionophore that uncouples the potential $\Delta\Psi$ across the inner mitochondrial membrane and thereby inhibits the import of all proteins of the main mitochondrial import pathway [30]. The radiolabelled 8.2 kDa protein again cofractionates with the reisolated organelles (Fig. 4, lane 5) but retained complete sensitivity towards the proteinase (lane 6). Consequently import of the 8.2 kDa protein into isolated mitochondria takes place, if the ionophore is absent. In agreement with the sequencing data, the size of the translation product prior and after import is identical.

4. Discussion

While the respiratory subunits of cytochrome *c* reductase from bovine, yeast and potato exhibit sequence conservation in the range of 45–60% [21,31,32], the 'core' proteins and the small subunits of the enzyme complex show a lower similarity, that lies between 25%

and 40% [19,24,29]. The sequence of the potato 8.2 kDa protein and the sequences of the 9.5/11 kDa proteins from bovine/yeast even have a smaller identity of – depending on the stringency of the alignment – 14–32%. The equivalence of the 9.5 kDa subunit from bovine and the 11 kDa protein from yeast was discussed previously [6,33]: the sequence identity was judged to be significant, but the secondary structure prediction and some results on the localization of the proteins in the enzyme complex differ. However, the primary structures of all other subunits of cytochrome *c* reductase from bovine were reported to have counterparts in yeast [2,33], making it unlikely that the 9.5/11 kDa proteins reflect unique components of the enzyme complexes of the two organisms. Meanwhile, sequence data on all subunits of cytochrome *c* reductase from potato are available [20]. Each subunit was shown to have similarity to one protein of the enzyme complex from yeast and bovine, respectively. The 8.2 kDa subunit only exhibits significant homology to the 9.5/11 kDa proteins of bovine/yeast. The three proteins have a polarity profile with striking similarities: a hydrophilic N-terminus with 3–5 basic amino acids (potato: amino acids 1–9), a short hydrophobic region (potato: aa 10–20), a second hydrophilic area comprising 5–6 basic residues (potato: aa 21–40), a large hydrophobic region (potato: aa 41–60) and a highly hydrophilic C-terminus with basic and acidic residues (potato: aa 61–72). The large hydrophobic area was reported to be of functional importance, as it is photoaffinity labelled upon incubation of isolated cytochrome *c* reductase with a quinone derivative in bovine [7]. Additionally, mutations in this area disturb the function and assembly of cytochrome *c* reductase in yeast [8,9]. A stretch of five consecutive aromatic residues in the large hydrophobic region of the yeast 11 kDa protein is assumed to be critical for the mentioned effects. However, these amino acids are not conserved in the 9.5/8.2 kDa proteins from bovine/potato (Fig. 2) and therefore their functional importance might be restricted to yeast. The only region that is highly conserved in all three organisms is the short hydrophobic stretch close to the N-terminus of the subunits (Fig. 2).

Transport of the 8.2 kDa protein into mitochondria is independent of a cleavable presequence. Also the mature 9.5/11 kDa proteins from bovine/yeast only lack the initiator methionine [6,34]. Absence of cleavable mitochondrial targeting sequences was reported for many of the small subunits of the respiratory protein complexes and seems to be rather a rule than an exception [33,35]. Typical presequences have a preponderance of basic and hydroxylated amino acids and comprise a helical secondary structure that has amphiphilic properties [36]. Proteins without a presequence presumably have internal targeting information with similar features. The 8.2 kDa protein from potato and the equivalent proteins from bovine and yeast exhibit two basic regions, that might be responsible for targeting to mitochondria: one at the extreme N-terminus and one in front of the large hydrophobic region. Import experiments with truncated forms of the protein will allow to further define the potential targeting domains.

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