THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 21, Issue of May 22, pp. 13143–13149, 1998
Printed in U.S.A.

New Insights into the Co-evolution of Cytochrome *c* Reductase and the Mitochondrial Processing Peptidase*

(Received for publication, December 8, 1997)

Stefanie Brumme‡, Volker Kruft§, Udo K. Schmitz‡, and Hans-Peter Braun‡¶

From the ‡Institut für Angewandte Genetik, Universität Hannover, Herrenhäuser Strasse 2, 30419 Hannover and §Applied Biosystems GmbH, Brunnenweg 13, 64331 Weiterstadt, Germany

The mitochondrial processing peptidase (MPP) is a heterodimeric enzyme that forms part of the cytochrome *c* reductase complex from higher plants. Mitochondria from mammals and yeast contain two homologous enzymes: (i) an active MPP within the mitochondrial matrix and (ii) an inactive MPP within the cytochrome *c* reductase complex. To elucidate the evolution of MPP, the cytochrome *c* reductase complexes from lower plants were isolated and tested for processing activity. Mitochondria were prepared from the staghorn fern *Platycerium bifurcatum*, from the horsetail *Equisetum arvense*, and from the colorless algae *Polytomella*, and cytochrome *c* reductase complexes were purified by a micro-isolation procedure based on Blue-native polyacrylamide gel electrophoresis and electroelution. This is the first report on the subunit composition of a respiratory enzyme complex from a fern or a horsetail. The cytochrome *c* reductase complexes from *P. bifurcatum* and *E. arvense* are shown to efficiently process mitochondrial precursor proteins, whereas the enzyme complex from *Polytomella* lacks proteolytic activity. An evolutionary model is suggested that assumes a correlation between the presence of an active MPP within the cytochrome *c* reductase complex and the occurrence of chloroplasts.

The mitochondrial cytochrome *c* reductase (also called the bc_1 complex or complex III) is a multisubunit enzyme of the respiratory chain that catalyzes reduction of cytochrome *c* by oxidation of ubiquinol. Coupled to this reaction, it translocates protons from the mitochondrial matrix to the space between the two mitochondrial membranes and thereby contributes to the chemiosmotic gradient across the inner mitochondrial membrane. The function of cytochrome *c* reductase is based on three subunits, which are directly involved in electron transport: cytochromes *b* and c_1 and the "Rieske" iron-sulfur protein (reviewed in Refs. 1 and 2). In several bacteria, the bc_1 complex only contains these three so-called "respiratory" subunits. However, all mitochondrial cytochrome *c* reductase complexes characterized so far comprise six to eight additional subunits, the functions of which are not entirely understood. Two of them are large and have molecular masses around 50 kDa, whereas four to six are small (<15 kDa). The primary structures of the large subunits, which are traditionally called the "core I" and "core II" proteins, are known for man, beef, yeast, potato, and *Euglena* (reviewed in Ref. 3). Both core proteins are located on the matrix exposed side of cytochrome *c* reductase, as revealed by electron microscopy of membrane crystals from *Neurospora crassa* (4) and more recently by x-ray analysis of cytochrome *c* reductase crystals from beef (5). Mutational analysis of the two core proteins from yeast revealed involvement of both subunits in assembly of the multisubunit complex (6–8).

The two core subunits exhibit some sequence similarity. Furthermore their primary structures resemble the sequences of the two subunits of the mitochondrial processing peptidase (MPP[1]; α- and β-subunits), which is a heterodimeric matrix-localized enzyme that removes presequences of nuclear encoded mitochondrial proteins upon their transport into the organelle (reviewed in Refs. 9–11). In fact, in potato, the two core subunits of cytochrome *c* reductase even represent the subunits of MPP and the cytochrome *c* reductase complex from this plant is a highly active processing peptidase (12). The same results have been shown for cytochrome *c* reductase from spinach and wheat (13, 14). An intermediate situation was found in *N. crassa*; the core I protein has β-MPP activity, whereas the α-subunit of MPP is localized in the mitochondrial matrix (15, 16). These molecular data can be explained by an evolutionary model, which was suggested recently (3, 17). (i) Early in evolution, MPP developed starting from a preexisting bacterial protease and became part of the cytochrome *c* reductase complex; (ii) later in evolution, the two subunits of MPP became detached from the enzyme complex to allow independent regulation of protein processing and respiration in some organisms; (iii) the detachment was realized by gene duplications, because due to the co-evolution of cytochrome *c* reductase and MPP, the two subunits of MPP became indispensable for assembly of this respiratory protein complex. From the perspective of this model, the core subunits are relics of an ancient processing peptidase and the bifunctional cytochrome *c* reductase complex in plants represents a situation that was originally present in the mitochondria from all organisms.

To test this model on the co-evolution of the cytochrome *c* reductase complex and the mitochondrial processing peptidase, we decided to analyze the processing activity of cytochrome *c* reductase from lower plants. A micro-isolation procedure is employed for the purification of cytochrome *c* reductase from the staghorn fern *Platycerium bifurcatum*, from the horsetail *Equisetum arvense*, and from the alga *Polytomella*, which is based on organelle preparations, Blue-native-polyacrylamide gel electrophoresis (BN-PAGE), and electroelution. We present data on the subunit composition and the function of the purified cytochrome *c* reductase complexes. Interestingly, the en-

* This work was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 49-511-7622674; Fax: 49-511-7623608; E-mail: braun@mpimg-berlin-dahlem.mpg.de.

[1] The abbreviations used are: MPP, mitochondrial processing peptidase; BN, Blue-native; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-tris(hydroxymethyl)methylglycine; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

zyme complexes from *P. bifurcatum* and *E. arvense* exhibit highly specific processing activity, whereas cytochrome *c* reductase from *Polytomella* is proteolytically inactive. Evolutionary implications of these findings are discussed.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria from Solanum tuberosum, Polytomella spp., P. bifurcatum, and E. arvense

S. tuberosum (Potato)—Mitochondria from potato tubers were isolated as described in Ref. 18. The organelles were suspended in 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH_2PO_4, pH 7.2, at a concentration of 10 mg of mitochondrial protein/ml. For the purification of the bc_1 complex by BN-PAGE, 100 μl of purified mitochondria were used per lane.

Polytomella spp.—Polytomella ssp. (198.80, E. G. Pringsheim) was obtained from the "SAG-Sammlung von Algenkulturen" at the University of Göttingen (Göttingen, Germany) and was grown in 2.5-liter culture flasks without shaking in the dark using the medium described by Schlösser (19) at 25 °C. Mitochondria were isolated basically as described in Ref. 20. The resulting crude mitochondria were washed with 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH_2PO_4, pH 7.2, and purified by Percoll step gradient centrifugation (14%, 22%, 45% Percoll in 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH_2PO_4, pH 7.2) at 70,000 $\times$ *g* for 45 min. Pure mitochondrial fraction was washed twice; resuspended in 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH_2PO_4, pH 7.2, at a concentration of 2 mg of mitochondrial protein/ml; and divided into aliquots of 200 μl.

P. bifurcatum (Staghorn Fern)—Plants were obtained from the greenhouse of the "Berggarten" at the University of Hannover. The young round, barren leaves ("mantle" leaves) were separated from the plants and cut into small pieces. All subsequent steps of mitochondria isolation were carried out at 4 °C. Pieces of leaves were homogenized three times in two volumes of 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 15.0 mM β-mercaptoethanol, 0.05 mM PMSF, 25 mM MOPS, pH 7.8, for 3 s using a Waring blender. The homogenate was filtered through two layers of muslin. The resulting suspension was centrifuged at 3000 $\times$ *g* for 5 min. To obtain the mitochondrial fraction, the supernatant was centrifuged at 18,000 $\times$ *g* for 30 min. The mitochondrial fraction was resuspended in 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH_2PO_4, pH 7.2; homogenized in a Dounce homogenizer; and purified by Percoll step gradient centrifugation (14%, 26%, 45% Percoll in 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH_2PO_4, pH 7.2, at 70,000 $\times$ *g* for 45 min). Mitochondria formed two light brown bands in the 26% phase. They were washed twice in resuspension buffer, solved at a concentration of 1 mg of mitochondrial protein/ml, and divided into aliquots of 600 μl.

E. arvense (Horsetail)—Wild plants were used. Mitochondria were isolated from the etiolated part of the stems from vegetative shoots, which are located near to the surface of the soil. All steps were carried out at 4 °C. The brown, silicified epidermis was peeled off. Stems were homogenized by grinding in a mortar in 10 volumes of 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 15.0 mM β-mercaptoethanol, 0.05 mM PMSF, 25 mM MOPS, pH 7.8. Subsequent steps of differential centrifugation and Percoll step gradient centrifugation were carried out as described above for isolating mitochondria from *P. bifurcatum*. Mitochondria formed a light, reddish brown band in the 26% phase of the gradient. The mitochondria were removed, washed twice, and finally solved in 0.4 M mannitol, 0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH_2PO_4, pH 7.2, at a concentration of ~1.5 mg of mitochondrial protein/ml. Aliquots of 300 μl were used as starting material of sample preparation for BN-PAGE.

Purification of Mitochondrial Protein Complexes by BN-PAGE

Blue-native-polyacrylamide gel electrophoresis and sample preparation for BN-PAGE was carried out as described in Ref. 21. Starting points for sample preparation were the obtained aliquots of mitochondria from *S. tuberosum*, *Polytomella* spp., *P. bifurcatum*, and *E. arvense*. The mitochondrial protein complexes from each organism were resolved on a separate gel consisting of a stacking gel (4% acrylamide) comprising 10 slots and a separating gel (4.95–12.6% acrylamide), respectively.

Electroelution of Cytochrome c Reductase Complexes

The blue protein bands representing cytochrome *c* reductase complexes were cut out after BN-PAGE and electroeluted as described in Ref. 22. Electroelution was performed with the electroelutor/concentrator ECU-040 (CBS Scientific Co., Del Mar, CA) overnight at 150 V and 4 °C with electrode buffer (25 mM Tricine, 7.5 mM Bis-Tris, 0.1 mM PMSF, pH 7.0).

Tricine-SDS-PAGE for Second Gel Dimension

Entire lanes from BN-PAGE with separated native-mitochondrial protein complexes were used for the resolution of subunits and identification of complexes by Tricine-SDS-PAGE as described in Ref. 21.

Analysis of Purified Protein Complexes by SDS-PAGE and Immunoblotting

Tricine-SDS-PAGE was carried out in the PROTEAN II cell from Bio-Rad (gel dimensions: 20 $\times$ 16 $\times$ 0.1 cm) according to the protocol published by Schägger and von Jagow (23). Approximately 1 μg of electroeluted enzyme complex was mixed with equal volumes of 2$\times$ loading buffer (10% SDS, 30% glycerol, 100 mM Tris, 4% β-mercaptoethanol, 0.006% bromphenol blue) and loaded into the slots. The electrophoresis was carried out at 20 °C; it was started at 30 V for 45 min and continued at 30 mA for 16 h. Proteins were either silver-stained or blotted onto nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) and incubated overnight with 1000-fold diluted antiserum. Visualization of immunopositive proteins was performed as described in Ref. 24.

Processing Assays

In vitro processing of radiolabeled precursor proteins was carried out for 1 h at 28 °C in a final volume of 120 μl, including 88 μl of processing buffer (22 mM Tris-HCl, 25 mM NaCl, 0.6% Triton X-100, 1 mM PMSF, 300 μM $ZnCl_2$), 1–4 μl of radiolabeled precursor protein, and about 2 μg of electroeluted cytochrome *c* reductase. The processing reaction was stopped with an equal volume of 2-fold concentrated loading buffer (10% SDS, 30% glycerol, 100 mM Tris-HCl, 4% β-mercaptoethanol, 0.006% bromphenol blue). Inhibition of processing activity was carried out by addition of 3 mM EDTA. Processing products were separated by SDS-PAGE as described by Laemmli (25). The gel was fixed with 50% acetic acid, incubated with Amplify (Amersham, Little Chalfont, United Kingdom) and exposed to X-OMAT™ films (Eastman Kodak Corp.).

RESULTS

Isolation of Cytochrome c Reductase Complexes from S. tuberosum, Polytomella spp., P. bifurcatum, and E. arvense—The isolation of highly pure mitochondria from plants devoid of chloroplast contaminations is facilitated by the use of etiolated starting material like tubers or dark grown seedlings. Hence, most biochemical studies on plant mitochondria were carried out with organelles from potato tuber or etiolated seedlings from different higher plants. However, lower plants lack suitable etiolated tissues for the preparation of mitochondria. The starting materials for our investigation were mantle leaves from the staghorn fern *P. bifurcatum*, which have a reduced content of chloroplasts, and subterranean parts of stems from the horsetail *E. arvense*. Furthermore, the colorless algae *Polytomella* spp. was used for isolating mitochondria, which is related to *Chlamydomonas* but lacks chloroplasts. Mitochondria from potato tuber were prepared as control organelles. Procedures for the isolation of mitochondria from potato and *Polytomella* were adapted from preexisting protocols (18, 20), whereas the method for the purification of fern and horsetail mitochondria was newly established (see "Experimental Procedures"). Owing to limited starting material, the overall amount of organelles was low for the fern and the horsetail. Therefore, a micro-isolation procedure was used for the purification of respiratory enzyme complexes, which is based on BN-PAGE and electroelution. BN-PAGE is a powerful tool for the separation of membrane-bound protein complexes (26); proteins are solubilized under mild conditions using non-ionic detergents, and Coomassie dyes are employed to introduce charge shifts on the polypeptides prior to electrophoresis. A two-dimensional resolution of mitochondrial protein complexes from *P. bifurcatum*, *E. arvense*, and *Polytomella* spp. is shown in Fig. 1.

Subunit Compositions of the Purified Cytochrome c Reductase Complexes—The cytochrome *c* reductase complexes were

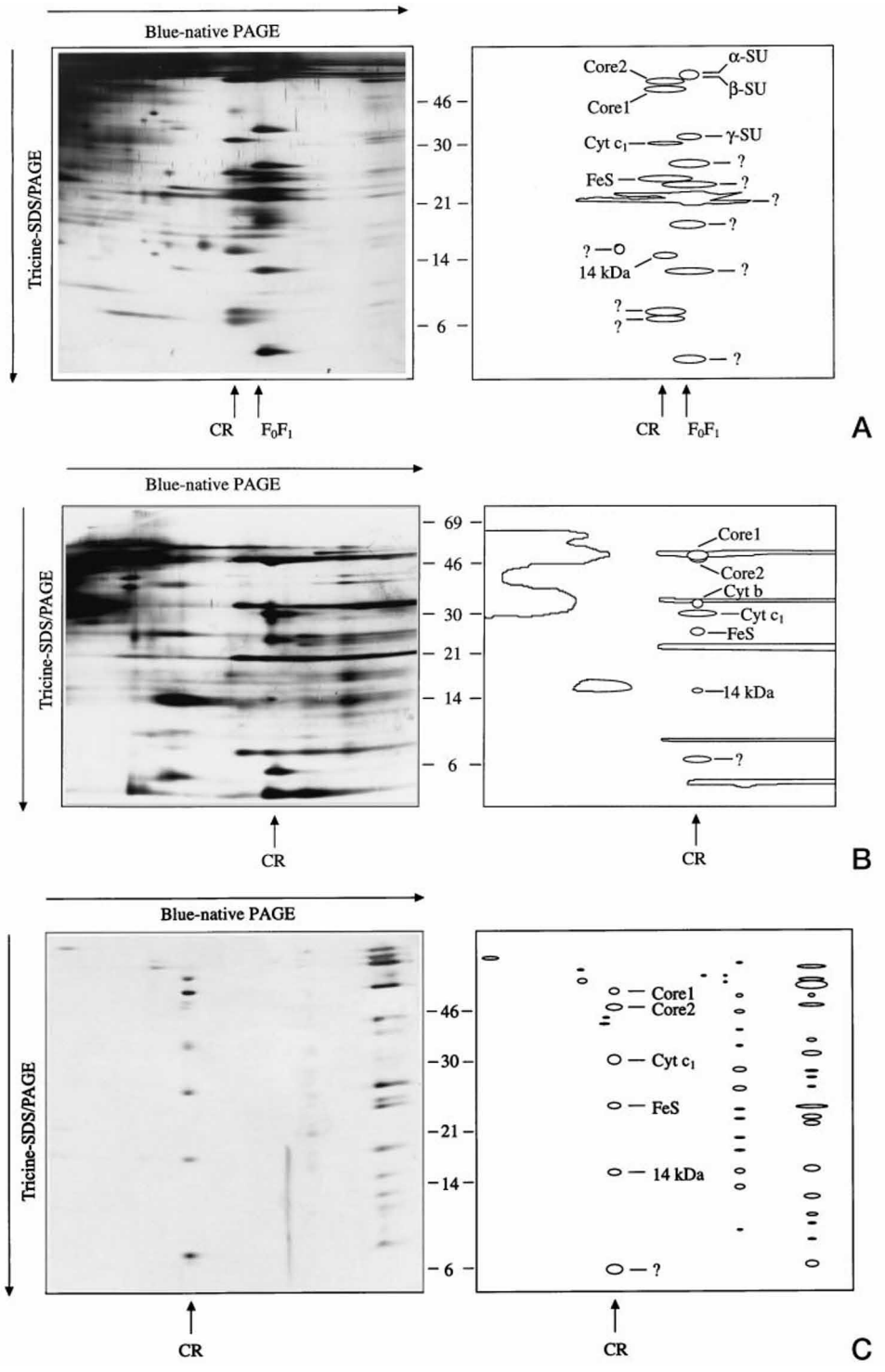

FIG. 1. **Two-dimensional resolution of the mitochondrial protein complexes from *P. bifurcatum* (*A*), *E. arvense* (*B*), and *Polytomella* spp. (*C*) by Blue-native-polyacrylamide gel electrophoresis and Tricine-SDS-PAGE.** The organelles from all three organisms were isolated as described under "Experimental Procedures," and the protein complexes were solubilized by 1.5% *n*-dodecylmaltoside. The gels were silver-stained. Schemes of the gels are given on the *right*, and the molecular sizes of standard proteins are given in the *middle* (in kDa). *CR*, cytochrome *c* reductase; *Cyt*, cytochrome; *FeS*, iron-sulfur protein; *SU*, subunit; F_0F_1, F_0F_1-ATP synthase.

identified by their subunit compositions and by immunostaining (see below). Protein bands corresponding to the native cytochrome *c* reductase complexes from potato, *P. bifurcatum*, *E. arvense*, and *Polytomella* spp. were cut out from one-dimensional Blue-native gels and the enzyme complexes were electroeluted. Subsequently, the obtained fractions were analyzed by Tricine-SDS-PAGE and silver staining. Fig. 2 shows the subunits of cytochrome *c* reductase complexes from all four organisms after the electroelution step. The resolved cytochrome *c* reductase complexes were blotted onto membrane filters and analyzed by immunostaining with antibodies directed against the core I protein and the iron-sulfur protein (Fig. 3). Some additional further protein bands were identified by direct protein sequencing (Table I). The combined data from Figs. 1–3 and Table I allow us to draw the following conclusions on the subunit compositions of the purified protein complexes.

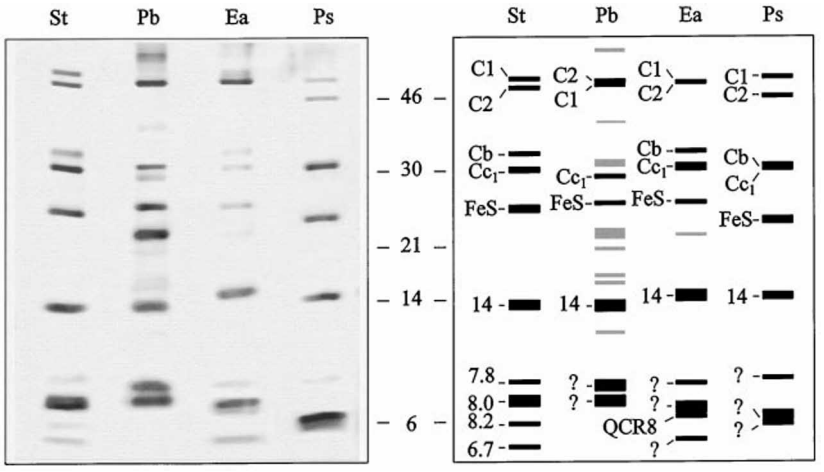

FIG. 2. **Tricine-SDS-PAGE of the cytochrome *c* reductase complexes from *S. tuberosum* (*St*), *P. bifurcatum* (*Pb*), *E. arvense* (*Ea*), and *Polytomella* spp. (*Ps*).** All protein complexes were isolated by Blue-native-polyacrylamide gel electrophoresis and electroelution as described under "Experimental Procedures." The gel was silver-stained. A scheme of the gel is given on the *right*, and the sizes of standard proteins are given in the *middle* (in kDa). *C1*, core I protein; *C2*, core II protein; *Cb*, cytochrome *b*; Cc_1, cytochrome c_1; *FeS*, iron-sulfur protein; *QCR8*, protein homologous to the yeast qcr8 gene product. The *numbers* indicate small subunits according to their calculated molecular mass in kDa; ? indicates subunits that could not be unambiguously identified. Bands shown in *gray* in the scheme are contaminations. The 8-kDa subunit of cytochrome *c* reductase from potato appears to be a doublet on this particular gel for unknown reasons.

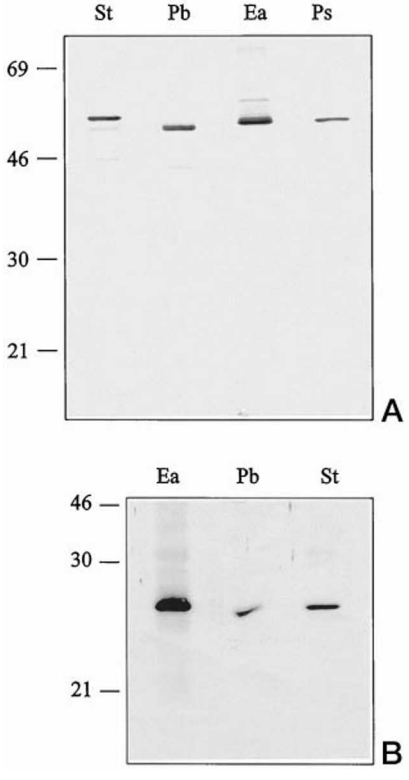

FIG. 3. **Identification of the core I and the iron-sulfur subunit by immunoblotting.** The cytochrome *c* reductase complexes from *S. tuberosum* (*St*), *P. bifurcatum* (*Pb*), *E. arvense* (*Ea*), and *Polytomella* spp. (*Ps*) were resolved by Tricine-SDS-PAGE as shown in Fig. 2 and subsequently blotted onto nitrocellulose membranes. *A*, incubation of a blot with an antiserum directed against the core I protein from *N. crassa*; *B*, incubation of a blot with an antiserum directed against the iron-sulfur protein from *N. crassa*. Molecular masses of standard proteins are given on the *left* of the blots (in kDa).

TABLE I
N-terminal sequences of subunits of the cytochrome c reductase complexes from P. bifurcatum and E. arvense

Amino acids are given in the one-letter-code; *X* stands for amino acids that could not be unambiguously identified.

Protein	Organism	N-terminal sequence
Cytochrome c_1	*P. bifurcatum*	DEAEEXLXA . . .
Cytochrome c_1	*E. arvense*	DEAEHSLAA . . .
FeS protein	*P. bifurcatum*	DTEVALQPSDDPTVA . . .
FeS protein	*E. arvense*	STNAVTQSLDXAV . . .
QCR8 protein	*E. arvense*	GKVAVRLKEVVYTLSPHQQN . . .

(i) Cytochrome *c* reductase from potato, which is a well characterized protein complex (2) and which was analyzed as a control enzyme complex, comprises 10 subunits (Fig. 2): the core I protein (apparent molecular mass: 56 kDa), the core II protein (52 kDa), cytochrome *b* (35 kDa), cytochrome c_1 (31 kDa), the FeS protein (24 kDa), a "14-kDa" subunit, and four subunits below 10 kDa.

(ii) The cytochrome *c* reductase from *P. bifurcatum* is contaminated by some subunits of the F_0F_1-ATP synthase complex (both enzyme complexes have a very similar size on the native gel dimension, see Fig. 1). The core I protein (apparent molecular mass: 53 kDa) was identified by immunoblotting (Fig. 3*A*, *lane 2*). The core II protein is assumed to migrate just above (Fig. 2, *lane 2*), as (*a*) the band at 53 kDa appeared to be a doublet on several gels and (*b*) the upper part of the band has a brown color on silver-stained gels typical for core II proteins, whereas the lower part of the band has a red-brown color typical for core I proteins. The N-terminal sequence of the 30-kDa band (Table I) exhibits significant sequence identity to the mature N terminus of cytochrome c_1 from potato. The band at 26 kDa strongly cross-reacts with an antiserum directed against the iron-sulfur protein from *N. crassa* (Fig. 3*B*). The N-terminal sequence of the 26-kDa protein was determined by direct protein sequencing but only exhibits low sequence identity to iron-sulfur proteins from other species. However, this result is not unexpected due to the low degree of sequence conservation at the N terminus of iron-sulfur subunits from different organisms. At least three small proteins (apparent molecular masses 14 kDa, two proteins <10 kDa) form part of the cytochrome *c* reductase complex from *P. bifurcatum* (Fig. 2, *lane 2*). For unknown reasons, the cytochrome *b* subunit of *P. bifurcatum* could not be detected on the gel in Fig. 2. However, it is highly unlikely that cytochrome *b* is absent, as it forms the core of the cytochrome *c* reductase complexes and as the apparent molecular mass of the dimeric protein complex from *P. bifurcatum* lies at 500 kDa on native gels (data not shown) in accordance with the molecular mass of cytochrome *c* reductase

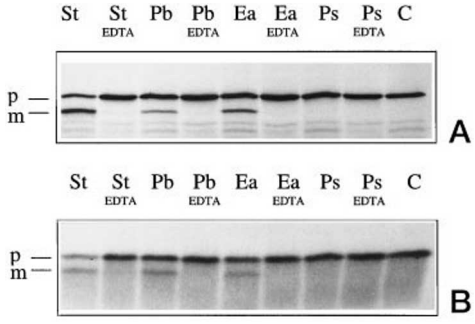

FIG. 4. **Processing activity of isolated cytochrome *c* reductase from *S. tuberosum* (*St*), *P. bifurcatum* (*Pb*), *E. arvense* (*Ea*), and *Polytomella* spp. (*Ps*).** The precursor of the β-subunit of the F_1-ATP synthase from tobacco (*A*) and the precursor of the iron-sulfur protein from potato (*B*) were synthesized *in vitro* in the presence of [^{35}S]methionine and treated with isolated cytochrome *c* reductase in the presence or absence of EDTA as indicated. The radiolabeled proteins were subsequently resolved by SDS-PAGE and visualized by fluorography. *C*, control; *p*, precursor protein; *m*, mature protein.

complexes from other organisms (2).

(iii) The cytochrome *c* reductase complex from the horsetail *E. arvense* could be obtained in highly pure form (Fig. 2) and comprises 10 subunits: the core I and core II proteins (apparent molecular masses: 53 kDa), cytochrome *b* (36 kDa), cytochrome c_1 (32 kDa), the FeS protein (26 kDa), the 14-kDa protein (which runs at 15 kDa), and four proteins below 10 kDa. The core I protein was identified by immunoblotting (Fig. 3*A*), cytochrome c_1 by direct protein sequencing (Table I), and the FeS protein by protein sequencing and immunoblotting (Table I, Fig. 3*B*). The core II protein most likely has a molecular mass identical to the core I protein, as the 53-kDa band seemed to be a doublet on several gels (data not shown). The first 20 amino acids of the second smallest subunit of cytochrome *c* reductase from *E. arvense* could be determined by cyclic Edman degradation. The protein exhibits 65% sequence identity to the potato 8.2-kDa subunit of the cytochrome *c* reductase complex, which is homologous to the yeast qcr8 gene product.

(iv) Likewise, the cytochrome *c* reductase complex from *Polytomella* spp. was isolated in very pure form (Fig. 2) and could be resolved into nine different subunits: core I protein (53 kDa), core II protein (49 kDa), cytochromes *b* and c_1 at 32 kDa, FeS protein (24 kDa), 14-kDa protein, and three or more subunits below 12 kDa. Cytochrome *c* reductase from this organism was purified before by González-Halphen and co-workers (20), and similar values were reported for the molecular weights of the subunits. The identities of the subunits were determined by direct protein sequencing and immunoblotting (20). However, González-Halphen and co-workers were able to resolve one more small subunit below 12 kDa.

Processing Activities of the Purified Cytochrome c Reductase Complexes—To investigate whether the purified cytochrome *c* reductase complexes include the activity of the mitochondrial processing peptidase, an *in vitro* processing assay was carried out using two radiolabeled mitochondrial precursor proteins: the β subunit of the ATP synthase complex and the FeS protein of cytochrome *c* reductase (Fig. 4). Both precursor proteins are converted into their mature form when incubated with cytochrome *c* reductase complex from potato, which was shown previously to be an efficient processing peptidase (12). Potato MPP is a metallopeptidase, and the processing reaction can be inhibited by the metal chelator EDTA (Fig. 4, *lanes 2*). Identical results were obtained with the isolated cytochrome *c* reductase complexes from *P. bifurcatum* and *E. arvense*. Both enzyme complexes efficiently process the tested precursor proteins into their mature forms in the absence of EDTA (Fig. 4, *lanes 3–6*). In contrast, cytochrome *c* reductase from *Polytomella* spp. did not exhibit detectable processing activity. Hence, the cytochrome *c* reductase complexes from potato, *P. bifurcatum*, and *E. arvense* do contain both subunits of MPP, whereas *Polytomella* spp. lacks at least one of the MPP proteins.

DISCUSSION

Blue-native-polyacrylamide gel electrophoresis has been shown here to be a suitable method for the isolation of mitochondrial protein complexes from *P. bifurcatum*, *E. arvense* and *Polytomella*. The procedure is especially valuable if the starting material for organelle preparations is limited.

To our knowledge, this is the first report on a biochemical preparation of a mitochondrial enzyme from a fern or a horsetail. The cytochrome *c* reductase complexes from *E. arvense* and *Polytomella* could be obtained in highly pure form, whereas the cytochrome *c* reductase complex from the fern *P. bifurcatum* was contaminated by subunits of the ATP synthase complex.

Cytochrome *c* reductase complexes from three very different eukaryotes (beef, yeast, and potato) have been studied extensively (2, 27, 28). All three enzyme complexes comprise 10 subunits: 3 respiratory proteins, 2 large core proteins, and 5 small polypeptides. In beef, the presequence of the Rieske iron-sulfur protein is retained in the cytochrome *c* reductase complex after proteolytic cleavage of the precursor protein and is considered to be an eleventh subunit (29, 30). Additionally, the cytochrome *c* reductase complex from *E. arvense* comprises 10 subunits, which have molecular weights very similar to the one reported for potato. The same number of subunits was reported for the cytochrome *c* reductase complex from *Polytomella* (20). Hence, the structure of the mitochondrial cytochrome *c* reductase complex, which differs remarkably from the structure of the corresponding prokaryotic protein complex, seems to be highly conserved in very different organisms and most likely evolved early in the development of the eukaryotic cell.

The electron transfer activities of the cytochrome *c* reductase complexes from potato, *P. bifurcatum*, *E. arvense*, and *Polytomella* prepared by Blue-native-polyacrylamide gel electrophoresis and electroelution were not measured, but there are several indications that the enzyme complexes are intact and most likely active. (i) The iron-sulfur protein, which easily dissociates from cytochrome *c* reductase complexes causing low enzymatic activity, forms part of all four cytochrome *c* reductase complexes as monitored by direct protein sequencing and immunoblotting; (ii) the apparent molecular masses of the cytochrome *c* reductase complexes from potato, *P. bifurcatum*, and *E. arvense* complexes during native gel electrophoresis was in the range of 500 kDa (data not shown), which is similar to the values reported for the intact dimeric cytochrome *c* reductase complexes from yeast and beef (2). Only the enzyme complex from *Polytomella* had a smaller size, which is in line with previous reports (20).

The activity of the mitochondrial processing peptidase was shown to reside within the mitochondrial matrix in yeast, *Neurospora*, and rat (31–33). *In vitro* processing assays with the isolated cytochrome *c* reductase complexes from yeast, *Neurospora*, and beef revealed no detectable processing activity of this respiratory enzyme complex (14). In contrast, the MPP activity forms part of cytochrome *c* reductase in all plants investigated so far: potato, spinach, and wheat (12–14). These experimental findings are in line with the sequence data presently available for the large subunits of the cytochrome *c* reductase complex, the so-called core subunits (Fig. 5). The core I proteins from man, beef, and yeast exhibit some sequence similarity to β-MPP subunits from different organisms, but they have an incomplete inverse zinc-binding motif, which was

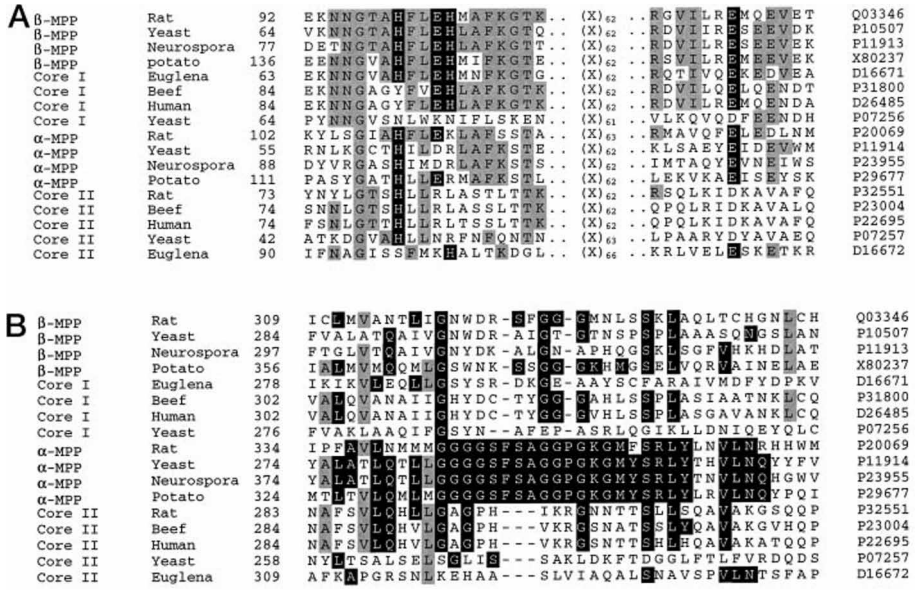

FIG. 5. **Alignment of two sequence stretches of MPP and core proteins from different organisms.** *A*, alignment of the zinc-binding region. Residues directly involved in metal binding are in *black boxes*; other residues conserved in at least six organisms are in *gray boxes*. *B*, alignment of the α-MPP domain. Residues that are identical in at least three of the four α-MPP proteins are in *black boxes* (α-MPP domain); other residues conserved in at least six organisms are in *gray boxes*. The *numbers* on the *left* of the sequences indicate the position of the first amino acid of the sequence stretches as deduced from the open reading frame of the corresponding nucleotide sequences. Accession numbers of the proteins are given on the *right* of the sequences.

shown to be essential for the proteolytic activity of MPP (34, 35) (Fig. 5*A*). Similarily, the sequences of the core II subunit from man, beef, and yeast resemble α-MPP sequences from various organisms, but lack a highly conserved stretch of uncharged amino acids, which is present in all α-MPP proteins sequenced so far and which is believed to be a prerequisite for the function of α-MPP in protein processing (Fig. 5*B*). In contrast, the core I protein from potato includes a complete inverse zinc-binding motif and the core II protein from this organism comprises the characteristic uncharged "α-MPP domain" (12, 36). The fungus *Neurospora* represents an intermediate situation; the core I protein has an inverse zinc-binding motif, but the core II protein lacks the α-MPP domain.[2] Hence, the cytochrome *c* reductase complex from *Neurospora* does not exhibit processing activity (14, 15). Additionally, the cytochrome *c* reductase complex from *Euglena*, which was not tested for processing activity so far, seems to represent such an intermediate situation; a zinc-binding motif is present in the the core I protein but no α-MPP domain in the core II protein (Fig. 5, *A* and *B*).

Processing activity of cytochrome *c* reductase complexes from three other plants: the fern *P. bifurcatum*, the horsetail *E. arvense*, and the colorless alga *Polytomella*, was tested. In agreement with the data from other plants, cytochrome *c* reductase from the fern and the horsetail comprise processing activity, whereas the enzyme complex from the colorless alga *Polytomella* has no proteolytic activity. These data allow us to draw some new conclusions on the evolution of the MPP subunits and the core proteins. To date, experimental or structural data on the proteolytic activity of the large subunits of cytochrome *c* reductase complexes from 12 organisms are known: the vertebrata man, beef, and rat; the fungi yeast and *Neurospora*; the plants potato, spinach, wheat, *P. bifurcatum*, and *E. arvense*; and the nongreen algae *Polytomella* and *Euglena*. It is generally accepted that mitochondria arose from purple bacteria and have a monophyletic origin (37, 38). As MPP is a heterodimer in all organisms investigated so far, the early mitochondria most likely contained α- and β-MPP, which probably both formed part of cytochrome *c* reductase. This situation is still valid in all plants. In other organisms, one or two gene duplications occurred, which were the prerequisite for the development of a MPP enzyme in the soluble mitochondrial fraction: (i) a duplication of the α-MPP protein, which gave rise to the core II protein, and (ii) a duplication of the β-MPP protein, which gave rise to the core I protein. Both gene duplications occurred in mammals and yeast, one duplication in *Neurospora*, *Euglena*, and probably *Polytomella*. Considering the current knowledge about the phylogeny of eukaryotes, these duplications must have taken place several times independently. This is in line with very recent findings that duplication of entire genomes occurred in the evolution of several higher eukaryotes like vertebrata or yeast (39, 40). The question is: why did these genome duplications lead to the evolution of core subunits in some organisms but not in others? Interestingly, all organisms that comprise proteolytically inactive core subunits and a soluble matrix-localized MPP are non-green organisms, which generally or temporarily lack chloroplasts. Possibly, the presence of chloroplasts, a second organelle involved in the bioenergetics of the cell, alleviated the evolutionary pressure on plant mitochondria, so that a soluble MPP was dispensable. Plant mitochondria have unique functions in the metabolism of the green cell, which seem not to require an independent regulation of respiration and protein processing. Investigation of cytochrome *c* reductase from further organisms may give further insights into the co-evolution of this respiratory enzyme complex and the subunits of MPP.

Acknowledgments—The staghorn fern *P. bifurcatum* was kindly provided by the Berggarten of the Hannover University. We thank Elke Buchsteiner for helpful discussions.

REFERENCES

1. Trumpower, B. L. (1990) *Microbiol. Rev.* **54,** 101–129
2. Braun, H. P., and Schmitz, U. K. (1995) *J. Bioenerg. Biomembr.* **27,** 423–436
3. Braun, H. P., and Schmitz, U. K. (1995) *Trends Biochem. Sci.* **20,** 171–175
4. Leonard, K., Wingfield, P., Arad, T., and Weiss, H. (1981) *J. Mol. Biol.* **149,** 259–274
5. Xia, D., Yu, C. A., Kim, H., Xia, J. Z., Kachurin, L. Z., Yu, L., and Deisenhofer, J. (1997) *Science* **277,** 60–66
6. Oudshoorn, P., van Steeg, H., Swinkels, B. W., Schoppink, P., and Grivell, L. A. (1987) *Eur. J. Biochem.* **163,** 97–103

[2] S. Brumme, U. K. Schmitz, and H.-P. Braun, unpublished data.

7. Crivellone, M. D., Wu, M. A., and Tzagoloff, A. (1988) *J. Biol. Chem.* **263,** 14323–14333
8. Gatti, D. L., and Tzagoloff, A. (1990) *J. Biol. Chem.* **265,** 21468–21475
9. Brunner, M., Klaus, C., and Neupert, W. (1994) in *Signal Peptidases* (von Heijne, G., ed) pp. 73–86, Landes Co., Austin, TX
10. Luciano, P., and Geli, V. (1996) *Experientia* (*Basel*) **52,** 1077–1082
11. Braun, H. P., and Schmitz, U. K. (1997) *Int. J. Biochem. Cell Biol.* **29,** 1043–1045
12. Braun, H. P., Emmermann, M., Kruft, V., and Schmitz, U. K. (1992) *EMBO J.* **11,** 3219–3227
13. Eriksson, A. C., Sjöling, S., and Glaser. E (1994) *Biochim. Biophys. Acta* **1186,** 221–231
14. Braun, H. P., Emmermann, M., Kruft, V., Bödicker, M., and Schmitz, U. K. (1995) *Planta* **195,** 396–402
15. Schulte, U., Arretz, M., Schneider, H., Tropschug, M., Wachter, E., Neupert, W., and Weiss, H. (1989) *Nature* **339,** 147–149
16. Weiss, H., Leonard, K., and Neupert, W. (1990) *Trends Biochem. Sci.* **15,** 178–180
17. Braun, H. P., Jänsch, L., and Schmitz, U. K. (1997) in *Proteolysis and Protein Turnover* (Hopsu-Havu, V. K., Järvinen, M., and Kirschke, H., eds) pp. 290–297, IOS Press, Amsterdam
18. Braun, H. P., and Schmitz, U. K. (1995) *Biochim. Biophys. Acta* **1229,** 181–186
19. Schlösser, U. G. (1994) *SAG-Sammlung von Algenkulturen Catalogue of Strains 1994: BOACEJ*, Vol. 3, pp. 113–186, University of Göttingen, Göttingen, Germany
20. Gutiérrez-Cirlos, E. B., Antaramian, A., Vázquez-Acevedo, M., Coria, R., and González-Halphen, D. (1994) *J. Biol. Chem.* **269,** 9147–9154
21. Jänsch, L., Kruft, K., Schmitz, U. K., and Braun, H.-P. (1996) *Plant J.* **9,** 357–368
22. Schägger, H. (1995) *Methods Enzymol.* **260,** 190–201
23. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379
24. Braun, H. P., Emmermann, M., Kruft, V., and Schmitz, U. K. (1992) *Mol. Gen. Genet.* **231,** 217–225
25. Laemmli, U. K. (1970) *Nature* **227,** 680–685
26. Schägger, H., and von Jagow, G. (1991) *Anal. Biochem.* **199,** 223–231
27. Schägger, H., Link, T. A., Engel, W. D., and von Jagow, G. (1986) *Methods Enzymol.* **126,** 181–191
28. Brandt, U., Uribe, S., Schägger, H., and Trumpower, B. L. (1994) *J. Biol. Chem.* **269,** 12947–12953
29. Borchart, U., Machleidt, W., Schägger, H., Link, T. A., and von Jagow, G. (1985) *FEBS Lett.* **191,** 125–130
30. Brandt, U., Yu, L., Yu, C.-A., and Trumpower, B. L. (1993) *J. Biol. Chem.* **268,** 8387–8390
31. Yang, M., Jensen, R. E., Yaffe, M. P., Oppliger, W., and Schatz, G. (1988) *EMBO J.* **7,** 3857–3862
32. Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F. U., and Neupert, W. (1988) *Cell* **53,** 795–806
33. Ou, W. J., Ito, A., Okazaki, H., and Omura, T. (1989) *EMBO J.* **8,** 2605–2612
34. Kitada, S., Shimokata, K., Niidome, T., Ogishima, T., and Ito, A. (1995) *J. Biochem.* (*Tokyo*) **117,** 1148–1150
35. Striebel, H. M., Rysavy, P., Adamec, J., Spizek, J., and Kalousek, J. (1996) *Arch. Biochem. Biophys.* **335,** 211–218
36. Emmermann, M., Braun, H. P., Arretz, M., and Schmitz, U. K. (1993) *J. Biol. Chem.* **268,** 18936–18942
37. Gray, M. W. (1993) *Curr. Opin. Genet. Dev.* **3,** 884–890
38. Lang, B. F., Burger G., O'Kelly, C. J., Cedergren, R., Golding, G. B., Lemieux, C., Sankoff, D., Turmel, M., and Gray, M. W. (1997) *Nature* **387,** 493–497
39. Spring, J. (1997) *FEBS Lett.* **400,** 2–8
40. Wolfe, K. H., and Shields, D. C. (1997) *Nature* **387,** 708–713