Plant Physiol. (1995) 107: 1217–1223

Molecular Features and Mitochondrial Import Pathway of the 14-Kilodalton Subunit of Cytochrome *c* Reductase from Potato[1]

Hans-Peter Braun and Udo K. Schmitz*

Institut für Genbiologische Forschung GmbH, Ihnestrasse 63, D-14195 Berlin, Germany

The cytochrome *c* reductase complexes from fungi and mammals both contain a 14-kD protein (yeast, 14.4 kD; bovine, 13.4 kD) that does not directly participate in electron transfer but possibly is indirectly involved in the function of the complex and has a role in assembly of the multimeric enzyme. A subunit of comparable size was identified for the bc_1 complex of higher plants. The 14-kD protein from potato (*Solanum tuberosum*) was specifically separated from the isolated protein complex in the presence of 6 M urea and is, therefore, assumed to be a peripheral component. Direct sequence analysis of the proteins from potato and wheat (*Triticum aestivum*) and isolation of corresponding cDNA clones for the subunit from potato revealed clear similarity to the equivalent proteins from yeast and bovine. The wheat 14-kD protein seems to occur in two isoforms. The 14-kD protein from plants is very hydrophilic, has a characteristic charge distribution, and contains no potential membrane-spanning helices. In vitro import of the radiolabeled 14-kD protein from potato into isolated mitochondria depends on the membrane potential across the inner mitochondrial membrane. The protein seems to lack a cleavable mitochondrial presequence, because it is not processed upon translocation. Possible intramolecular regions involved in targeting of the 14-kD protein to plant mitochondria are discussed.

The mitochondrial bc_1 complex (EC 1.10.2.2.) is the middle segment of the respiratory chain and catalyzes the reduction of Cyt *c* by the oxidation of ubiquinol. Coupled with this reaction it contributes to the chemiosmotic gradient across the inner mitochondrial membrane by translocating protons from the matrix to the intermembrane space. In fungi and mammals the Cyt *c* reductase is an oligomeric protein complex comprising 9 to 11 subunits (reviewed by Trumpower, 1990; Bechmann et al., 1992): two large "core" proteins, three respiratory proteins that directly participate in electron transport (Cyt *b*, Cyt c_1, and the "Rieske" iron sulfur protein), and four to six small proteins with molecular masses of less than 20 kD. Since bacterial bc_1 complexes that contain only the respiratory subunits have the same activity as the eukaryotic enzymes (Trumpower, 1990), the role of the supplementary subunits is not quite understood. One of them is the bovine subunit VI (13.4 kD), which was shown to be similar to subunit VII from yeast (14.4 kD). Originally, this protein was called a "ubiquinone-binding protein" because it was thought to bind arylazido ubiquinone derivatives upon photoaffinity labeling (Yu and Yu, 1982; Yu et al., 1986). Later, a slightly smaller subunit with different properties was identified as the labeled protein (Usui et al., 1991; bovine subunit VII, 9.5 kD). Therefore, we refer to the bovine 13.4-kD subunit and the yeast 14.4-kD subunit as the "14-kD protein" of Cyt *c* reductase.

The sequence of the 14-kD protein is known for bovine (Wakabayashi et al., 1985), human (Suzuki et al., 1988, 1989), and yeast (de Haan et al., 1984). Like all other subunits of Cyt *c* reductase except Cyt *b*, it is nuclear encoded and posttranslationally transported into the mitochondrion. Since the in vitro synthesized translation product of the 14-kD protein from yeast, *Neurospora*, and rat has the same molecular mass as the mature protein (Teintze et al., 1982; van Loon et al., 1983; Nishikimi et al., 1986), the subunit has either no cleavable targeting sequence for mitochondrial import or only a very short one.

The 14-kD protein of Cyt *c* reductase from yeast and bovine is located on the matrix side of the enzyme complex as shown with antibodies directed against the subunit (Japa et al., 1987; Hemrika and Berden, 1990; Usui et al., 1991). Mutations of Cyt *b* affect the steady-state level of the 14-kD protein together with an 11-kD subunit in yeast (de Haan et al., 1984). Cyt *b* and the 14- and 11-kD proteins also behave as a distinct subset of subunits as judged by their responses to mutations in other components of the complex (Crivellone et al., 1988). If the gene for the yeast 14-kD subunit is inactivated by gene disruption, the resulting mutant has no Cyt *c* reductase activity, is respiratory deficient, has reduced steady-state levels for other subunits of the protein complex, and lacks spectroscopically detectable Cyt *b* (Grivell, 1989; Schoppink et al., 1989). There are indications that the 14-kD protein has a role in assembly of the bc_1 complex (Hemrika et al., 1994). In bovine the 14-kD subunit is part of a "Cyt *b*-linked fraction" upon cleavage of the bc_1 complex into subcomplexes (Link et al., 1986; Schägger et al., 1986). It can be cross-linked with core protein II, which is also localized on the matrix side of the protein complex (Gonzalez-Halphen et al., 1988). Digestion of isolated Cyt *c* reductase from bovine with papain or trypsin affects mainly core protein II and the 14-kD protein and leads to a decrease of the H^+/electron ratio for proton pumping (Lorusso et al., 1989; Cocco et al., 1991).

[1] This work was supported by the Deutsche Forschungsgemeinschaft, grant Schm. 698/2.

* Corresponding author; e-mail schmitz@mpimg-berlin-dahlem.mpg.de; fax 49–30–83000736.

In plants little is known about the small subunits of the bc_1 complex. Cyt *c* reductase from potato (*Solanum tuberosum*) comprises 10 subunits (Berry et al., 1991; Braun and Schmitz, 1992; Braun et al., 1994) and was shown to include the activity of the general mitochondrial processing peptidase, which cleaves off the presequences of nuclear-encoded mitochondrial precursors upon their import into the organelle (Braun et al., 1992a, 1994; Emmermann and Schmitz, 1993; Emmermann et al., 1993). Here we report the identification, topography, sequence, and import pathway of the 14-kD protein from higher plants.

MATERIALS AND METHODS

Purification and Analysis of Cyt *c* Reductase from Potato and Wheat

Mitochondria from potato (*Solanum tuberosum* var Bintje) and wheat (*Triticum aestivum* var Nandu) were isolated as described by Braun et al. (1992b, 1995). The mitochondria were subfractionated into a soluble and a membrane fraction by sonication and ultracentrifugation (Linke and Weiss, 1986) and Cyt *c* reductase was prepared by Cyt *c* affinity chromatography, ultrafiltration, and gel filtration as originally reported by Weiss and Juchs (1978) and Weiss and Kolb (1979) for the purification of this enzyme complex from *Neurospora*. With some modifications, the isolation procedure is applicable to higher plants (Braun and Schmitz, 1992; Braun et al., 1995). Purified Cyt *c* reductase was analyzed by SDS-gel electrophoresis (Laemmli, 1970; Schägger and von Jagow, 1987). Antibodies were raised against the 14-kD protein from potato and used for immunological identification of the corresponding subunits of Cyt *c* reductase from wheat. The proteolytic fragmentation of the 14-kD proteins from potato and wheat, the separation of the generated peptides by reverse-phase HPLC, and the N-terminal sequencing of peptides by cyclic Edman degradation are described elsewhere (Braun et al., 1994).

Separation of the 14-kD Subunit from the Cyt *c* Reductase Complex

Cyt *c* reductase from potato (1.5 mg/0.1 mL) was incubated with 6 M urea in the presence of 2% Triton X-100 for 5 min at 4°C. Subunits detached from the complex were purified by gel filtration (Ultrogel AcA 34; Serva, Heidelberg, Germany) using a 0.7- × 30-cm column, an elution buffer with 50 mM Tris-acetate (pH 7.2), 0.05% Triton X-100, 0.2 mM PMSF, and a flow rate of 1.2 mL/h.

Screening of cDNA Libraries and DNA Sequence Analysis

Two oligonucleotide mixtures with the lowest degeneracy were derived from internal sequences of the 14-kD protein of Cyt *c* reductase from potato (Braun et al., 1994). The mixtures contained the full complement of sequences that could potentially encode the two octapeptides Glu-Asp-Leu-Gln-Ala-Met-Gln-Thr (384 combinations) and Glu-Ile-Val-Asp-Ala-Arg-Asn-Gln (2304 combinations). The oligonucleotides were end labeled with T4 polynucleotide kinase and [γ-^{32}P]dATP and used for screening of a λgt11 cDNA library of potato tuber (*S. tuberosum*, var Desirée). DNA cloning and sequencing were performed according to standard procedures (Sambrook et al., 1989). Sequences were analyzed on a VAX computer using the Genetics Computer Group software package (Devereux et al., 1984).

In Vitro Import of the 14-kD Protein from Potato into Isolated Mitochondria

In vitro transcription of clone pCR14–1, which encodes the complete open reading frame of the potato 14-kD protein, was carried out with a transcription kit (Stratagene) according to the supplier's instructions. Translation was performed in the presence of [^{35}S]Met with rabbit reticulocyte or wheat germ lysate (Promega, Madison, WI). Both systems allowed translation of the 14-kD protein but the reticulocyte lysate turned out to be more efficient. The isolation of mitochondria from potato for experiments on in vitro import of the 14-kD protein was described by Emmermann et al. (1994). The conditions for the import experiments are detailed elsewhere (Braun and Schmitz, 1995).

RESULTS

Cyt *c* reductase from potato was shown to contain four proteins below 15-kD and Cyt *c* reductase from wheat at least three (Pfeiffer et al., 1990; Berry et al., 1991; Braun and Schmitz, 1992; Braun et al., 1995). Although antibodies

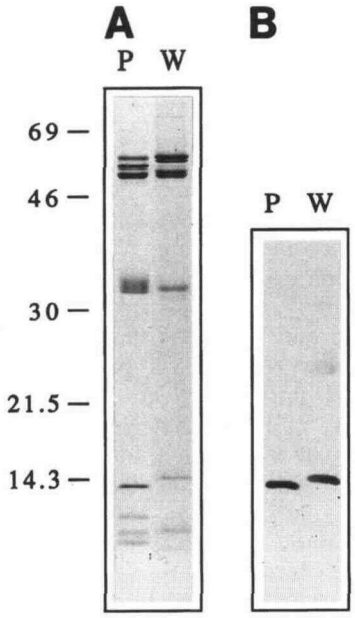


Figure 1. Immunological identification of the 14-kD proteins of Cyt *c* reductase from potato (P) and wheat (W). A, SDS/PAGE of the isolated protein complexes (the iron sulfur protein [25 kD] of both complexes is missing). B, Electroblot of the Cyt *c* reductase complexes detected with antibodies against the 14-kD protein from potato. The numbers on the left indicate the masses of standard proteins (in kD).

directed against Cyt *b*, Cyt c_1, the iron sulfur protein, and the core proteins from fungi strongly react with the equivalent subunits of Cyt *c* reductase from potato (Braun and Schmitz, 1992), no immunological cross-reaction with the small proteins was observed (not shown). To further analyze these subunits, antibodies were raised against the 14- and 12-kD subunits of the enzyme complex from potato. The 14-kD serum specifically recognizes the 14-kD protein of Cyt *c* reductase from potato and strongly cross-reacts with a 14.5-kD protein of the respiratory complex from wheat (Fig. 1).

The topographical arrangement of the 14-kD subunit of Cyt *c* reductase from potato was tested by specific destabilization of the protein complex in the presence of detergent and salt. As reported previously Cyt *c* reductase from potato is exceptionally stable. It remains intact under conditions in which the protein complex from *Neurospora* becomes dissected into three subcomplexes, e.g. in the presence of 2 M NaCl (Linke and Weiss, 1986; Emmermann et al., 1993). Various other conditions were tested to determine whether it is possible to separate single polypeptides of the enzyme complex from potato. Six molar urea specifically detached the 14- and the 12-kD subunits (Fig. 2, lanes 18–28), whereas all other subunits of Cyt *c* reductase were still present as a complex (Fig. 2, lanes 1–17). The 14- and 12-kD subunits, therefore, most likely have a peripheral position in the respiratory complex.

To obtain information concerning the primary structure of the 14-kD subunit of Cyt *c* reductase from higher plants, the protein was subjected to Edman degradation. However, the N termini of the 14-kD proteins from potato and wheat were blocked for direct amino acid sequencing. Since the N-terminal amino acids of other subunits of Cyt *c* reductase from potato have been determined (Braun et al., 1992b; Emmermann et al., 1993, 1994), an artificial blockage due to the isolation procedure of the enzyme complexes seems unlikely. Also, the 13.4-kD subunit of Cyt *c* reductase from bovine and the 14.4-kD subunit from yeast have been reported to be blocked (de Haan et al., 1984; Wakabayashi et al., 1985). To obtain internal sequence information regarding the 14-kD proteins from potato and wheat, both subunits were digested with endoprotease Lys C and the peptides generated were separated by reverse-phase HPLC. Sequence data for the subunit from potato were published previously (Braun et al., 1994); the data for wheat are presented in Figure 3. The sequence of six of nine peptides of the 14-kD protein from wheat could be determined, constituting a total of 95 amino acids. Interestingly, two peptides have an identical sequence except for the second residue, which is either Phe or Tyr. Therefore, the 14-kD protein of Cyt *c* reductase from wheat seems to occur in two isoforms. Similarities between the peptide sequences obtained from the wheat 14-kD subunit and the equivalent proteins from other organisms are discussed below.

The amino acid sequences of the 14-kD protein from potato were used to derive degenerative oligonucleotides for screening a cDNA library from potato and to analyze corresponding clones. Five positively reacting clones with an insert size of 0.65 kb were isolated and sequenced on both strands. The sizes of the inserts were slightly variable (620–662 bp) but the sequences were identical for all clones. The nucleotide and deduced amino acid sequence of the insert of one clone, termed pCR14–1, is shown in Figure 4. It comprises 662 bp, including an open reading frame of 369 bp that encodes a protein of 123 amino acids with a calculated molecular mass of 14,461 D. Because an in-frame stop codon is located upstream of the ATG, the open reading frame most likely encodes the entire protein. The 3′ noncoding region comprises 263 bp and is followed by a short poly(A) tail. The amino acid sequences of peptides for the 14-kD protein from potato that were reported in Braun et al. (1994) are identical with four stretches of the deduced amino acid sequence of clone pCR14–1 except for one residue (Met at position 71 of the open reading frame was identified as Val by direct protein sequencing).

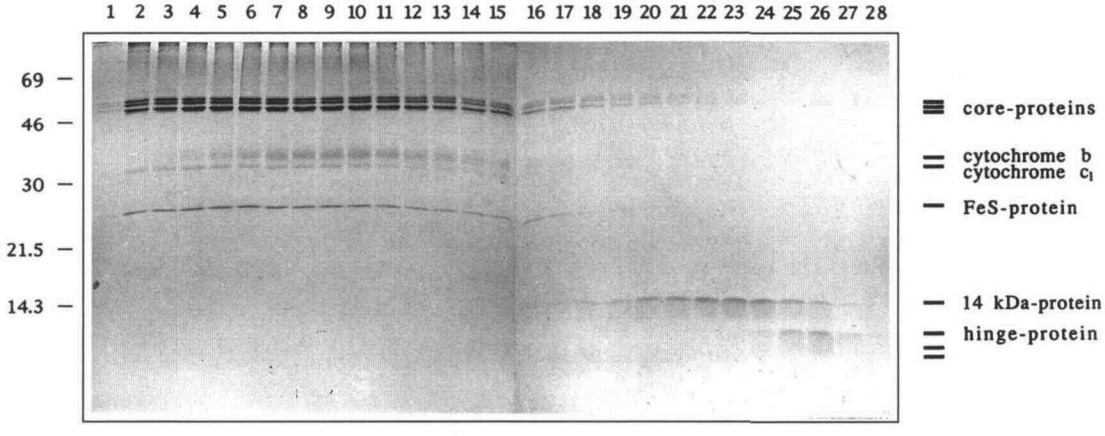
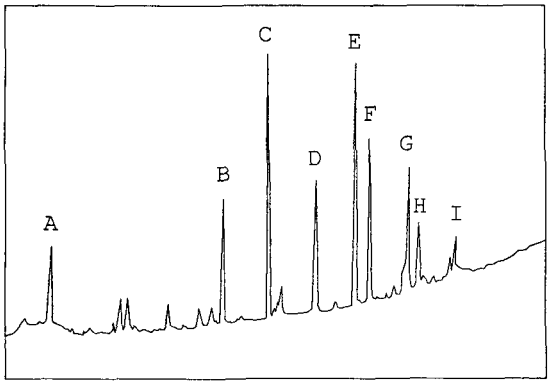


Figure 2. Separation of the 14-kD protein of Cyt *c* reductase from potato. The enzyme complex was incubated with 6 M urea as described in "Materials and Methods" and the cleavage products were separated by gel-filtration chromatography. Fractions eluting from the column (lanes 1–28) were analyzed by SDS/PAGE and Coomassie staining. The identities of the subunits of Cyt *c* reductase from potato are given on the right and the masses (in kD) of standard proteins are given on the left.



A: -
B: (K) E A L G R L P R E V V
C: (K) H Q Y L P D D V R A L Q Q P F K
D: (K) A L G A L P L Y E R T L P
E: (K) N Y G L R Y D D L Y D P Y F D L D I K
F: (K) N F G L R Y D D L Y D P Y F D L D I K
G: (K) S Y L S D M L A L V K
H: -
I: -

Figure 3. Direct amino acid sequence determination of the 14-kD protein of Cyt c reductase from wheat. Top, HPLC elution profile of the 14-kD subunit after digestion with endoprotease Lys C. The generated peptides (A–I) were separated by a linear gradient of water and acetonitrile (*x* axis, 5–60% acetonitrile; *y* axis, absorption). Bottom, Amino acid sequences of peptides B through G. The preceding amino acid of the peptides was assumed to be Lys (K). The sequences of peptides A, H, and I could not be determined.

The sequence of the potato 14-kD protein shows clear similarity to the 14.4-kD subunit of Cyt *c* reductase from yeast (also termed subunit VII), to the 13.4-kD subunit from bovine (also termed subunit VI), and to the peptide sequences of the 14-kD protein from wheat. Based on the alignment in Figure 5 the potato 14-kD subunit shares 35% amino acids with the protein from bovine and 24% with the one from yeast (33% conservation between the sequences from yeast and bovine). The sequence identity between the partial sequence from wheat (73 amino acids) and potato is about 75%. Comparison of the sequences from potato, yeast, and bovine indicates a small deletion in the potato subunit close to the N terminus and an insertion of seven amino acids after residue 25 (Fig. 5). At the C terminus the 14-kD protein from potato shows an extension. Interestingly, the sequence in this area (. . . REALGALP . . .) partially resembles an internal stretch of the potato subunit (. . . KEALNRLP . . . after amino acid 48). The potato 14-kD subunit is hydrophilic because it contains 31% charged amino acids (22 positively and 16 negatively charged residues), which are distributed along the whole protein. Like the bovine 13.4-kD protein the 14-kD subunit of Cyt *c* reductase from potato contains no Cys.

Both the 14.4-kD subunit from yeast and the 13.4-kD protein from bovine have no cleavable presequence for import into the mitochondrion or only a very short one. Since the calculated molecular mass of the 14-kD protein from potato (14,461 D) is nearly identical with the apparent

```
                                M  A  S  S  F  S  R  W  L      9
  TGTTTGTGTTAGCGATTGAAA ATG GCA TCG TCG TTC TCC AGA TGG CTC    48

 V  D  P  K  K  N  P  L  A  A  I  H  M  K  T                 24
GTG GAT CCG AAG AAG AAC CCT CTC GCC GCC ATC CAC ATG AAA ACC    93

 L  S  S  R  L  R  N  Y  G  L  R  H  D  D  L                 39
CTC TCC TCT CGC CTC CGT AAT TAC GGG CTC CGA CAT GAT GAT TTA   138

 Y  D  P  M  Y  D  L  D  V  K  E  A  L  N  R                 54
TAT GAT CCG ATG TAT GAT TTG GAC GTG AAG GAA GCT CTT AAT CGC   183

 L  P  R  E  I  V  D  A  R  N  Q  R  L  L  R                 69
CTT CCG AGG GAG ATT GTT GAT GCC AGA AAC CAG CGC CTT CTG CGT   228

 A  M  D  L  S  M  K  H  Q  Y  L  P  E  D  L                 84
GCC ATG GAC CTC TCC ATG AAG CAC CAG TAC CTC CCA GAG GAT CTT   273

 Q  A  M  Q  T  P  F  R  N  Y  L  Q  E  M  L                 99
CAG GCA ATG CAA ACA CCA TTT AGG AAC TAT CTT CAG GAA ATG CTG   318

 A  L  V  K  R  E  S  A  E  R  E  A  L  G  A                114
GCT CTT GTT AAA AGG GAG AGT GCA GAA CGT GAG GCT TTG GGA GCA   363

 L  P  L  Y  Q  R  T  L  P  *                               123
TTG CCT CTT TAT CAG CGT ACA CTC CCT TAA AGAAATCCCATCTCCCTTT   412

AAACTTTTTCCATTGAGAATAACTACTGTACTGGTTGTAGATCTCCGTGGCGGAGATAG   471

AAATAACAGGATGTTTGTTTGCTTCTATGATCCAATACTTCAAGTTAGTCATCCTGTGG   530

GCTTTATCATTGTAAACCTATATTTTTTCATATGGGACGTACATTTTCGCCAGAACTCC   589

CATATTGCTCTGGTTTGACAGGTTTTATTGGTGTTTCTGAGTATTATATATACATCCTG   648

GAACGTAATTTTTT                                              662
```

Figure 4. Nucleotide sequence and deduced amino acid sequence of the insert of clone pCR14–1.

molecular mass (14 kD, Fig. 1), a presequence seems to be absent in plants also. To verify the lack of a cleavable targeting sequence, the 14-kD subunit of Cyt *c* reductase from potato was imported into isolated organelles (Fig. 6). The protein contains seven Met residues and is, therefore, efficiently radiolabeled with [^{35}S]Met by in vitro translation (Fig. 6, lane 1). The translation product is sensitive to proteinase K (lane 2). Upon incubation with mitochondria, the protein binds to the organelles (lanes 3 and 6) but only becomes protected against proteinase K if the membrane potential is retained (lanes 4 and 7). This indicates that the protein is efficiently imported into the mitochondria and becomes degradable by proteinase K only if the mitochondria are lysed with Triton X-100 (lane 5). There is no detectable difference in size between the translation product and the imported form of the 14-kD protein.

```
wheat   ..........................................
potato  MASSF----------SRWLVDPKKNPLAAIH
bovine    AGRPAVSAS----------SRWLEGIKKWYYNAAG
yeast   MPQSFTSIARIGDYILKSPVLSKLCVPVANQFINLAG

........KNYGLRYDDLYDPYFDLDIKEALGRLPREVV..............
MKTLSSRLRNYGLRHDDLYDPMYDLDVKEALNRLPREIVDARNQFLLRAMDLS
FNKL-------GLMRDDTI-HEND-DVKEAIRRLPENLYDDRVFFIKRALDLS
YKKL-------GLKFDDLIAEENP-IMQTALRRLPEDESYARAYFIIRAHQTE

·KHQYLPDDVRALQQPFKSYLSDMLALVK·····KALGALPLYERTLP
MKHQYLPEDLQAMQTPFRNYLQEMLALVKRESAEREALGALPLYCRTLP
MRQQILPKEQWTKYEEDKSYLEPYLKEVIRERKEREEWAKK
LTEHILPRNQWIKAQEDVPYLLPYILEAEAAAKEKDELDNIEVSK
```

Figure 5. Sequence comparison of the 14-kD protein of Cyt *c* reductase from potato with the partial sequence from wheat and the corresponding sequences from yeast (de Haan et al., 1984) and bovine (Wakabayashi et al., 1985). Identical amino acids are boxed and dashes are introduced to increase sequence identities. The dots indicate gaps in the sequence of the protein from wheat.

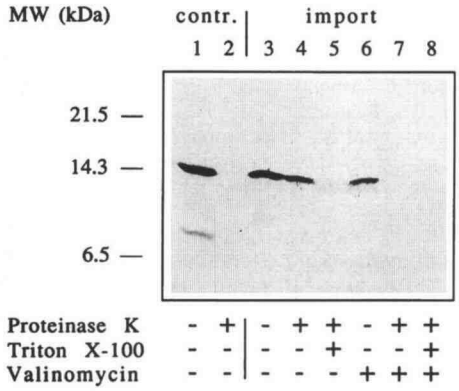


Figure 6. In vitro import of the 14-kD protein from potato into isolated mitochondria. The radiolabeled protein was analyzed by SDS/PAGE and fluorography. Synthesis of the untreated 14-kD protein and its sensitivity toward proteinase K are shown in lanes 1 and 2, respectively. Lanes 3 through 8 exhibit import experiments in the presence or absence of proteinase K, Triton X-100, and valinomycin as indicated. The masses of standard proteins are given on the left. The weak signal at 7 kD of lane 1 most likely represents a smaller version of the 14-kD protein due to an artificial translation start or premature stop of translation. contr., Control.

DISCUSSION

Molecular features of the 14-kD subunit of Cyt *c* reductase were characterized, both in potato, a dicotyledonous plant, and in wheat, a monocotyledonous plant. The plant sequences are clearly related to the sequences of 14-kD proteins of Cyt *c* reductase complexes from yeast and bovine. On the other hand, the identity of about 30% among the 14-kD proteins from plants, fungi, and mammals is smaller than the conservation of the respiratory subunits of Cyt *c* reductase from these organisms, which is approximately 50% (Zanlungo et al., 1991; Braun et al., 1992b; Emmermann et al., 1994). This also explains the absence of immunological cross-reactivity between the antibodies directed against small subunits of Cyt *c* reductase from fungi and the equivalent subunits from higher plants.

Because the function of the 14-kD subunit in electron transport and/or proton translocation is rather unclear, it is difficult to discuss domain-like structures of the protein. However, the distribution of charges in the 14-kD protein from different organisms is very characteristic. The N-terminal part (corresponding to amino acids 1–36 from potato, see alignment in Fig. 5) has a surplus of positive charges (potato, 9+/1−; yeast and bovine, 6+/1−). It is followed in all organisms by a highly negative stretch (in potato, amino acids 37–47) that includes two successive Asp residues at identical positions. The C-terminal half of the 14-kD proteins has a mixed charge distribution and comprises five conserved Arg residues (at positions 54, 63, 66, 69, and 109 in the protein from potato). The 14-kD subunit from potato, yeast, and mammals has a net positive charge and is predicted to have a high helical content. As reported previously these helices have amphiphilic properties (Wakabayashi et al., 1985; Link et al., 1987; Suzuki et al., 1988) that might enable this hydrophilic subunit to interact with the mitochondrial membrane or with ubiquinol. The 14-kD protein lacks potential membrane-spanning helices and is, therefore, most likely anchored by protein-protein interactions with other components of the bc_1 complex. These findings are in line with the assumption of a peripheral localization of the 14-kD subunit of Cyt *c* reductase.

The sequence data for the 14-kD protein from wheat reveal the presence of isoforms for this subunit of the bc_1 complex. Isoforms for the 14-kD protein possibly also occur in potato, since 1 of the 71 residues that were identified by direct protein sequencing (Braun et al., 1994) differs from the sequence encoded by cDNA clone pCR14–1. Alternatively, this inconsistency may be due to a sequencing error. However, the occurrence of isoforms was also reported for other subunits of Cyt *c* reductase from potato (Braun et al., 1992b; Emmermann et al., 1993) and seems to be a rule rather than an exception. Whether these isoforms have an important biological role or simply reflect the polyploidy of many crops (potato is tetraploid, wheat is hexaploid) remains to be established.

The 14-kD protein from potato lacks a cleavable presequence for mitochondrial import. Recently, an increasing number of nuclear-encoded mitochondrial proteins that are imported without cleavable targeting signals have been described: subunits VIII and IX of Cyt *c* reductase from yeast (Trumpower, 1990), the 9.5-kD protein of the bovine bc_1 complex (Yu and Yu, 1993), subunit Vc of Cyt *c* oxidase from sweet potato (Nakagawa et al., 1990), subunit VIb of the same enzyme complex from yeast (LaMarche et al., 1992), 14 or more subunits of NADH-ubiquinol oxidoreductase from bovine (Walker et al., 1992), and at least 4 polypeptides of the large ribosomal subunit from yeast mitochondria (Grohmann et al., 1991) as well as chaperonin 10 from rat (Ryan et al., 1994). Most of these proteins are rather small and the mechanism of their import has rarely been investigated. Import of proteins with cleavable presequences was shown to depend on the positive charge of the N-terminal extensions and on the membrane potential across the inner mitochondrial membrane with the negative side facing the matrix (Schleyer et al., 1982). Interestingly, import of the 14-kD protein from potato into mitochondria also requires the membrane potential and, therefore, most likely also needs a positively charged domain as prerequisite for translocation. This postulated domain could be the N-terminal part (amino acids 1–36), which has typical features of a mitochondrial presequence: it comprises 9 positively charged amino acids, only 1 negative residue, and 5 Ser residues. Alternatively, one of the internal amphiphilic helices may be involved in import of the 14-kD subunit into mitochondria. Further investigations using import experiments with truncated versions of the 14-kD protein will clarify this issue.

ACKNOWLEDGMENTS

We wish to thank Professor H. Weiss, Düsseldorf, Germany, for providing antibodies against the small subunits of Cyt *c* reductase, and H. Mentzel for excellent technical assistance.

Received September 15, 1994; accepted December 16, 1994.
Copyright Clearance Center: 0032-0889/95/107/1217/07.
The EMBL accession number for the sequence reported in this article is X79276.

LITERATURE CITED

Bechmann G, Schulte U, Weiss H (1992) Mitochondrial ubiquinol-cytochrome c oxidoreductase. *In* L Ernster, ed, Molecular Mechanisms in Bioenergetics. Elsevier, Amsterdam, The Netherlands, pp 199–216

Berry EA, Huang L, DeRose VJ (1991) Ubiquinol-cytochrome *c* oxidoreductase of higher plants. J Biol Chem **266:** 9064–9077

Braun HP, Emmermann M, Kruft V, Bödicker M, Schmitz UK (1995) The general mitochondrial processing peptidase from wheat is integrated into the cytochrome bc_1 complex of the respiratory chain. Planta **195:** 396–402

Braun HP, Emmermann M, Kruft V, Schmitz UK (1992a) The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain. EMBO J **11:** 3219–3227

Braun HP, Emmermann M, Kruft V, Schmitz UK (1992b) Cytochrome c_1 from potato: a protein with a presequence for targeting to the mitochondrial intermembrane space. Mol Gen Genet **231:** 217–225

Braun HP, Kruft V, Schmitz UK (1994) Molecular identification of the 10 subunits of cytochrome c reductase from potato mitochondria. Planta **193:** 99–106

Braun HP, Schmitz UK (1992) Affinity purification of cytochrome c reductase from plant mitochondria. Eur J Biochem **208:** 761–767

Braun HP, Schmitz UK (1995) Molecular structure of the 8 kDa subunit of cytochrome c reductase from potato and its $\Delta\Psi$-dependent import into isolated mitochondria. Biochim Biophys Acta (in press)

Cocco T, Lorusso M, Sardanelli AM, Minuto M, Ronchi S, Tedeschi G, Papa S (1991) Structural and functional characteristics of polypeptide subunits of the bovine heart ubiquinol-cytochrome-c reductase complex. Eur J Biochem **195:** 731–734

Crivellone MD, Wu M, Tzagoloff A (1988) Assembly of the mitochondrial membrane system. J Biol Chem **263:** 14323–14333

de Haan M, van Loon APGM, Kreike J, Vaessen RTMJ, Grivell LA (1984) The biosynthesis of the ubiquinol-cytochrome c reductase complex in yeast. DNA sequence analysis of the nuclear gene coding for the 14-kDa subunit. Eur J Biochem **138:** 169–177

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:** 387–395

Emmermann M, Braun HP, Arretz M, Schmitz UK (1993) Characterization of the bifunctional cytochrome *c* reductase/processing peptidase complex from potato mitochondria. J Biol Chem **268:** 18936–18942

Emmermann M, Clericus M, Braun HP, Mozo T, Heins L, Kruft V, Schmitz UK (1994) Molecular features, processing and import of the Rieske-iron-sulfur protein from potato mitochondria. Plant Mol Biol **25:** 271–281

Emmermann M, Schmitz UK (1993) Unique properties of the cytochrome *c* reductase integrated processing peptidase from potato mitochondria. Plant Physiol **103:** 615–620

Gonzalez-Halphen D, Lindorfer MA, Capaldi RA (1988) Subunit arrangement in beef heart complex III. Biochemistry **27:** 7021–7031

Grivell LA (1989) Nucleo-mitochondrial interactions in yeast mitochondrial biogenesis. Eur J Biochem **182:** 477–493

Grohmann L, Graack HR, Kruft V, Choli T, Goldschmidt-Reisin S, Kitakawa M (1991) Extended N-terminal sequencing of proteins of the large ribosomal subunit from yeast mitochondria. FEBS Lett **284:** 51–56

Hemrika W, Berden JA (1990) Membrane topography of the subunits of ubiquinol-cytochrome-c oxidoreductase of *Saccharomyces cerevisiae*. Eur J Biochem **192:** 761–765

Hemrika W, de Jong M, Berden JA, Grivell LA (1994) The C-terminus of the 14-kDa subunit of ubiquinol-cytochrome-c oxidoreductase of the yeast *Saccharomyces cerevisiae* is involved in the assembly of a functional enzyme. Eur J Biochem **220:** 569–576

Japa S, Zhu QS, Beattie DS (1987) Subunit VII, the ubiquinone-binding protein, of the cytochrome bc_1 complex of yeast mitochondria is involved in electron transport at center o and faces the matrix side of the membrane. J Biol Chem **262:** 5441–5444

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

LaMarche AEP, Abate MI, Chan SHP, Trumpower BL (1992) Isolation and characterization of COX12, the nuclear gene for a previously unrecognized subunit of *Saccharomyces cerevisiae* cytochrome *c* oxidase. J Biol Chem **267:** 22473–22480

Link TA, Schägger H, von Jagow G (1986) Analysis of the structure of the subunits of the cytochrome bc_1 complex from beef heart mitochondria. FEBS Lett **204:** 9–15

Link TA, Schägger H, von Jagow G (1987) Structural analysis of the bc_1 complex from beef heart mitochondria by the sided hydropathy plot and by comparison with other bc complexes. *In* S Papa, B Chance, Ernster L, eds, Cytochrome Systems: Molecular Biology and Bioenergetics. Plenum, New York, pp 289–301

Linke P, Weiss H (1986) Reconstitution of ubiquinol-cytochrome-c reductase from *Neurospora* mitochondria with regard to subunits I and II. Methods Enzymol **126:** 201–210

Lorusso M, Cocco T, Boffoli D, Gatti D, Meinhardt S, Ohnishi T, Papa S (1989) Effect of papain digestion on polypeptide subunits and electron-transfer pathways in mitochondrial bc_1 complex. Eur J Biochem **179:** 535–540

Nakagawa T, Maeshima M, Nakamura K, Asahi T (1990) Molecular cloning of a cDNA for the smallest nuclear-encoded subunit of sweet potato cytochrome c oxidase. Eur J Biochem **191:** 557–561

Nishikimi M, Shimomura Y, Ozawa T (1986) Cell-free synthesis of ubiquinone-binding protein of mitochondrial cytochrome bc_1 complex. Biochem Biophys Res Commun **138:** 1291–1297

Pfeiffer WE, Ingle RT, Ferguson-Miller S (1990) Structurally unique plant cytochrome c oxidase isolated from wheat germ, a rich source of plant mitochondrial enzymes. Biochemistry **29:** 8696–8701

Ryan MT, Hoogenraad NJ, Høj PB (1994) Isolation of a cDNA clone specifying rat chaperonin 10, a stress-inducible mitochondrial matrix protein synthesized without a cleavable presequence. FEBS Lett **337:** 152–156

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schägger H, Link TA, Engel WD, von Jagow G (1986) Isolation of the eleven protein subunits of the bc_1 complex from beef heart. Methods Enzymol **126:** 181–191

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **166:** 368–379

Schleyer M, Schmidt B, Neupert W (1982) Requirement of a membrane potential for the posttranslational transfer of proteins into mitochondria. Eur J Biochem **125:** 109–116

Schoppink PJ, Berden JA, Grivell LA (1989) Inactivation of the gene encoding the 14-kDa subunit VII of yeast ubiquinol cytochrome c oxidoreductase and analysis of the corresponding mutant. Eur J Biochem **181:** 475–483

Suzuki H, Hosokawa Y, Toda H, Nishikimi M, Ozawa T (1988) Cloning and sequencing of a cDNA for human mitochondrial ubiquinone-binding protein of complex III. Biochem Biophys Res Commun **156:** 987–994

Suzuki H, Hosokawa Y, Toda H, Nishikimi M, Ozawa T (1989) Isolation of a single nuclear gene encoding human ubiquinone-binding protein in complex III of mitochondrial respiratory chain. Biochem Biophys Res Commun **161:** 371–378

Teintze M, Slaughter M, Weiss H, Neupert W (1982) Biogenesis of mitochondrial ubiquinol-cytochrome *c* reductase (cytochrome bc_1 complex). J Biol Chem **257:** 10364–10371

Trumpower BL (1990) Cytochrome bc_1 complexes of microorganisms. Microbiol Rev **54:** 101–129

Usui S, Yu L, Harmon J, Yu CA (1991) Immunochemical study of subunit VI (M_r 13, 400) of mitochondrial ubiquinol-cytochrome c reductase. Arch Biochem Biophys **289:** 109–117

van Loon APGM, Kreike J, de Ronde A, van der Horst GTJ, Gasser SM, Grivell LA (1983) Biosynthesis of the ubiquinol-cytochrome c reductase complex in yeast. Eur J Biochem **135:** 457–463

Wakabayashi S, Takao T, Shimonishi Y, Kuramitsu S, Matsubara H, Wang T, Zhang Z, King TE (1985) Complete amino acid sequence of the ubiquinone binding protein (QP-C), a protein similar to the 14,000-dalton subunit of the yeast ubiquinol-cytochrome *c* reductase complex. J Biol Chem **260:** 337–343

Walker JE, Arizmendi JM, Dupuis A, Fearnley IM, Finel M, Medd SM, Pilkington SJ, Runswick MJ, Skehel JM (1992) Sequences of 20 subunits of NADH-ubiquinone oxidoreductase from bovine heart mitochondria. J Mol Biol **226:** 1051–1072

Weiss H, Juchs B (1978) Isolation of a multiprotein complex containing cytochrome b and c_1 from *Neurospora crassa* mitochondria by affinity chromatography on immobilized cytochrome c. Eur J Biochem **88:** 17–28

Weiss H, Kolb J (1979) Isolation of mitochondrial succinate: ubiquinone reductase, cytochrome c reductase and cytochrome c oxidase from *Neurospora crassa* using nonionic detergent. Eur J Biochem **99:** 139–149

Yu CA, Yu L (1993) Mitochondrial ubiquinol-cytochrome c reductase complex: crystallization and protein-ubiquinone interaction. J Bioenerg Biomembr **23:** 259–273

Yu L, Yang FD, Yu CA, Tsai AL, Palmer G (1986) Identification of ubiquinone-binding proteins in yeast mitochondrial ubiquinol-cytochrome c reductase using an azido-ubiquinone derivative. Biochim Biophys Acta **848:** 305–311

Yu L, Yu CA (1982) The interaction of arylazido ubiquinone derivative with mitochondrial ubiquinol-cytochrome *c* reductase. J Biol Chem **257:** 10215–10221

Zanlungo S, Litvak S, Jordana X (1991) Isolation and nucleotide sequence of the potato mitochondrial gene for apocytochrome b. Plant Mol Biol **17:** 527–530