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The ubiquinol cytochrome *c* oxidoreductase complex of spinach leaf mitochondria is involved in both respiration and protein processing

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Abstract

Nuclear encoded mitochondrial precursor proteins are cleaved to mature size products by the general mitochondrial processing peptidase (MPP). In contrast to non-plant tissues where MPP is located in the matrix, the general processing activity of potato tuber (storage tissue) mitochondria has been shown to constitute an integral part of the isolated cytochrome *c* reductase complex of the respiratory chain. Here we show isolation of MPP from photosynthetic tissue, spinach leaf mitochondria, starting from the total membrane processing extract using extraction with dodecyl-β-maltoside followed by FPLC anion-exchange and gel filtration chromatography. The total spinach leaf MPP is found in the fractions containing the cytochrome *c* reductase complex and is shown to be an integral part of the complex. No processing activity has been found in any other fractions. The isolated cytochrome *c* reductase complex is shown to process three precursor proteins of different intramitochondrial localisation, the $F_1\beta$ subunit of ATP synthase (extrinsic membrane protein on matrix side), the Rieske FeS protein (integral membrane protein facing intermembrane space) and the malate dehydrogenase (matrix protein). The processing activity is totally inhibited by EDTA and orthophenanthroline. Our results together with the results in potato mitochondria show that integration of MPP into the cytochrome *c* reductase is a general phenomenon for plants. The complex consists of ten protein bands on SDS-PAGE of 61, 54, 52, 34, 32, 26, 15, 12, 11 and 10 kDa. The 61, 54 and 52 kDa bands correspond to Core proteins, the 32 kDa band to cytochrome *b* and the 26 kDa band to Rieske FeS protein as estimated by immunological methods. The three Core proteins are shown to be immunologically related to MPP from other sources, the Core 1 protein corresponding to β-MPP and the Core 2 and Core 3 proteins corresponding to α-MPP, which in comparison to MPP in potato mitochondria indicates species-dependent differences in the appearence of the processing components. Furthermore, the processing activity of the isolated and membrane-bound spinach cytochrome *c* reductase complex is shown to be inhibited by antimycin A and myxothiazol, electron transfer inhibitors of the complex. The inhibition of processing is, however, not correlated to the inhibition of electron transfer through the complex or to the redox state of the complex.

Key words: Protein processing; Precursor protein; Mitochondrial processing peptidase; Protein import; Ubiquinol cytochrome *c* oxidoreductase complex

1. Introduction

Mitochondrial biogenesis requires cooperation between the mitochondrial and nuclear genetic systems. The mitochondrial DNA encodes for only a limited amount of mitochondrial proteins and therefore mitochondrial biogenesis requires a specific trafficking and import system for nuclear encoded proteins. The mitochondrial proteins encoded by the nucleus are synthesised in the cytosol as precursors. Most of these precursors contain amino-terminal presequences that are required for targeting the protein into the organelle.

Abbreviations: MPP, mitochondrial processing peptidase; IMP 1, inner membrane peptidase; cytochrome *c* reductase, ubiquinol cytochrome *c* oxidoreductase; MIP, mitochondrial intermediate peptidase; PMSF, phenylmethylsulphonyl fluoride; ATP synthase, F_0F_1-adenosine triphosphate synthase; Rieske FeS protein, *Neurospora crassa* non-heme iron sulphur protein of the cytochrome *c* reductase complex; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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During or after translocation into the mitochondria, the presequences are proteolytically cleaved off by a highly specific processing system. Removal of the presequences is not essential for import but is needed for assembly of the imported polypeptides into functional oligomeric protein complexes (for reviews see [1,2]).

Most mitochondrial precursor proteins are cleaved to their mature form in a single cut by the mitochondrial general processing peptidase (MPP), (for new nomenclature see [3]), which has been purified and characterised in *Neurospora crassa* [4], *Saccharomyces cerevisiae* [5] and rat liver [6]. MPP consists of two structurally related proteins that cooperate in processing, α-MPP and β-MPP. Both subunits are soluble proteins found in the mitochondrial matrix except for β-MPP from *N. crassa* which is to 70% attached to the inner face of the mitochondrial inner membrane. The processing activity is inhibited by EDTA and orthophenanthroline, indicating that MPP is dependent on metal ions for catalytic activity.

Some mitochondrial precursors are cleaved to mature form in two sequential steps involving a second matrix peptidase, mitochondrial intermediate peptidase (MIP) [7–9]. The precursors are initially processed to the intermediate form by MPP. Formation of mature form is then catalysed by MIP that removes eight amino acids (octapeptide) from the amino-terminus of the intermediate form [10]. The reason for this two-step cleavage has been suggested to be that the mature portion of twice-cleaved precursors are structurally incompatible with cleavage by MPP and that the octapeptides function to supply the structural requirements for cleavage [11–13]. Import of some proteins destined to the mitochondrial intermembrane space also involves two proteolytic steps. The matrix targeting signal is first removed by MPP, followed by cleavage of the intermembrane targeting signal by the inner membrane peptidase (IMP 1) which is located in the inner membrane [14]. IMP 1 from *S. cerevisiae* shows high sequence similarity to *Escherichia coli* leader peptidase [15].

In *N. crassa*, the processing enhancing subunit of MPP, β-MPP, is identical to the Core 1 subunit of the cytochrome *c* reductase complex of the respiratory chain. This indicates that the protein has a bifunctional role and is involved in both processing of precursor proteins and in electron transport [16]. In *S. cerevisiae* and rat liver, β-MPP shows sequence similarity but not identity to the corresponding Core 1 proteins. Based on sequence similarity studies, the two proteins of the general processing enzyme, α-MPP and β-MPP and the Core 1 and Core 2 proteins of the cytochrome *c* reductase complex have been grouped as a new family of proteins involved in both bioenergetics and biogenesis of the mitochondrion [16].

Our previous work has shown that in contrast to the results for fungi and mammals, the general processing peptidase of plant mitochondria in spinach leaf and potato tubers is a membrane-bound enzyme [17]. In spinach, the processing peptidase cannot be disassociated from the membrane by high pH, high ionic strength, chaotropic reagents or weak detergent treatment, indicating that the enzyme is an integral membrane protein. The processing activity of mitochondrial membranes is stimulated by divalent metal ions and inhibited by metal chelators, indicating that metal ions are needed for the catalytic function. No effect on processing was seen with PMSF, bestatin, leupeptin or pepstatin [17]. Most interestingly, when the work reported here was in progress, it was shown by Braun et al. [18] that the purified cytochrome *c* reductase complex from potato tuber contained processing activity and that the processing peptidase constitutes an integral part of the complex. Furthermore, the three high molecular mass subunits of the complex were sequenced [18,19] and shown to exhibit high degree of sequence similarity to subunits of MPP from other sources and to the Core proteins of the cytochrome *c* reductase complex. Partial purification of the enzyme from spinach leaf mitochondria showing that MPP co-fractionates with the cytochrome *c* reductase complex has been presented as a preliminary report [20].

In the work presented here, we describe a purification procedure of the general processing peptidase from spinach leaf mitochondria starting from whole-membrane processing extract. The total processing activity is shown to be located within the cytochrome *c* reductase complex and constitutes an integral part of the complex, thereby indicating that the localisation of MPP in the cytochrome *c* reductase complex is a general feature for plants. We show that the three Core proteins of the complex are immunologically related to the two subunits of MPP from other sources, indicating that these proteins are responsible for proteolytic processing. We also show that the processing activity is sensitive to antimycin A and myxothiazol, inhibitors of electron transfer in the cytochrome *c* reductase complex, but is not correlated to the electron transfer or to the redox state of the complex.

2. Materials and methods

2.1. Fractionation of spinach leaf mitochondria – purification of the cytochrome c reductase complex

Spinach (*Spinacia oleracea* L.) leaf mitochondria were isolated according to Hamasur et al. [21]. The mitochondria were diluted with 0.3 M sucrose/10 mM MOPS (pH 7.5) to a protein concentration of 5–8 mg/ml and disrupted in the presence of 30 mM $MgCl_2$ and 1 mM PMSF using a Branson Sonifier (equipped

with a microtip, setting 3) four times for 30 s at 4°C. Membranes were separated from matrix by centrifugation at 130000 × *g* for 45 min. Purification of the processing activity was achieved by dodecyl-β-maltoside extraction followed by anion-exchange and gel filtration chromatography according to the modified method by Peiffer et al. [22]. The isolated mitochondrial membranes were solubilised in 50 mM dodecyl-β-maltoside, 5% sucrose and 20 mM KH_2PO_4 (pH 7.5) at protein concentration of 15 mg/ml in a total volume of 2 ml, for 15 min on ice and 15 min during agitation at 4°C and centrifuged at 40000 × *g* for 20 min. The dodecyl-β-maltoside extract was filtrated through a 0.22 μm membrane and loaded onto a Pharmacia FPLC Mono Q 5/5 HR anion-exchange column equilibrated with 5% sucrose, 20 mM KH_2PO_4 (pH 7.5), 0.01% dodecyl-β-maltoside at a flow rate of 1 ml/min. A discontinuous KCl-gradient (0–1 M KCl in 5% sucrose, 20 mM KH_2PO_4 (pH 7.5), 0.01% dodecyl-β-maltoside (described in detail in the legend to Fig. 1A) was applied to the column. Fractionation procedure resulted in the separation of different protein complexes. Fractions were desalted by dialysis and tested for processing activity and immunological cross-reactivity. Fractions containing processing activity were pooled and concentrated using Centricon ultrafiltration tubes (cut-off 3000 Da). Further purification of the complex was achieved with gel filtration using Pharmacia FPLC Superose 6 HR 10/30 column equilibrated with 0.1 M KCl in 5% sucrose, 20 mM KH_2PO_4 (pH 7.5), 0.01% dodecyl-β-maltoside at a flow rate of 0.2 ml/min. The cytochrome *c* reductase complex was eluted after approximately 60 min. Active fractions were pooled and concentrated. All the chromatographic steps were performed at 4°C.

2.2. SDS-PAGE

The polypeptide content of eluted fractions from Mono Q anion-exchange column was evaluated by SDS-PAGE using 12–17% polyacrylamide gels in the presence of 4 M urea in the buffer system of Laemmli [23]. Protein was precipitated with 10% trichloroacetic acid (final concentration 5%), incubated for 5 min on ice and centrifuged for 20 min in an Eppendorf centrifuge at 13000 rpm at 4°C. The resulting pellets were solubilised in water and double-strength sample buffer 1:1 and sonicated for 15 min at 15°C. After electrophoresis protein bands were stained with Coomassie brilliant blue.

2.3. In vitro transcription and translation of mitochondrial precursor proteins

N. crassa Rieske FeS protein transcripts were produced with linearised pGem3 plasmid vector using Ambion MAXIscript™ in vitro transcription kit with SP6 RNA polymerase. Translation of mRNAs was carried out in the presence of [^{35}S]methionine in rabbit reticulocyte lysate [24]. *Nicotiana plumbaginifolia* $F_1\beta$ transcription and translation was carried out with unlinearised pTZ18U vector using Promega TNT™ coupled reticulocyte lysate systems with T7 RNA polymerase. *Citrullus vulgaris* malate dehydrogenase was transcribed and translated in accordance to [25] and [26].

2.4. Processing of precursor proteins

The reaction for processing contained 0.5 μl of [^{35}S]methionine-labelled translation product (approx. 10000–15000 cpm) and 0.5 μg (purified cytochrome *c* reductase) or 10 μg (membranes) protein in 0.5% Triton X-100, 15 mM Tris-HCl (pH 8.0), 2.5 mM $MnCl_2$ in a final volume of 20 μl. Triton X-100 was excluded when the effect of inhibitors of the respiratory chain on processing activity was investigated and the isolated cytochrome *c* reductase complex was incubated with the inhibitor for 5 min before translation product was added. Processing was carried out for 25 min at optimal temperature for each precursor, at 30°C for the Rieske FeS precursor and at 20°C for the precursors of $F_1\beta$ and malate dehydrogenase. The reaction was stopped by addition of double-strength sample buffer 1:1 [23] and solubilised by boiling for 2 min. The processing reaction was analysed on SDS-PAGE using 12% polyacrylamide gels in the presence of 4 M urea in the buffer system of Laemmli [23]. Gels were fixed, impregnated with Amplify™ (Amersham), and dried. Flourography was carried out at −70°C.

2.5. Oxidoreductase activity

NADH oxidase activity of spinach mitochondrial membranes was measured spectrophotometrically at 340 nm according to [27].

2.6. Immunological characterisation

Proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes, Hybond-C Super (Pharmacia) using Western blot technique and immunological characterisation was performed by immunodecoration of Western blots [28]. Immunological cross-reactivity was detected by horseradish peroxidase-labelled secondary antibodies and enhanced chemiluminescence [29,30].

2.7. Protein determination

Protein was determined with Bio-Rad protein assay reagent according to the method of Bradford [31]. Bovine immunoglobulin was used as a standard.

A

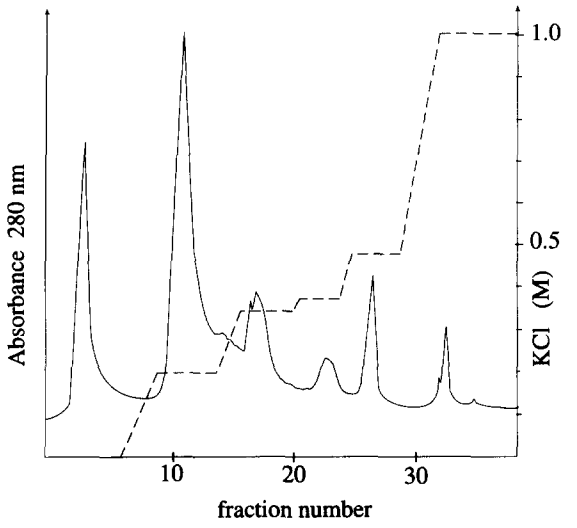
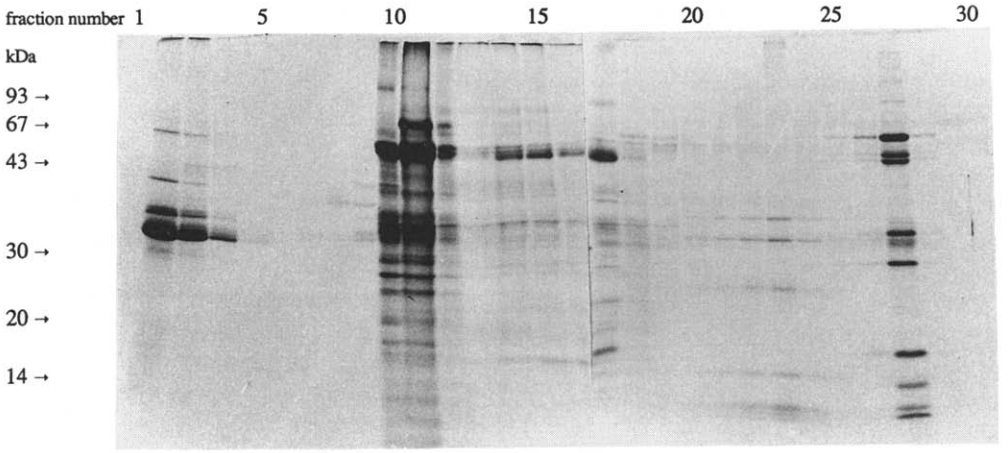


Fig. 1. (A) Elution profile from FPLC Mono Q 5/5 HR anion-exchange column. Spinach mitochondrial membranes were solubilised in 30 mM dodecyl-β-maltoside and the extract was loaded onto the column. Unbound protein was removed by washing with 5% sucrose, 20 mM KH_2PO_4 (pH 7.5), 0.01% dodecyl-β-maltoside. Bound protein was then eluted at a flow rate of 1 ml/min by applying a discontinuous KCl-gradient onto the column: 0–0.24 M in 2.5 ml, 0.24 M for 5.5 ml, 0.24–0.37 M in 1.5 ml, 0.37 M for 4.5 ml, 0.37–0.39 M in 0.5 ml, 0.39 M for 3.5 ml, 0.39–0.49 M in 1 ml, 0.49 M for 4 ml, 0.49 M–1.00 M in 3.5 ml and 1 M for 2.5 ml. A_{280} is indicated by the solid line and the KCl-gradient by the dashed line. (B) SDS-PAGE of fractions eluted from FPLC Mono Q anion-exchange column. Equal volume of each fraction was precipitated with trichloroacatic acid (see Section 2 for details). Proteins were stained with Coomassie brilliant blue. (C) Immunological analysis of fractions eluted from FPLC Mono Q anion-exchange column using antibodies directed against (A) F_1-ATP synthase from spinach leaves, (B) cytochrome *c* oxidase from *S. cerevisiae*, (C) β-MPP from *N. crassa* (β-MPP and Core 1 protein are identical in *N. crassa*).

B



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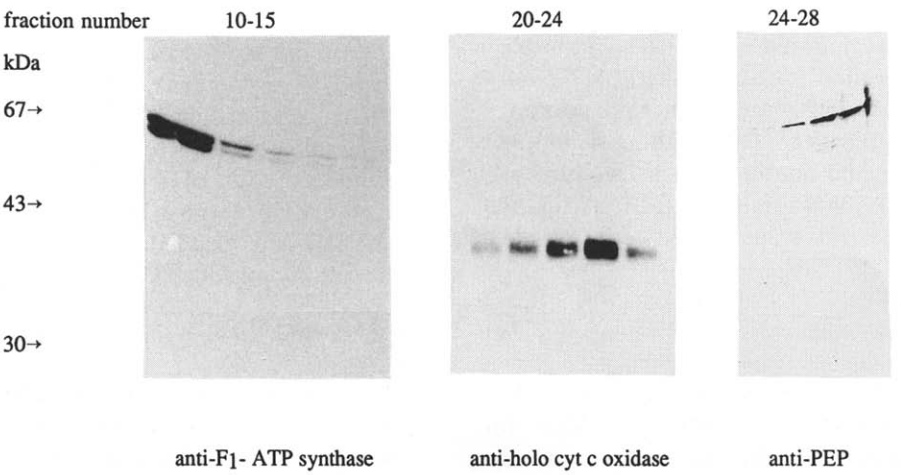

3. Results

3.1. Isolation of the membrane-bound processing peptidase from spinach leaf mitochondria

In order to isolate the membrane-bound processing activity from spinach leaf mitochondria, a simple two-step procedure is used, based on extraction of mitochondrial membranes with non-ionic detergent, dodecyl-β-maltoside, followed by fractionation of the extract using anion-exchange chromatography. The method was originally developed by Peiffer et al. [22] and used for isolation of the cytochrome *c* oxidase complex from wheat germ. The method also results in good separation of other protein complexes of the respiratory chain. Spinach leaf mitochondrial membranes are extracted with 50 mM dodecyl-β-maltoside which extracts about 95% of the processing activity. The concentration of dodecyl-β-maltoside was adjusted after observations that at detergent concentration lower than 50 mM, nearly all processing activity was extracted from the membrane whereas at concentrations of detergent above 70 mM, the fractionation profile on the FPLC column was diffuse and the processing activity was difficult to recover. The extract containing the processing activity is fractionated on a FPLC Mono Q 5/5 HR anion-exchange column. The elution profile is shown in Fig. 1A and shows that good separation of different protein complexes is achieved. The protein content of the eluted fractions analysed on SDS-PAGE is shown in Fig. 1B. In order to identify the eluted protein complexes, fractions were tested for immunological cross-reactivity using antibodies raised against different complexes of the mitochondrial inner membrane: ATP synthase from spinach leaves, cytochrome *c* oxidase from *S. cerevisiae* and different individual subunits of cytochrome *c* reductase from yeast and *N. crassa*, Core proteins, cytochrome *b* and Rieske FeS protein (Figs. 1C and 3A). The immunological cross-reactivity studies showed that ATP synthase is eluted at 0.24 M KCl, cytochrome *c* oxidase at 0.39 M KCl and cytochrome *c* reductase at 0.49 M KCl.

3.2. The membrane-bound general processing peptidase of spinach leaf mitochondria is an integral part of the cytochrome c reductase complex of the respiratory chain

To localise the general processing activity in the fractions eluted from the anion-exchange column and to reveal if the enzyme was able to cleave precursors of different intramitochondrial location, we used the precursor of *N. plumbaginifolia*, $F_1\beta$ subunit of ATP synthase (Fig. 2A) which is an extrinsic membrane-bound protein, *N. crassa* Rieske FeS protein (Fig. 2B) which is an integral membrane protein, and the matrix protein, malate dehydrogenase from *C. vulgaris* (Fig. 2C).

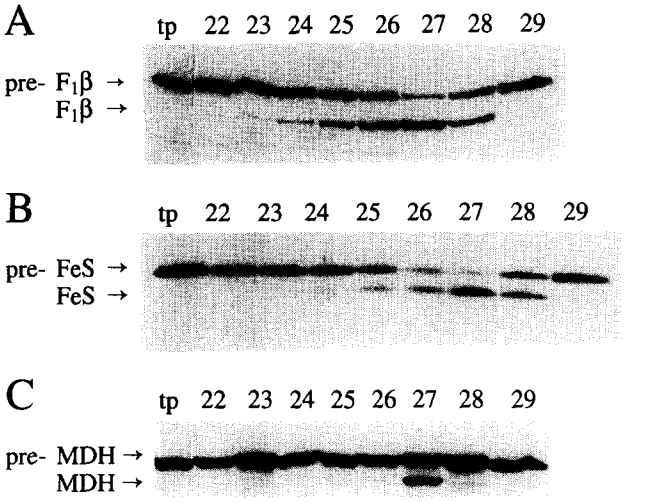

Fig. 2. (A) Processing of the precursor of *N. plumbaginifolia* $F_1\beta$ subunit of the ATP synthase with different fractions from FPLC Mono Q anion-exchange column. Translation mixture alone contained precursor of 59 kDa (pre-$F_1\beta$). Processing was revealed by appearence of an additional band of 51 kDa corresponding to the mature form of the precursor ($F_1\beta$). (B) As in A, but with the precursor of *N. crassa* Rieske FeS of 29 kDa. Processing was revealed by appearence of an additional band of 26 kDa. (C) As in A, but with the precursor of *C. vulgaris* malate dehydrogenase of 32 kDa. Processing was revealed by appearance of an additional band of 29 kDa.

All three precursors are cleaved to the mature forms by dialysed fractions containing the cytochrome *c* reductase complex and the processing activity is inhibited by EDTA and orthophenanthroline (not shown). The processing activity profiles differ for the precursors. The processing reaction was not run simultanously for all precursors and therefore the observed difference is probably due to partial inactivation of MPP in fractions with lower protein content, especially in the experiments with malate dehydrogenase. Anyhow, no processing activity is seen with any other fraction from the anion-exchange column. The fact that three precursors of different intramitochondrial location are cleaved by the enzyme proves that the peak contains the general processing activity.

The cytochrome *c* reductase complex eluted from the Mono Q anion-exchange column analysed on SDS-PAGE contains mainly ten polypeptides of 61, 54, 52, 34, 32, 26, 15, 12, 11 and 10 kDa (Figs. 1B and 3B). All fractions containing processing activity were pooled, dialysed and applied to a FPLC Superose 6 HR 10/30 gel filtration column. All ten polypeptides and the processing activity were recovered in the major protein peak after elution, indicating that all polypeptides constitute an integral part of the cytochrome *c* reductase complex (not shown).

In order to identify the different polypeptides of the cytochrome *c* reductase complex, immunological cross-reactivity was tested using antibodies raised against

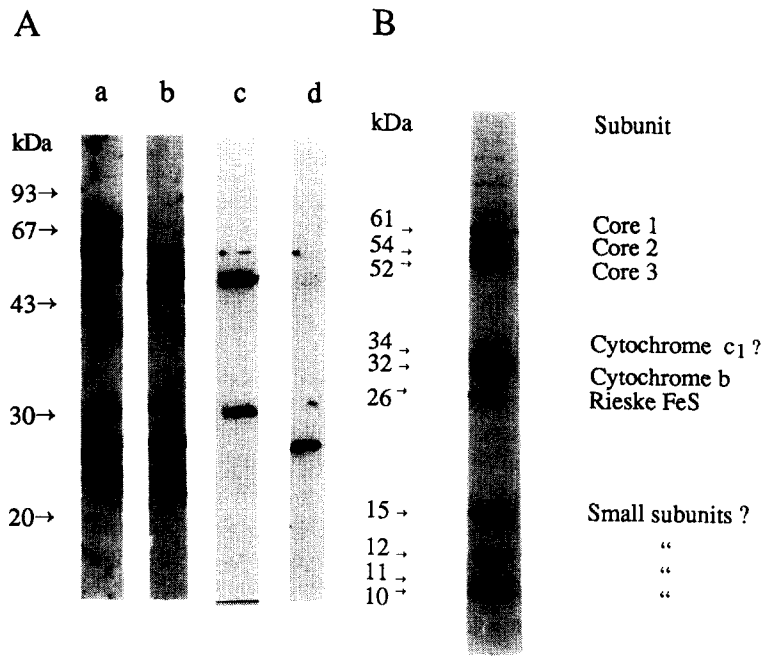

Fig. 3. (A) Immunological analysis of purified cytochrome *c* reductase using antibodies directed against subunits of cytochrome *c* reductase from *S. cerevisiae*: A, Core 1; B, Core 2; C, cytochrome *b*; and D, Rieske FeS protein. (B) Purified cytochrome *c* reductase from Mono Q anion-exchange column analysed on SDS-PAGE. Proteins were stained with Coomassie brilliant blue (cytochrome *b* from other species have been reported to migrate faster than cytochrome c_1 because of its strongly hydrophobic character, indicating that the 34 kDa protein of the spinach leaf cytochrome *c* reductase complex corresponds to cytochrome c_1).

different subunits of cytochrome *c* reductase from *S. cerevisiae* (Fig. 3A). Antibodies directed against Core 1 protein cross-react with the 61 kDa protein, whereas antibodies directed against Core 2 protein cross-react predominantly with the 54 and 52 kDa proteins. Cross-reactivity is also seen with the 61 and 26 kDa proteins, indicating that the antibodies were not monospecific. Antibodies directed against cytochrome *b* recognise the 32 kDa protein and antibodies raised against Rieske FeS protein recognise the 26 kDa protein. Cytochrome *b*, which is a very hydrophobic protein, has been previously shown to migrate on SDS gels as a broad band [38] as observed in Fig. 3A. The antibodies raised against cytochrome *b* also show cross-reactivity with the 61, 54 and 52 kDa proteins.

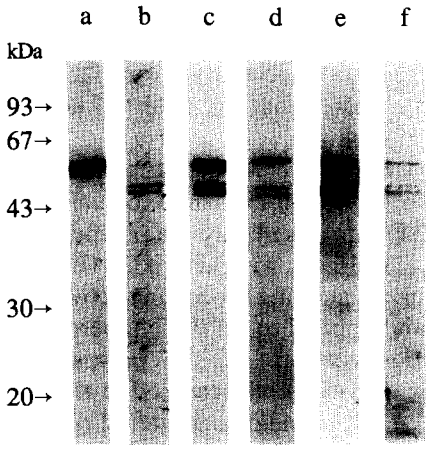

Fig. 4. Immunological analysis of purified cytochrome *c* reductase using antibodies raised against (A) β-MPP and (B) α-MPP from *S. cerevisiae*, (C) β-MPP and (D) α-MPP from *N. crassa*, (E) β-MPP and (F) α-MPP from *S. tuberosum*.

To investigate whether any of the polypeptides of the cytochrome *c* reductase complex from spinach leaf mitochondria correspond to MPP from other species, immunological cross-reactivity was tested with antibodies raised against: β-MPP (Fig. 4A) and α-MPP (Fig. 4B) from *S. cerevisiae*, β-MPP (Fig. 4C) and α-MPP (Fig. 4D) from *N. crassa* and β-MPP (Fig. 4E) and α-MPP (Fig. 4F) from *Solanum tuberosum*. Antibodies directed against β-MPP from *S. cerevisiae* specifically recognise the 61 kDa protein, whereas antibodies directed against β-MPP from *N. crassa* cross-react predominantly with the 61 kDa protein but also with the 54 and 52 kDa proteins. Antibodies directed against α-MPP from *S. cerevisiae* show strongest cross-reactivity with the 54 and 52 kDa proteins but cross-reactivity is also seen with the 61 kDa protein. Antibodies directed against α-MPP from *N. crassa* and α-MPP and β-MPP from *S. tuberosum* cross-react to the same extent with the 61, 54 and 52 kDa proteins.

3.3. Effect of different inhibitors of the respiratory chain on processing

We have investigated if there exists interdependence between the protein processing reaction and the redox state or the electron transfer reaction within the cytochrome *c* reductase complex. We have tested the effect of DTT, which reduces the complex completely, and the effect of inhibitors of the respiratory chain, on the processing activity. Processing activity of the isolated cytochrome *c* reductase complex and of the mitochondrial membrane fraction was investigated with the precursor of *N. plumbaginifolia* $F_1\beta$ subunit of the ATP synthase. No effect on processing is seen when the isolated complex is reduced by 10 mM DTT (Fig. 5), or when the NADH induced electron transfer in mitochondrial membranes is inhibited with KCN (not shown). This indicates that processing is not dependent on the redox state of the isolated cytochrome *c* reductase complex or on respiration.

The processing activity of the isolated complex is, however, inhibited by antimycin A, an inhibitor of the ubiquinone reductase site of the cytochrome *c* reductase complex (Fig. 6). The titrations with the inhibitor are performed with the same concentration of ethanol (0.3%) in all samples. Triton X-100 has been excluded from the assays for processing activity in order to preserve intact subunit–subunit interactions in the complex. Fig. 6A shows autoradiograms of the processing at increasing concentrations of antimycin A. Values shown in Fig. 6B are calculated by densitometric scanning of the autoradiograms in Fig. 6A and expressed as a ratio between mature form and the sum of the precursor and the mature form. This calculation was chosen in order to minimize differences of the radioactivity recovery in the autoradiograms. Upon titration of the processing activity with antimycin A in the range 1–22 μM, there is a gradual increase of the inhibition with 50% at 10 μM antimycin A and 70% at 22 μM. In the presence of Triton X-100 during measurements of the processing activity, no inhibitory effect of antimycin A (or myxothiazol) was observed (not shown).

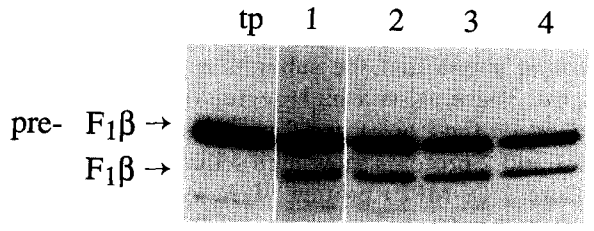

Fig. 5. Effect of DTT on the processing activity of the purified cytochrome *c* reductase complex using the precursor of *N. plumbaginifolia* $F_1\beta$ subunit of the ATP synthase. Translation product alone (tp). (1) Control containing no DTT; the concentrations of DTT are as follows: (2), 1 mM; (3), 5 mM; (4), 10 mM.

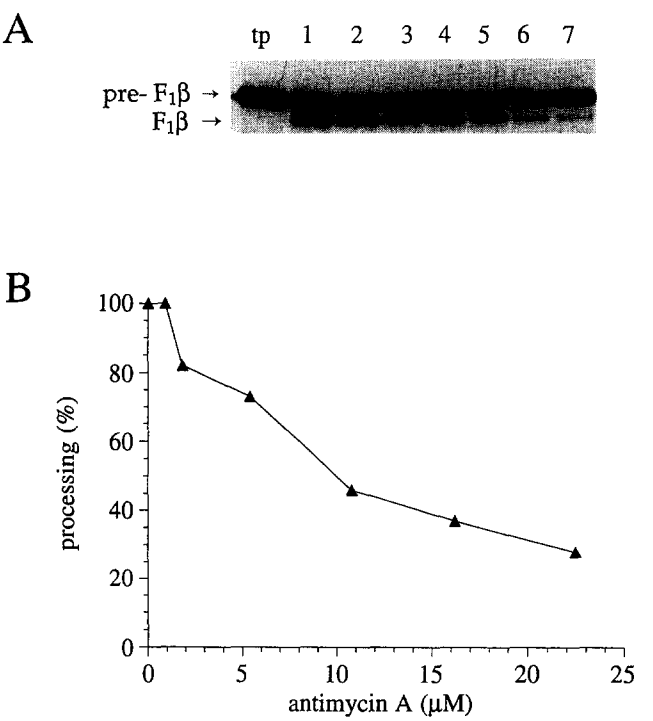

Fig. 6. (A) Effect of antimycin A on the processing activity of the purified cytochrome *c* reductase complex using the precursor of *N. plumbaginifolia* $F_1\beta$ subunit of the ATP synthase. No Triton X-100 was present in the reaction buffer. Translation product alone (tp). (1) No antimycin A added; the concentrations of antimycin A are as follows: (2), 0.9 μM; (3), 1.8 μM; (4), 5.5 μM; (5), 10.9 μM; (6), 16.4 μM; (7), 22.8 μM. (B) Inhibition profile of antimycin A on processing activity of the purified cytochrome *c* reductase. Degree of inhibition is measured by scanning autoradiograms with laser densitometer and is calculated as a ratio of the mature form to the sum of the precursor and the mature form.

Fig. 7 shows titrations of the processing activity and the electron transfer activity in total membrane fraction isolated from spinach leaf mitochondria. The membrane fraction constitutes a more intact system in comparison to the isolated cytochrome *c* reductase complex. Processing activity was measured as above with the precursor of *N. plumbaginifolia* $F_1\beta$ subunit of the ATP synthase. Electron transfer activity was measured spectrophotometrically as oxidation of NADH. When both activities were titrated either with antimycin A (Fig. 7A) or myxothiazol, an inhibitor of ubiquinol oxidase site of the cytochrome *c* reductase (Fig. 7B), no correlation of the inhibition profiles of the processing and respiration was observed. Whereas respiration was almost totally inhibited at 0.01 μM antimycin A and 0.1 μM myxothiazol, processing activity was only slightly inhibited (about 10%) at these concentrations of the inhibitors. At 5 μM concentra-

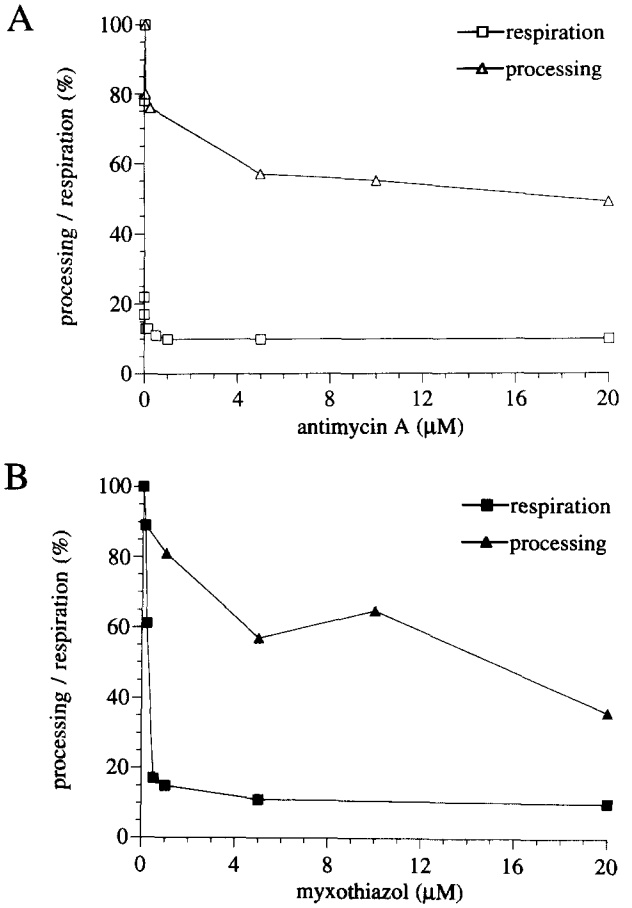

Fig. 7. Effect of antimycin A and myxothiazol on the NADH-induced reduction of cytochrome *c* and on the processing activity in spinach mitochondrial membrane fraction using the precursor of *N. plumbaginifolia* $F_1\beta$ subunit of the ATP synthase. No Triton X-100 was present in the reaction buffer. Measurements were performed as described in Section 2. 100% of NADH oxidation corresponds to 3.97 μmol (mg protein)$^{-1}$ min^{-1}. Degree of inhibition of the processing activity is measured by scanning autoradiograms with laser densitometer and is calculated as a ratio of the mature form to the sum of the precursor and the mature form.

tion for both antimycin A and myxothiazol, about 40% of the processing activity was inhibited. At 20 μM of inhibitor, the processing activity was inhibited by 50% by antimycin A and 65% by myxothiazol.

4. Discussion

In contrast to non-plant sources where the general mitochondrial processing peptidase is a matrix protein, MPP of plant mitochondria in spinach leaves and potato tubers has been shown to be an integral membrane protein [17,18,32]. Most interestingly, it has been shown that MPP of potato tuber mitochondria constitutes an integral part of the cytochrome *c* reductase complex (purified by affinity chromatography) [18,19].

Here we describe an independent purification procedure of the processing activity from plant photosynthetic tissue, spinach leaf mitochondria (in contrast to storage tissue of potato tubers), starting from the total mitochondrial membrane processing extract. The isolation procedure is based on extraction of the processing activity from the mitochondrial membrane with non-ionic detergent, dodecyl-β-maltoside, followed by fractionation of the extract on FPLC Mono Q anion-exchange column which results in good separation of the different protein complexes of the mitochondrial inner membrane. The total processing activity is found only in the fractions containing the cytochrome *c* reductase complex. Our findings show that the processing activity of spinach leaf mitochondria located in the cytochrome *c* reductase complex catalyses cleavage of three mitochondrial precursor proteins of different intramitochondrial localisation and is inhibited by EDTA and orthophenanthroline, suggesting that the activity corresponds to the general mitochondrial processing peptidase. Our results together with the results of Schmitz and collegues [18,19] show that integration of MPP in the cytochrome *c* reductase complex is a general feature for plants.

The purified cytochrome *c* reductase complex of spinach leaf mitochondria comprises ten proteins. Ten subunits were also found in two different preparations of the cytochrome *c* reductase complex from potato tuber mitochondria [33,34]. In contrast to the cytochrome *c* reductase in fungi [35,36] and in mammals [37] where only two Core proteins are found, the plant mitochondrial complex of spinach leaf and potato tubers appears to contain three Core proteins. In the report of Berry et al. [33] it was discussed that the three high molecular mass components may constitute allelic or tissue specific forms of one of the Core proteins. In cytochrome *c* reductase from *S. cerevisiae*, Core 2 protein consistently migrates as a doublet on SDS-PAGE [38]. The author suggests that this microheterogeneity may be due to the presence of some incompletely processed Core 2 protein and that there exists the possibility that the purified complex does consist of two populations with a completely and an incompletely processed Core 2 protein.

Sequence analysis of the three Core proteins of the cytochrome *c* reductase complex isolated from *S. tuberosum* shows that the subunits represent different proteins. There exists a high degree of sequence identity between the Core 1 and 2 proteins (70%) [19]. The Core 1 and 2 proteins show high sequence similarity to the Core 1 (β-MPP) protein of *N. crassa* (49%), but lower to the Core 2 proteins of yeast, bovine and humans (about 25%). The Core 3 protein shows 24% identity with Core 2 protein of *N. crassa* and 15 and 12% with Core 1 and 2 proteins from yeast. In spinach

leaf mitochondria, the Core 2 and 3 proteins cannot be products of incomplete processing of the Core 1 protein, as antibodies raised against yeast Core 1 protein cross-reacts only with spinach Core 1 protein and antibodies against yeast Core 2 protein cross-react only with spinach Core 2 and 3 proteins. It is also unlikely that the Core 3 protein is a product of proteolysis of the Core 2 protein, as the stoichiometry of the proteins as judged by Coomassie staining and immunological data (see also below) seems to be equimolar and is constant from preparation to preparation.

Furthermore, the Core 1 protein of the spinach leaf complex is specifically recognised by monospecific antibodies raised against *S. cerevisiae* β-MPP, whereas the Core 2 and 3 proteins are recognised by antibodies against α-MPP. Antibodies against α-MPP and Core 2 protein from any source tested, do consistently recognise both the Core 2 and 3 proteins from spinach. No specific recognition of one of the components has been observed. These results indicate that in the spinach cytochrome *c* reductase complex there are two proteins related to α-MPP and one protein related to β-MPP. These results are in contrast to the situation in potato tuber mitochondria, where it has been found that two proteins, Core 1 and 2 of the cytochrome *c* reductase complex, correspond to β-MPP [39] and one, Core 3 protein, corresponds to α-MPP [18,19]. As discussed above, there exists high sequence homology (70%) between potato Core 1 and 2 proteins, whereas there is only low sequence similarity (20%) between the Core 3 protein and Core 1 or Core 2 proteins. No high sequence identity regions have been found between the Core 3 protein and any of the Core 1 or 2 proteins. On the basis of sequence analysis in potato and immunological cross-reactivity in spinach, as discussed above, it is unlikely that in spinach, the 54 kDa or the 52 kDa protein would correspond to the second β-MPP of potato. These results suggest species- and/or tissue-dependent differences in the occurrence of protein processing components within the cytochrome *c* reductase complex. It will be highly interesting to analyse amino acid sequences of the 54 and 52 kDa proteins and to investigate sequence similarity to other MPP proteins, as well as to study occurrence of these peptides in other tissues of spinach, e.g., roots. Studies of tissue-specific differences and sequence analysis are in progress in our laboratory.

The cytochrome *c* reductase complex of plant mitochondria is thus bifunctional, catalysing both electron transport and protein processing. What is the role of Core proteins in these functions? Core proteins belong to the group of subunits of the cytochrome *c* reductase complex without redox groups [40–42]. They are suggested to be extrinsic membrane proteins facing the matrix [43], and are probably held to the complex through interactions with other subunits of the complex. The disassociability of the Core proteins was shown to differ between different organisms [44]. Core proteins are not required for the electron transfer or proton translocation per se, since they are not found in the mechanistically very similar prokaryotic cytochrome *c* reductase complexes. Yeast deletion mutants lacking subunits corresponding to the Core proteins can form only trace amounts of the cytochrome *c* reductase complex, indicating that these subunits are required for assembly of the complex [45,46]. In *N. crassa*, the cytochrome *c* reductase complex is inactive without Core proteins [47]. This shows that the Core proteins are required for activity, stability and assembly of the complex in eukaryotes. Results from sequence analysis of the different regions of β-MPP from *N. crassa* and Core 1 and β-MPP from *S. cerevisiae* suggest that in *N. crassa* the amino-terminal domain is preferentially involved in the processing function, whereas the carboxyl-terminal domain is involved in the Core function [47]. In α-MPP, which corresponds to Core 3 protein in potato, a highly conserved hydrophobic region within the otherwise hydrophylic interior of the protein and an internal acidic region have been suggested to be important for the processing function [18]. Although both in spinach and in potato mitochondria ([18,19], this report) three proteins correspond to MPP (and Core proteins), it still remains to be elucidated if all these three subunits are required for processing activity and if they function as Core proteins in the cytochrome *c* reductase complex.

Does there exist interdependence between respiration and protein processing? When the isolated complex is reduced with DTT or when electron transfer in mitochondrial membranes is inhibited by KCN, no effect on processing is seen. This indicates that the processing activity is not dependent on the redox state of the complex or on respiration. However, the processing activity of both the isolated and membrane bound complex is inhibited by antimycin A and myxothiazol, inhibitors of the oxidoreductase function of the cytochrome *c* reductase complex binding to two different ubiquinone binding sites of the complex [49–51]. Lack of sensitivity of the processing reaction to antimycin A and myxothiazol has been reported for the potato tuber cytochrome *c* reductase complex [52], however in this study the processing activity was measured in the presence of Triton X-100, which prevents the inhibitory effect (unpublished results). We have compared titration profiles with antimycin A and myxothiazol of the oxidoreductase function and the protein processing function measured under the same conditions. These studies clearly show that the inhibition of electron transport through the cytochrome *c* reductase complex does occur at much lower concentrations of the inhibitors than the inhibition of the processing reaction. This suggests that inhibition of the

processing activity by respiratory chain inhibitors is not a result of the inhibition of electron transfer through the complex. On the other hand, the fact that the inhibitory effect on processing is seen in the isolated cytochrome *c* reductase complex indicates that binding sites for the inhibitors are located within the complex. The inhibitors of other mitochondrial inner membrane protein complexes, as oligomycin or rotenon at similar concentrations (5–20 μM), do not show an inhibitory effect on processing measured with the isolated complex (not shown). It is conceivable that there exist high and low affinity binding sites for the inhibitors within the complex, either on the same protein or on different subunits of the complex, the high affinity binding site being involved in electron transfer and low affinity binding site in processing. Binding of antimycin A has been reported to induce a conformational change in the cytochrome *c* reductase complex [53–55]. The inhibitory effect on processing may also be a result of antimycin A-induced conformational changes in the complex which influence the processing ability of the Core proteins. This would indicate that cooperation of several subunits of the cytochrome *c* reductase complex in spinach leaf mitochondria is required for optimal efficiency of the processing reaction. The effect of Triton X-100 supports this interpretation. Direct studies of the inhibitor binding are required to understand the effect of inhibitors of the electron transfer on processing activity.

Present studies relate to an interesting evolutionary development of the mitochondrial processing peptidase. In yeast there exists only low sequence similarity between the Core proteins and MPP subunits; in *N. crassa*, Core 1 protein is identical to β-MPP, whereas in plants three Core proteins show high sequence similarity to both subunits of MPP and seem to be involved in both the oxidoreductase function and in the protein processing function. These differences are probably related to the fact that yeast can grow anaerobically and therefore it may be advantageous in this situation to express processing and respiration independently. On the other hand, in obligate aerobic organisms, integration of the biogenetic and respiratory events may be important for the regulation of both biogenetic and metabolic events in mitochondria. What is the topology of the cytochrome *c* reductase complex in comparison to import sites? Is the precursor processed during import? Is import of the precursors dependent on processing? Does the respiratory function of the oxidoreductase complex influence import and processing? The physiological and biogenetic significance of the association of the respiratory function and the processing activity as well as the regulation and mutual interdependence of the translocation of the precursor, processing and electron transfer in plants are under investigation in our laboratory.

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