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Characterization of the Bifunctional Cytochrome c Reductase-processing Peptidase Complex from Potato Mitochondria*

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In potato, cytochrome c reductase, a protein complex of the respiratory chain, exhibits processing activity toward mitochondrial precursor proteins. One of the two cooperating components of the processing peptidase was shown to be identical with subunit III of the complex. Here we report that two additional proteins of the complex (subunit I and II) share 40–50% sequence identity with the processing enhancing protein, the other component of the processing enzyme from fungi and mammals. Thus the composition and structure of the complex integrated processing peptidase seems to be different from its fungal and mammalian counterparts. Cytochrome c reductase from potato is extraordinarily stable, and separation of subunit III from the complex leads to aggregation of the remaining subcomplex and irreversible loss of processing activity. Expression of the three high molecular weight subunits of the complex allowed purification of each individual protein. Neither the individual subunits nor their combinations are active in *in vitro* processing assays suggesting that they may need the structural support of the complex for activity. In contrast to mitochondrial processing peptidases from other organisms, the purified potato enzyme is active in the presence of high salt (above 1 M NaCl) and works efficiently without addition of metal ions. These data indicate that potato cytochrome c reductase is a bifunctional protein complex with unique features. Possibly, there is a more general evolutionary relationship between cytochrome c reductases and mitochondrial processing peptidases than hitherto assumed.

Proteins destined to the mitochondrial compartment usually have signal sequences at their amino terminus which direct them to the organelle and to the right intramitochondrial location (Verner and Schatz, 1988; Hartl and Neupert, 1990). In most cases these so-called presequences are removed upon import into the organelle probably because they might interfere with proper folding and assembly or with the function of the protein. In fungi and mammals an endoproteinase located in the mitochondrial matrix performs complete or partial cleavage of the presequences from proteins targeted to the matrix, the inner membrane or the intermembrane space (Böhni *et al.*, 1980; Pfanner and Neupert 1990). This enzyme has been purified from *Neurospora* (Hawlitschek *et al.*, 1988), yeast (Yang *et al.*, 1988) and rat (Ou *et al.*, 1989; Kleiber *et al.*, 1990) and was termed "matrix processing protease" or "general matrix processing peptidase" (Kalousek *et al.* 1988) as it acts on the majority of imported proteins. It consists of two subunits, the presumptive catalytically active component "matrix processing peptidase" (MPP)[1] (termed Mas2 in yeast (Yaffe and Schatz, 1984)) and the so-called "processing enhancing protein" (PEP, termed Mas1 in yeast). More recently two other mitochondrial processing peptidases involved in the formation of mature proteins which undergo two-step proteolytic cleavage have been characterized. The mitochondrial intermediate processing peptidase (MIP) cleaves off the eight COOH-terminal amino acids of the presequences of some soluble proteins like ornithine transcarbamylase (Kalousek *et al.*, 1992). While MIP and the general matrix processing peptidase are soluble proteins, another protease removing the sorting signals of proteins targeted to the intermembrane space is localized in the inner mitochondrial membrane (Schneider *et al.*, 1991; Behrens *et al.*, 1991).

In *Neurospora* one component of the matrix processing peptidase, the processing enhancing protein, has been shown to be identical with subunit I of cytochrome c reductase (also known as cytochrome bc_1 complex or complex III of the respiratory chain), a protein complex of the inner mitochondrial membrane (Schulte *et al.*, 1989). As PEP is also found in the soluble fraction of *Neurospora* mitochondria, the matrix localized molecules are supposed to cooperate with the hydrophilic MPP during processing (Arretz *et al.*, 1991). In potato mitochondria, however, the activity of the general mitochondrial processing peptidase is entirely associated with cytochrome c reductase (Braun *et al.*, 1992b). The complex from potato contains 10 subunits including three with a molecular mass above 50 kDa while in fungi this complex is composed of nine subunits with not more than two of them being larger than 45 kDa. The high molecular mass subunits (traditionally termed "core" proteins) do not contain redox centers but nevertheless are important for ubiquinol cytochrome c oxidoreductase activity (Linke and Weiss, 1986) and assembly of the complex (Tzagoloff *et al.*, 1986; Oudshorn *et al.*, 1987).

As subunit III (51 kDa) of cytochrome c reductase from potato shares significant sequence similarity with MPP, we have asked whether plant mitochondria also contain a PEP homologue and, if so, which of the subunits of cytochrome c reductase it may be. Here we report properties of the three high molecular mass subunits of potato cytochrome c reductase. Protein sequencing and structural analysis of cDNA clones reveals that two different polypeptides with highest similarity to PEP form part of the complex. We present data on the activity of the presumptive proteolytic subunits after

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[1] The abbreviations used are: MPP, matrix processing peptidase; PEP, processing enhancing protein; MIP, mitochondrial intermediate processing peptidase; PAGE, polyacrylamide gel electrophoresis.

their separation from the complex and show that the complex integrated processing enzyme has unique properties.

MATERIALS AND METHODS

Isolation of Potato Mitochondria and Purification of Cytochrome c Reductase—Mitochondria were isolated from potato tuber (var. Marfona) as described previously (Braun *et al.*, 1992a). Cytochrome *c* reductase was purified by affinity chromatography and gel filtration as outlined in Braun and Schmitz (1992). The purified protein complex was stored at −70 °C.

Cleavage of Cytochrome c Reductase—Cytochrome *c* reductase was concentrated by ultrafiltration to about 15 mg/ml protein and 100-μl aliquots (1.5 mg of protein) were used for each cleavage reaction. Depending on the experiment different amounts of detergent, of EDTA, and of several salts with variable chaotropic properties were used: a solution of 10% Triton X-100 was added to a final concentration of 0.5, 1, 1.5, or 2%; a solution of 0.5 M EDTA (pH 8.0) was added to a final concentration of 3, 5, or 50 mM; 5 M stock solutions of NaCl, NaBr, NaI, and guanidinium hydrochloride were added to a final concentration of 0.5, 0.8, 1, or 2 M. The assay was gently shaken and kept on ice for 10 min. Afterward it was gel-filtrated through an Ultrogel AcA 34 column (0.7 × 30 cm) with a flow rate of 1.2 ml/h in a buffer containing 50 mM Tris-acetate (pH 7.2), 0.05% Triton X-100, and 0.2 mM phenylmethylsulfonyl fluoride (elution buffer). The eluate was collected in 10-min intervals and analyzed by SDS-PAGE. For processing assays the eluate was concentrated about 5-fold, dialyzed against elution buffer, and incubated with radiolabeled mitochondrial precursor proteins as outlined below.

Incubation of Cleavage Products with groEL/ES from Escherichia coli—To allow refolding of proteins denatured during the cleavage of cytochrome *c* reductase a 1–2 molar excess of groEL/ES proteins from *E. coli* was added to the concentrated fractions. These proteins were a kind gift of Dr. F.-U. Hartl, New York. The process was performed in a buffer containing 40 mM Tris (pH 8.0), 2 mM $MgCl_2$, 0.05% Triton X-100, 1 mM ATP, and 0.2% phenylmethylsulfonyl fluoride. Incubation with groEL/ES was carried out for 10 min at 0 °C and subsequently at 25 °C for 30 min prior to their use in an *in vitro* processing assay.

In Vitro Processing of Radiolabeled Precursor Proteins—The cDNA of the $F_1\beta$-subunit of ATPase from tobacco (Boutry and Chua, 1985) was transcribed *in vitro* at 37 °C for 40 min as described previously (Braun *et al.*, 1992b). The precursor protein was synthesized in rabbit reticulocyte lysate containing radiolabeled methionine. *In vitro* processing of radiolabeled protein was carried out in the presence of 0.5% Triton X-100, 25 mM NaCl, 0.1 mM $MnCl_2$, 0.1 mM $ZnCl_2$, 0.1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 22 mM Tris/HCl at pH 8.0 in a final volume of 100 μl. The buffer was supplemented with purified cytochrome *c* reductase or its cleavage products as indicated. The processing reaction was carried out for 1 h at 28 °C; it was stopped with an equal volume of Laemmli (1970) buffer (2-fold concentrated and incubated for 10 min at 60 °C prior to loading onto the gel. Processing products were analyzed by SDS-PAGE and fluorography.

Analysis of Proteins—Protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. Proteins were separated in 14% polyacrylamide gels according to Laemmli (1970). For protein sequencing, the polypeptides of cytochrome *c* reductase were fractionated by SDS-PAGE and blotted onto poly(vinylidene fluoride) membranes (Millipore). Individual protein bands were stained with Ponceau S, cut out, and digested overnight with endoproteinase Lys-C as outlined in Graack *et al.* (1991). The resulting peptides were purified by reverse phase high performance liquid chromatography and subjected to Edman degradation in an Applied Biosystems pulsed-liquid phase sequencer (Lottspeich, 1985). Phenylthiohydantoin amino acids were separated on-line in an Applied Biosystems model 120A analyzer and identified by manual interpretation of the data.

Expression of Polypeptides in E. coli—Using appropriate primers the genes encoding the 51- and 53-kDa subunit from cytochrome *c* reductase were amplified by polymerase chain reaction without the regions encoding the presequences. Taq polymerase and assay buffer were purchased from Böhringer Mannheim and used according to the manufacturer's instructions. The genes encoding the mature proteins were cloned into a pMALc2 vector (Biolabs) using *Bam*HI sites which had been introduced by the polymerase chain reaction primers. *E. coli* cells transformed with the recombinant plasmids were grown to an OD of 0.5 and induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside for expression of the proteins. Cells were harvested after 3 h and lysed as described in Sambrook *et al.* (1989). The fusion proteins formed inclusion bodies which were purified. The isolated inclusion bodies were subsequently denatured in breaking buffer (6 M urea, 100 mM NaCl, 10 mM dithiothreitol, 50 mM β-mercaptoethanol, 10 mM Hepes, pH 7.5) and shaken at room temperature for 2–3 h. They were dialyzed several times at 4 °C in an excess volume of 10 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol. The resulting protein solution was applied to an amylose resin column for affinity purification of the fusion protein (see Biolabs instructions). Fractions containing the fusion protein were concentrated by ultrafiltration (XM 30 filters, Amicon) and the maltose binding protein was cleaved off with endoprotease Xa as described by the supplier (Biolabs).

Screening of cDNA Libraries—Copy DNA libraries from potato tubers and leaves were constructed as described elsewhere (Emmermann *et al.*, 1991). Two sets of oligonucleotides with the lowest degeneracy were derived from peptide sequences which had been obtained from the 55- and 53-kDa polypeptides of cytochrome *c* reductase as described above. The oligonucleotide mixtures contained the full complement of sequences that could potentially encode a nonapeptide (Pro-Pro-Asp-Ala-Met-Ile-Tyr-Asp-Arg) and a decapeptide (Ilu-Glu-lu-Ile-Glu-Asn-Met-Gly-Gly-His) of the 55-kDa protein and two decapeptides (Lys-Phe-Glu-Glu-Arg-Lys-Ile-Glu-Arg-Glu and Ala-Asn-Arg-Phe-Ile-Phe-Asp-Glu-Asp-Val) of the 53-kDa protein. The oligonucleotides were end labeled with T4 polynucleotide kinase and [γ-^{32}P]dATP (Amersham Corp.) and used for screening the cDNA library (Sambrook *et al.* 1989).

Cloning, Sequencing, and Computer Analysis of DNA—DNA from phages hybridizing to the labeled oligonucleotides was isolated with Qiagen columns (Diagen) according to the manufacturers instructions. The cDNA inserts were cloned into Bluescript vectors (Stratagene) and sequenced on both strands following the didesoxynucleotide method (Sanger *et al.*, 1977). Overlapping subclones were produced with the exonuclease III deletion strategy. DNA and protein sequences were analyzed with programs of the Staden package on a VAX computer.

RESULTS

Cleavage of Cytochrome c Reductase from Potato—To determine which subunits of potato cytochrome *c* reductase are involved in the processing of mitochondrial precursor proteins, we tried to separate individual polypeptides, especially the high molecular mass subunits, from the complex. As detailed under "Materials and Methods" the cleavage reactions were carried out in the presence of 0.5–2% Triton X-100 and 0.5–2 M NaCl, NaBr, NaI, or guanidinium hydrochloride; the reactions contained optionally 5–50 mM EDTA as the processing enzyme is dependent on divalent metal ions. Cleavage products were separated by gel filtration and subsequently analyzed by SDS-PAGE. While cytochrome *c* reductase from *Neurospora* can easily be dissected into three subcomplexes in the presence of 2% Triton X-100 and 2 M NaCl (Linke and Weiss, 1986) these conditions do not lead to the dissociation of polypeptides from the potato complex (not shown). More chaotropic ions like bromide and iodide (Hatefi and Hanstein, 1974) lead to a partial dissociation of the 51- (identified as MPP (Braun *et al.*, 1992b)), 14-, and 12-kDa subunits of potato cytochrome *c* reductase from the complex if concentrations around 2 M are used. A complete dissociation of the same polypeptides from the complex occurs in the presence of 0.8–1 M guanidinium hydrochloride (Fig. 1). Upon separation of these subunits from the complex the major part of the remaining subcomplex aggregates, probably due to the disclosure of hydrophobic faces.

A variety of other conditions were tested to find out whether it is possible to dissect other polypeptides, especially the two largest subunits which might be involved in processing, from the complex. Higher concentrations of guanidinium hydrochloride (2 M) cause complete precipitation of the complex while lower concentrations have little effect. Also urea in concentrations up to 6 M does not effectively disintegrate the

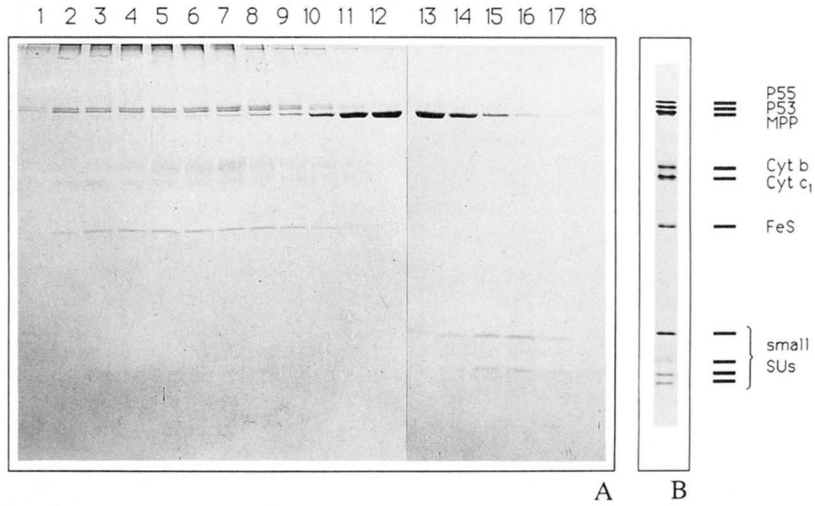

FIG. 1. **Cytochrome *c* reductase from potato and its cleavage with 1 M guanidinium HCl.** *A*, cleavage products were separated by gel filtration chromatography and equal volumes of 18 subsequent fractions were analyzed by SDS-PAGE. Only three distinct subunits are dissected from the complex (*lanes 11–18*). They are present in relatively higher amount than the remaining complex which may be aggregated as indicated by stainable material entering only partly the gel (*lanes 1–9*). In *lanes 1–4* where MPP is nearly quantitatively separated from the complex, the amount of aggregated material is higher than in the subsequent fractions. *B*, intact cytochrome *c* reductase complex separated by SDS-PAGE. Subunits previously identified (Braun *et al.*, 1992b) are indicated as *MPP*, *Cyt b* (cytochrome b), *Cyt* c_1 (cytochrome c_1), *FeS* (Rieske-iron sulfur protein), and *small SUs* (small subunits); subunit I and II have been termed *P55* and *P53*, respectively, according to their molecular mass.

complex. In summary these data indicate that we were successful in separating 3 of the 10 subunits from the complex, but most of the conditions leading to the complete disintegration of cytochrome c reductase from *Neurospora* (Linke and Weiss, 1986) or bovine (Rieske *et al.*, 1967; Schägger *et al.*, 1986) have limited effect on the enzyme from potato.

Destabilization of Cytochrome c Reductase Complex Causes Irreversible Loss of Processing Activity—Fractions containing the dissected MPP subunit (51 kDa) or the remaining complex including the two largest subunits (55 and 53 kDa) were tested separately or combined for processing activity. All proteins were desalted by gel filtration and dialysis before use. As shown in Fig. 2, neither MPP alone nor the P55/P53 proteins alone nor their combination exhibit processing activity. Thus the proteolytically active subunits may either be denatured and inactivated by the guanidinium treatment or they may require the structural support of the complex for activity. As groEL/ES proteins from *E. coli* are known to achieve refolding of partly denatured proteins (Georgopoulos *et al.*, 1990; Martin *et al.*, 1991) the 51-kDa protein and fractions containing the other subunits of the complex were incubated with a molar excess of purified *E. coli* heat shock proteins in the presence of ATP and Mg^{2+} ions. While groEL/ES increases the amount of processed precursor protein in assays containing the uncleaved cytochrome *c* reductase (Fig. 2*A*, *lane 3*) it has no effect on the processing activity of subunits separated from the complex (not shown). Furthermore treatment of the mitochondrial processing peptidase from *Neurospora* with 1 M guanidinium hydrochloride does not significantly reduce its processing activity, if the salt is removed. These results may be interpreted in support of a model suggesting that the proteolytically active subunits of potato cytochrome *c* reductase need the structural support of the complex for proper function.

Unique Properties of the Cytochrome c Reductase Integrated Mitochondrial Processing Peptidase from Potato—The high structural resistance of potato cytochrome *c* reductase toward salt and nonionic detergent prompted us to analyze processing

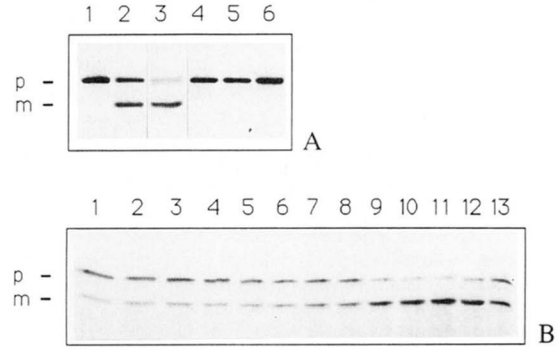

FIG. 2. **Processing activity of cytochrome *c* reductase and of cleavage products of the complex.** A radiolabeled precursor of the β-subunit of F_1-ATPase was used in all experiments and visualized after SDS-PAGE by fluorography; *p* indicates the precursor protein and *m* its mature form. *A*, processing activity is irreversibly lost upon separation of MPP (51 kDa) from the complex. *Lane 1*, control. *Lane 2*, a suboptimal amount of cytochrome *c* reductase (0.7 μg) was incubated with the precursor protein yielding about 50% mature protein. *Lane 3*, the same amounts of cytochrome *c* reductase and precursor as in *lane 2* were used but a molar excess of groEL/ES proteins was present during the assay. Less precursor than in *lane 1* remains after the reaction, possibly due to interaction of the heat shock proteins with improperly folded precursors or aggregated cytochrome *c* reductase complexes. No mature protein is produced in an assay containing 1 μg of MPP alone (*lane 4*; protein from fraction 13 of the cleavage reaction was used, see Fig. 1) or about 1 μg of P55 and P53 (*lane 5*; fraction 2 of the cleavage reaction, see Fig. 1). *Lane 6*, incubation of the combined fractions 13 and 2 with the radiolabeled precursor. *B*, processing activity is highly salt resistant. Incubation of 0.5 μg of cytochrome *c* reductase with radiolabeled precursor in the presence of different amounts of NaCl (*lane 1*, no salt; *lane 2*, 0.02 M; *lane 3*, 0.1 M; *lane 4*, 0.3 M; *lane 5*, 0.4 M; *lane 6*, 0.5 M; *lane 7*, 0.6 M; *lane 8*, 0.7 M; *lane 9*, 0.8 M; *lane 10*, 0.9 M; *lane 11*, 1 M; *lane 12*, 1.2 M; *lane 13*, 1.5 M).

activity under high salt conditions. Upon incubation of radiolabeled precursor of the β-subunit of ATPase with the purified complex in the presence of 0–1.5 M NaCl different amounts of mature protein are produced (Fig. 2*B*). Unexpect-

edly the processing enzyme has its optimal activity at concentrations around 900 mM NaCl and is still active at concentrations of 1.5 M NaCl. This does not only show that the cytochrome c reductase integrated protease is highly salt resistant but also provides evidence that the interaction between the mitochondrial processing peptidase and its substrate is insensitive to high sodium chloride. In contrast, the processing proteases from fungi and mammals are completely inactivated by 150 mM NaCl (Hawlitschek *et al.*, 1988; Ou *et al.*, 1989).

Protein Sequencing of High Molecular Mass Polypeptides of Cytochrome c Reductase—As cleavage of potato cytochrome c reductase was accompanied by loss of processing activity we isolated cDNA clones encoding the three largest subunits to determine their primary structure and to express them in *E. coli* for further functional analysis. The 55-, 53-, and 51-kDa polypeptides were separated by SDS-PAGE and blotted onto poly(vinylidene fluoride) membranes, and individual subunits were digested with endoproteinase Lys-C. The resulting peptides were analyzed by Edman degradation and oligonucleotide mixtures containing the full complement of sequences that could potentially encode two peptides from each subunit were employed to screen cDNA libraries from potato tubers and leaves. The characterization of cDNA clone pM1 encoding the 51-kDa polypeptide has been reported elsewhere (Braun *et al.*, 1992b).

The 53-kDa Subunit of Cytochrome c Reductase from Potato Is Highly Similar to PEP—Sequence analysis of cDNA clones giving specific signals with oligonucleotides derived from the 53-kDa protein revealed the presence of an open reading frame encoding a polypeptide of 530 amino acids. The initiation codon of clone p53.1 is preceded by two in frame stop codons at nucleotide 35 and 47 of the cDNA. The 3′-noncoding region is relatively short (180 nucleotides excluding the poly(A) tail). The protein encoded by the main open reading frame has a calculated molecular mass of 59.3 kDa indicating that the 53-kDa protein of cytochrome c reductase may be synthesized as a larger precursor. Three internal peptides from the 53-kDa polypeptide comprising a total of 61 amino acids are completely identical with three stretches of the amino acid sequence deduced from clone p53.1. This result confirmed that clone p53.1 really contains the genetic information of subunit II of the complex. A search for similarity between the amino acid sequence of the 53-kDa protein and sequences kept in the EMBL database revealed more than 50% sequence identity with the processing enhancing protein of the mitochondrial processing peptidase from *Neurospora* and 42% sequence identity with PEP from yeast. This significant homology suggests that the 53-kDa protein may form part of the processing peptidase from potato mitochondria. In *Neurospora* (Schulte *et al.*, 1989) and possibly also in bovine (Gencic *et al.*, 1991) the processing enhancing protein and subunit I of cytochrome c reductase are identical, while in yeast they are different proteins. Interestingly, there is 43% sequence identity between the 53-kDa polypeptide and subunit I of cytochrome c reductase from bovine but significantly less sequence identity with subunit I of cytochrome c reductase from yeast (25%).

Isolation and Sequencing of cDNA Clones Encoding the 55-kDa Subunit Reveals a Second PEP-like Protein in Potato Cytochrome c Reductase—As subunit II (53 kDa) of the complex turned out to be highly homologous to PEP and subunit III shows sequence similarities to MPP, we wondered about the identity of subunit I. To find out whether it may represent a homologue of the core II protein from other cytochrome c reductase complexes, oligonucleotide mixtures encoding two internal peptides of the 55-kDa protein were used to screen a cDNA library. Sequence analysis of several positively reacting clones revealed identical structures for their DNA inserts. They contain a long open reading frame encoding a protein of 534 amino acids. The initiation codon is preceded by a relatively short 5′-untranslated region of 18 base pairs. As no in frame stop codon is located in front of the ATG we cannot exclude that a longer open reading frame exists, but the sequence comparisons discussed below make this rather unlikely. Three peptides corresponding exactly to three parts of the amino acid sequence of the polypeptide encoded by clone p55.1 confirm that this clone really encodes subunit I of cytochrome c reductase. Surprisingly the 55-kDa protein has a similar amino acid composition as the 53-kDa protein. An alignment of both polypeptides (Fig. 3) shows 70% sequence identity and also the cDNA inserts encoding the 53- and the 55-kDa proteins are significantly similar at the DNA level (71% identical nucleotides). Consequently a database search reveals highest sequence identity of the 55-kDa protein with PEP from *Neurospora* (49%) and yeast (41%) and significantly less identical amino acids in comparison to the core II protein from yeast, bovine, and human (about 25% identity) or the core I protein from yeast (27% identity).

Both the 55 and 53-kDa proteins have a bipartite structure with a hydrophilic NH_2-terminal half which is highly similar to the NH_2-terminal half of the Mas1-encoded PEP from yeast (60% identity) and a more hydrophobic COOH-terminal part sharing only 33% sequence identity with the yeast protein. Compared with the NH_2-terminal half, the COOH-terminus is more similar to the core I protein from yeast. Also the hydrophobicity profiles of the 55- and 53-kDa proteins of potato cytochrome c reductase are highly similar to those of PEP/core I from *Neurospora* and the NH_2-terminal part of the Mas1-encoded PEP from yeast. Taken together these data suggest that the two largest subunits of the cytochrome c reductase complex perform a similar function during processing of mitochondrial precursors as the PEP/Mas1 proteins from fungi.

Subunits I (55 kDa) and II (53 kDa) Carry Cleavable Mitochondrial Presequences, and Their Mature Forms Differ Only Two Amino Acids in Length—To determine the exact length of the mature 55- and 53-kDa proteins gas phase sequencing of the amino termini from both polypeptides was performed. The mature 53-kDa protein starts with the sequence Ser-Ala-Ser-Ala-Ala-Val-Ala-Ala-Thr-Ser-Ser-Ser-Thr-Pro-Ala which corresponds exactly to residue 27–41 of the protein encoded by clone p53.1. The presequence of this subunit consists of 26 amino acids exhibiting typical features of mitochondrial targeting sequences: it contains 7 basic residues, 6 hydroxylated residues, but no acidic amino acids.

NH_2-terminal sequencing of the mature 55-kDa protein shows that it starts with the sequence Ser-Thr-Ser-Ile-Thr-Asn-Pro-Ser-Gln-Ser-Ser-Ser-Leu-Pro-Ser-Pro-Pro-Pro-Pro-Asp-Ala-Met-Ile-Tyr-Asp-Arg-Leu which is identical with a stretch of the amino acid sequence encoded by clone p55.1. Hence the 55-kDa protein is synthesized as a larger precursor with a calculated molecular mass of 59.9 kd containing a presequence of 32 amino acids. Interestingly the mature subunits I and II of potato cytochrome c reductase differ only two amino acids in length. There are several possible explanations for their different mobility in SDS-polyacrylamide gels, *e.g.* covalently attached groups or tightly bound phospholipids.

To find out whether the proteins encoded by clone p53.1 and p55.1 are processed by cytochrome c reductase, the precursors were synthesized by coupled *in vitro* transcription/

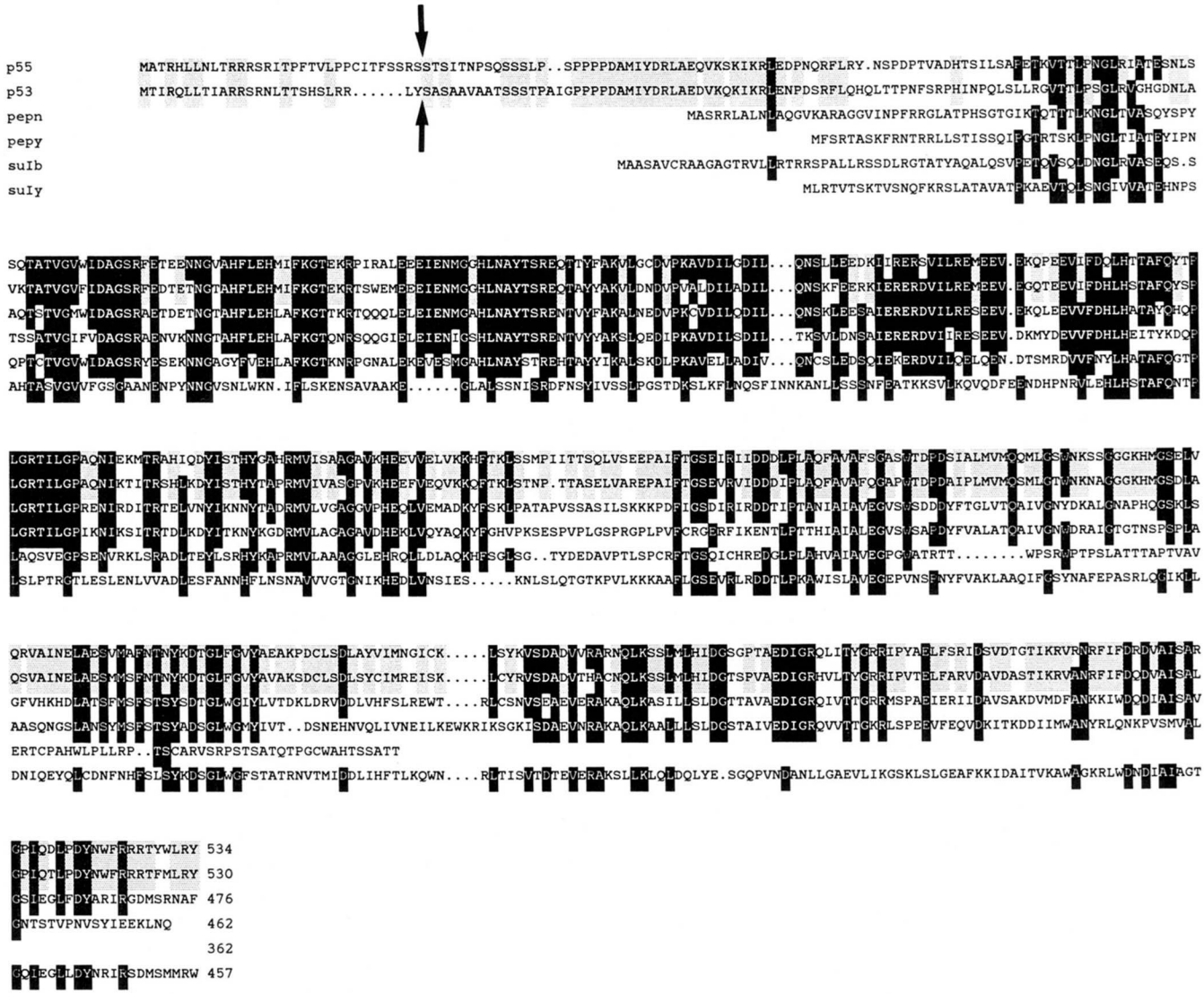

FIG. 3. **Comparison of subunit I (P55) and subunit II (P53) of potato cytochrome *c* reductase with PEP and subunit I of the complex from other organisms.** The alignment includes the amino acid sequences of the processing enhancing protein from *Neurospora* (*pepn*; Hawlitschek *et al.*, 1988) and yeast (*pepy*; Witte *et al.*, 1988) as well as the sequences of subunit I of cytochrome *c* reductase from bovine (*suIb*; Gencic *et al.*, 1991) and yeast (*suIy*; Tzagoloff *et al.*, 1986). Amino acids identical in at least four of the proteins shown are given in *white letters* and amino acids identical between P55 and P53 are *shaded*. The *arrows* indicate the cleavage sites within the precursor proteins of P55 and P53.

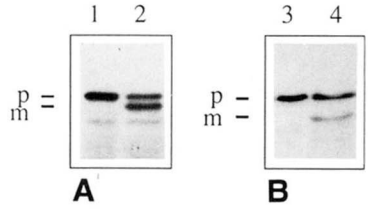

FIG. 4. **The precursors of subunit I and II of cytochrome *c* reductase are processed by the isolated complex to a 53- and 55-kDa protein.** The proteins encoded by clone p53.1 (*lanes 1* and *2*) and p55.1 (*lanes 3* and *4*) were synthesized in the presence of [^{35}S] methionine and incubated with 2 μg of purified cytochrome *c* reductase. In *lanes 1* and *3* Laemmli buffer was added at the beginning of the reaction. Apart from the precursors (*p*), the mature proteins (*m*) of 53 kDa (*lane 2*) and 55 kDa (*lane 4*) are visible.

translation in the presence of [^{35}S]methionine. Upon incubation of the radiolabeled proteins with purified cytochrome *c* reductase both precursors are converted to smaller products corresponding in size to subunit I and II of the potato complex (Fig. 4).

Functional Analysis of High Molecular Mass Subunits of Cytochrome c Reductase—As the proteolytically active subunits loose their function after separation from the complex we used alternative methods to characterize their activity. The complex was treated with antibodies raised against the 53- and 51-kDa subunit (Braun and Schmitz, 1992) prior to incubation with labeled precursor proteins. No inhibition of processing activity was observed upon addition of the individual or the combined antisera (not shown). Possibly the antibodies only recognize the SDS denatured proteins used for immunization or they do not bind efficiently to the subunits due to their integration in the complex. Another approach was the expression of the high molecular mass subunits of cytochrome *c* reductase in *E. coli*. As the cleavage sites of the three largest subunits have been determined (this report),[2] it was possible to synthesize the corresponding genes by polym-

[2] M. Emmermann and U. K. Schmitz, manuscript in preparation.

erase chain reaction without the coding region for the presequences. The genes for the 55-, 53-, and the 51-kDa protein were cloned into an *E. coli* expression vector and the recombinant proteins were isolated by affinity chromatography (see "Materials and Methods"). Incubation of the individual subunits with radiolabeled precursor of the β-subunit of ATPase did not generate the mature β-subunit protein. Similarly, the combined high molecular mass subunits of cytochrome *c* reductase did not exhibit processing activity (not shown). In contrast, the combined subunits of the mitochondrial processing enzyme from rat which have been expressed in the same *E. coli* strain and purified with exactly the same procedure exhibit strong processing activity.[3] Likewise, under conditions allowing functional reconstitution of the *Neurospora* enzyme the subunits from potato exhibit no processing activity. These data are in line with the above mentioned results on the inactivity of individual subunits separated from the complex. In summary our investigations indicate that the proteolytically active subunits are dependent on the structural support of the complex for proper functioning.

DISCUSSION

As in potato the polypeptides involved in the proteolytic removal of many mitochondrial presequences form an integral part of cytochrome *c* reductase, a respiratory complex located in the inner mitochondrial membrane, we propose to term this enzyme "general mitochondrial processing peptidase" instead of "matrix processing peptidase" (Hawlitschek *et al.*, 1988; Yang *et al.*, 1988). Apart from subunit III (51 kDa) of the complex which exhibits significant sequence similarity with MPP (Braun *et al.*, 1992b) the two largest subunits share a high degree of sequence identity with PEP/core I from *Neurospora* and the Mas1-encoded PEP from yeast (this report). Thus the "core" proteins from potato cytochrome *c* reductase seem to be more closely related to the general mitochondrial processing peptidases from other organisms than to the core proteins from yeast. Especially no homologue of the core II protein which has been characterized in yeast (Oudshorn *et al.*, 1987), human (Hosokawa *et al.*, 1989), and bovine (Gencic *et al.*, 1991) forms part of the potato complex as revealed by sequencing of peptides from all its subunits.[4] Although yeast mutants deficient in core II protein have reduced levels of cytochrome *b* and reduced cytochrome *c* reductase activity (Oudshoorn *et al.*, 1987) in potato the protein seems to be replaceable by a distantly related polypeptide. This view is in agreement with earlier suggestions that the core proteins may be important for the assembly and stability of the complex (Grivell, 1989). Nevertheless core proteins are essential for electron transfer from ubiquinol to cytochrome *c* as demonstrated in reconstitution experiments with cytochrome *c* reductase from *Neurospora* (Linke and Weiss, 1986). From the characterization of core proteins in *Neurospora* (Schulte *et al.*, 1989), bovine (Gencic *et al.*, 1991), and potato, a picture is emerging which suggests that the core I protein may be bifunctional allowing its participation in mitochondrial respiration and biogenesis. The proposed two-domain structure of core I from *Neurospora* and bovine which is also found in the two largest subunits of potato cytochrome *c* reductase is consistent with a possible role of these proteins in processing and electron transport.

It is not clear why potato cytochrome *c* reductase has three core proteins, and so far it has not been proven that all three of them are present twice in a cytochrome *c* reductase dimer. On the other hand, the molecular mass of the dimers from potato is about 100 kDa higher than the one of the dimeric enzyme from *Neurospora* (Braun and Schmitz, 1992) suggesting that an additional 50-kDa component forms part of each complex. It is conceivable that the complete integration of the processing peptidase in the complex requires an additional subunit for stabilization. Such a model would also provide some explanation for the extraordinary stability of cytochrome *c* reductase from potato. Probably due to protein-protein interactions the combined enzymes form a complex which is more stable than the individual cytochrome *c* reductase and processing peptidase complexes from yeast, *Neurospora*, and rat. In turn separation of proteolytically active subunits from the complex seems to be accompanied by loss of processing activity indicating that the protease may need the structural support of the complex. For the following reasons we suggest to consider cytochrome *c* reductase and the mitochondrial processing peptidase from potato as a bifunctional structural unity (Fig. 5). (i) The 10 subunits shown in the figure behave as one complex during all biochemical purification procedures applied (Braun and Schmitz, 1992); dilution of the complex during purification leads to loss of a respiratory subunit (*FeS*) but not of the proteolytic proteins (Braun *et al.*, 1992b). (ii) The isolated complex is extraordinary stable toward salt and detergent and exhibits a highly salt resistant processing activity. (iii) Treatment of the complex with strong chaotropic agents (1 M guanidinium HCl) leads to cleavage of three subunits (including MPP), to aggregation of the remaining complex and to irreversible loss of processing activity.

In comparison to the soluble processing peptidases from fungi and mammals the cytochrome *c* reductase integrated enzyme from potato has several unique features. Its activity is highly salt resistant and even enhanced at unphysiological levels of NaCl (900 mM) while the purified processing enzymes from *Neurospora* and rat mitochondria are inactivated by 150 mM salt (Hawlitschek *et al.*, 1988; Ou *et al.*, 1989). In view of these results the suggestion of Ou *et al.* (1989) that elevated levels of salt may disturb the interaction between the enzyme and the substrate rather than that between MPP and PEP is

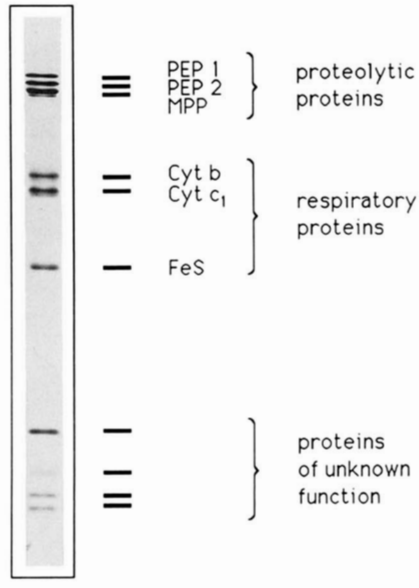

FIG. 5. **Illustration of the cytochrome *c* reductase-processing peptidase complex from potato.** Three arguments suggest that the 10 subunits are structurally tightly associated and functionally dependent on the supramolecular structure of one single complex (see "Discussion").

[3] F. Kalousek, personal communication.

[4] H. P. Braun, V. Kruft, and U. K. Schmitz, manuscript in preparation.

not very likely. The secondary and tertiary structure of presequences which has been proposed to be important for the recognition of cleavage sites (Verner and Schatz, 1988) seems to be maintained even at concentrations above 1 M salt.

The high salt resistance as well as the independence of the purified potato enzyme from externally added metal ions are probably a result of the integration of the processing enzyme into a respiratory complex. The heterodimeric processing peptidases from yeast and rat and the polypeptides of the protease from *Neurospora* which can only be obtained as monomers obviously lose most of their endogenous metal ion cofactors during purification. For this reason it is not clear which metal ions are bound to the native processing peptidases *in vivo* (Yang *et al.*, 1991).

What are the consequences of the integration of the processing peptidase from potato into cytochrome *c* reductase? Possibly protein import must occur close to complex III of the respiratory chain as the removal of presequences from imported precursors depends on the action of this bifunctional complex. This may lead to the problem of harmonizing spatial requirements of the respiratory chain and the import machinery. On the other hand there is growing evidence that the outer and inner mitochondrial membranes contain their own translocation machineries which can act independently (Glick *et al.*, 1991; Hwang *et al.*, 1991; Rassow and Pfanner, 1991) and are supposed to be laterally mobile (Pfanner *et al.*, 1992). For the membrane-bound processing protease it may be even easier to stay in contact with the protein import apparatus than for a soluble protease. At present we cannot exclude that an association between the protease and cytochrome *c* reductase is a more general phenomenon. The evolutionary relationship between components of the mitochondrial processing enzyme and the core proteins of cytochrome *c* reductase may be interpreted in support of this hypothesis. The identity of the core I protein and PEP may be more common than the situation in yeast where two distinct proteins evolved to function as core I protein and PEP. Yeast might be an exceptional case as it has the ability to repress the biogenesis of respiratory complexes under anaerobic conditions while maintaining mitochondrial protein import at the same time. The functional and evolutionary implications of the relation between the processing peptidase and cytochrome *c* reductase may be elucidated by further investigations on the physiology of the bifunctional potato complex and by purification of both enzymatic activities from other organisms.

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