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Characterization of the Bifunctional Mitochondrial Processing Peptidase (MPP)/*bc*$_1$ Complex in *Spinacia oleracea*

AnnaCarin Eriksson,[1] Sara Sjöling,[1] and Elzbieta Glaser[1]

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The mitochondrial general processing peptidase (MPP) in plant mitochondria constitutes an integral part of the cytochrome *bc*$_1$ complex of the respiratory chain. Here we present a characterization of this bifunctional complex from spinach leaf mitochondria. The purified MPP/*bc*$_1$ complex has a molecular mass of 550 kDa, which corresponds to a dimer. Increased ionic strength results in partial dissociation of the dimer as well as loss of the processing activity. Micellar concentrations of nonionic and zwitterionic detergents stimulate the activity by decreasing the temperature optimum of the processing reaction, whereas anionic detergents totally suppress the activity. MPP is a metalloendopeptidase. Interestingly, hemin, a potent regulator of mitochondrial and cytosolic biogenesis and inhibitor of proteosomal degradation, inhibits the processing activity. Measurements of the processing activity at different redox states of the *bc*$_1$ complex show that despite bifunctionality of the MPP/*bc*$_1$ complex, there is no correlation between electron transfer and protein processing.

KEY WORDS: Mitochondrial processing peptidase MPP; *bc*$_1$ complex; protein import; plant mitochondria; cytochrome *c* reductase; protein processing.

INTRODUCTION

The majority of the mitochondrial proteins are encoded by the nuclear genome and synthezised on cytosolic ribosomes as precursor proteins. Most of these precursors have amino-terminal presequences that contain information for intra- and inter-organellar sorting and targeting. Inside the mitochondrion the presequences are proteolytically cleaved off by a specific processing system. Most mitochondrial precursor proteins are cleaved to their mature form in a single cut by the general mitochondrial processing peptidase (MPP) (Böhni *et al.*, 1980; Schmidt *et al.*, 1984).

MPP has been purified from *Neurospora crassa* (Hawlitschek *et al.*, 1988), *Saccharomyces cerevisiae* (Yang *et al.*, 1988), rat liver (Ou *et al.*, 1989), potato tuber (Braun *et al.*, 1992), and spinach leaf mitochondria (Eriksson and Glaser, 1992; Eriksson *et al.*, 1993, 1994). MPP is a metal-dependent endopeptidase consisting of two structurally related subunits, α-MPP and β-MPP. In *S. cerevisiae* and rat liver both α- and β-MPP are located in the mitochondrial matrix and form a heterodimeric complex. In *N. crassa*, the subunits of MPP do not form a detectable complex and β-MPP has been shown to be identical to the Core 1 subunit of the *bc*$_1$ complex of the respiratory chain and 70% of β-MPP is associated to the inner membrane (Schulte *et al.*, 1989). In *S. cerevisiae* and mammals both α-MPP and β-MPP show sequence similarity but not identity to the respective Core proteins of the *bc*$_1$ complex (Schulte *et al.*, 1989).

Based on sequence similarity studies, the subunits of MPP and the Core proteins of the *bc*$_1$ complex are believed to belong to the same family of bifunctional proteins which are involved in both electron transfer and protein processing (Braun and Schmitz, 1995; Glaser *et al.*, 1994; Schulte *et al.*, 1989). The β-subunit of

[1] Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden.

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the MPP proteins shows significant sequence similarity with a family of insulin-degrading enzymes (Braun and Schmitz, 1995; Brunner *et al.*, 1994; Rawlings and Barrett, 1991) containing an inverse metal-binding site, His-X-X-Glu-His, defining a new family of metalloendopeptidases, the pitrilysin family (Rawlings and Barrett 1991, 1993). This metal-binding site is conserved in all β-MPP subunits but is degenerate in α-MPP and in the Core proteins that are not involved in protein processing. Mutations of the histidines and the glutamate in the His-X-X-Glu-His motif in rat β-MPP abolished processing, indicating that β-MPP is the catalytically active subunit (Kitada *et al.*, 1995). Genetic depletion studies and studies with overexpressed subunits show that β-MPP is unable to catalyze processing in the absence of α-MPP, indicating an essential function of α-MPP in processing (Arretz *et al.*, 1994; Géli *et al.*, 1990, Yang *et al.*, 1991).

In plants, the processing activity is entirely associated with the mitochondrial inner membrane (Eriksson and Glaser, 1992). Purification of plant MPP revealed that the enzyme constitutes an integral part of the bc_1 complex of the respiratory chain. The purified complex contains, when analyzed on glycine/SDS-PAGE, three high molecular weight proteins called Core proteins. Immunological and sequence analysis data showed that the Core proteins correspond to MPP subunits from other sources (Braun *et al.*, 1992; Eriksson and Glaser, 1992; Eriksson *et al.*, 1993, 1994). In spinach, Core protein 1 corresponds to β-MPP, and Core proteins 2 and 3 correspond to the α subunit of MPP (Eriksson *et al.*, 1994). In potato both β-MPP and α-MPP have been suggested to occur in isoforms, indicating that the plant bc_1 complex may contain different isoforms of the two Core proteins identified in other systems (Braun *et al.*, 1992; Emmermann *et al.* 1994, Jänsch *et al.*, 1995). The stoichiometry of the Core proteins in the functional bc_1 complex is, however, still unclear. Attempts to separate the Core proteins from the bc_1 complex resulted in loss of processing activity, which indicates functional integration of the Core proteins into the bc_1 complex and requirement of the other subunits of the complex for maintaining the Core proteins in a functional catalytic structure (Emmermann *et al.*, 1993).

The finding that the mitochondrial processing peptidase in plants is integrated into the bc_1 complex of the respiratory chain raises several interesting questions concerning interdependence between processing and electron transfer as well as the biochemical consequences of the integration of the peptidase into an oligomeric membrane-bound complex. Here we present characterization of the bifunctional MPP/bc_1 complex from spinach leaf mitochondria in order to address the above questions.

MATERIALS AND METHODS

Isolation and Gel Filtration Chromatography of the Integrated MPP/bc_1 Complex

Spinach (*Spinacia oleracea L.*) leaf mitochondria were isolated according to Hamasur *et al.* (1990). The mitochondria were diluted to a protein concentration of 5–8 mg/ml and disrupted by sonication in the presence of 30 mM $MgCl_2$. Membranes were separated from matrix by centrifugation at 130,000 × *g* for 45 min (Eriksson and Glaser, 1992). The integrated MPP/bc_1 complex was isolated by dodecyl-β-maltoside extraction of the mitochondrial membranes followed by anion-exchange and gel filtration chromatography as described by Eriksson *et al.* (1994).

The bc_1 complex isolated from FPLC Mono Q anion-exchange column was desalted and concentrated with Centricon ultrafiltration tubes (cut-off 3000 Da). A number of standard proteins (see figure legend) were loaded onto a Pharmacia FPLC Superose 6 HR 10/30 column equilibrated with 5% sucrose, 20 mM KH_2PO_4, pH 7.5, 0.01% dodecyl-β-maltoside at a flow rate of 0.2 ml/min in the presence or absence of 0.5 M KCl. The bc_1 complex was eluted under the same conditions as the standard proteins. Active fractions were pooled and concentrated. All the chromatographic steps were performed at 4°C.

In Vitro Transcription and Translation of *N. plumbaginifolia* $F_1\beta$ Precursor Protein

In vitro transcription and translation was carried out with the cDNA clone of *N. plumbaginifolia* $F_1\beta$ cloned in an unlinearized pTZ18U vector. The expression was performed using Promega TNT™ coupled reticulocyte lysate systems with T7 RNA polymerase in the presence of [^{35}S]-methionine.

Processing of *N. plumbaginifolia* $F_1\beta$ Precursor

The reaction for processing contained 0.5 μl of [^{35}S]-methionine labelled $F_1\beta$ precursor (ca. 10,000–

15,000 cpm) and 0.5 μg of the purified bc_1 complex or 15 μg mitochondrial membranes in 10 mM HEPES, pH 8.0, in a final volume of 20 μl. Processing was carried out for 25 min at 25°C. The processing activity was measured in the presence of different detergents and protease inhibitors and also at different pH and temperature as described in figure legends. The reaction was stopped by addition of double-strength sample buffer 1:1 (Laemmli, 1970) and solubilized at 90°C for 5 min. The processing reaction was analyzed on SDS-PAGE using 12% polyacrylamide gels in the presence of 4 M urea in the buffer system of Laemmli (Laemmli, 1970). Gels were fixed, impregnated with Amplify™ (Amersham), and dried. Fluorography was carried out at −80°C.

Protein Determination

Protein was determined with Bio-Rad protein assay reagent according to the method of Bradford (Bradford, 1976). Bovine immunoglobulin was used as a standard.

RESULTS

The molecular mass of the integrated MPP/bc_1 complex from spinach was calculated from its mobility through a FPLC Superose 6 gel filtration column compared to a number of standard proteins (Fig. 1). The bc_1 complex eluted close to thyroglobulin (669 kDa) and its molecular mass was estimated to be 605 kDa (Fig. 1A). Accounting for bound detergent the molecular mass is approximately 550 kDa, which corresponds to a dimer. This is in accordance with the situation in other organisms where the bc_1 complex has been shown to be a structural dimer in the mitochondrial membrane (Leonard *et al.*, 1981; Perkins and Weiss, 1983; Schmitt and Trumpower, 1990; Weiss and Kolb, 1979). Increasing the ionic strength by including 0.5 M KCl in the elution buffer resulted in partial dissociation of the dimer, giving rise to an extra protein peak corresponding in size to monomeric bc_1 complex (Fig. 1B). No activity was found in either peak in the presence of salt. After dialysis, the processing activity was partially restored in both peaks (Fig. 1C). When KCl is added directly to the processing reaction, the processing activity decreased with the increasing salt concentration and was totally inhibited at 1.2 M KCl (Fig. 1D). Our results are in contrast to the processing of the *N. plumbaginifolia* $F_1\beta$ precursor catalyzed by potato MPP which is stimulated by NaCl, with maximum activity at 1–1.2 M NaCl (Emmermann *et al.*, 1993), but in agreement with results reported for MPP from *N. crassa* showing that MPP is sensitive to addition of KCl and NaCl (Hawlitschek *et al.*, 1988).

The enzymatic activity catalyzed by the isolated spinach MPP/bc_1 complex or by mitochondrial membranes show similar pH and temperature profiles with optimal activity at pH 7–9 and at 35°C (Fig. 2B), indicating that the solubilization of the MPP/bc_1 complex from the membrane into dodecyl-β-maltoside micelles does not change the properties of the enzyme. The processing activity can be detected over a broad pH (6–11) (not shown) and temperature range (0–50°C) (Fig. 2B).

Figure 2A shows the effect of various detergents on processing activity catalyzed by the MPP/bc_1 complex. The anionic detergent SDS totally inhibited processing activity at both monomer and micelle concentrations. Triton X-100 (nonionic), dodecyl-β-maltoside (nonionic), and CHAPS (zwitterionic) stimulated processing activity about 2-fold above CMC, whereas monomer concentrations of the detergents did not affect the processing activity or were slightly inhibitory (Triton X-100). In order to examine the stimulatory effect of the detergents, processing was performed in the presence and absence of micellar concentrations of Triton X-100 at various temperatures. We found that in the presence of Triton X-100 micelles, the temperature optimum for processing catalyzed by the MPP/bc_1 complex (Fig. 2B) or by mitochondrial membranes (not shown) was changed from 35°C to 20°C.

The processing activity catalyzed by mitochondrial membranes has been shown to be dependent on divalent metal ions for catalytic activity (Eriksson and Glaser, 1992). Table I shows the effect of various protease inhibitors on processing activity catalyzed by the purified bc_1 complex. Metal chelators, *ortho*-phenanthroline, and EDTA totally inhibited the processing, whereas the activity was neither inhibited by any of the serine-, cysteine-, aspartic-, amino-, or thiol-type protease inhibitors nor by vanadate, an inhibitor of the ATP-dependent proteolysis. The processing activity was not dependent on ATP (not shown). Processing was, however, inhibited by hemin, which is an inhibitor of the ubiquitin-coupled degradative system in the cytosol (Fig. 3). 1 μM hemin totally inhibited the processing activity. The apo-form of heme, protoporphyrin IX, also inhibited processing at slightly higher concentrations. Adding $FeCl_3$ to the reaction buffer did not affect pro-

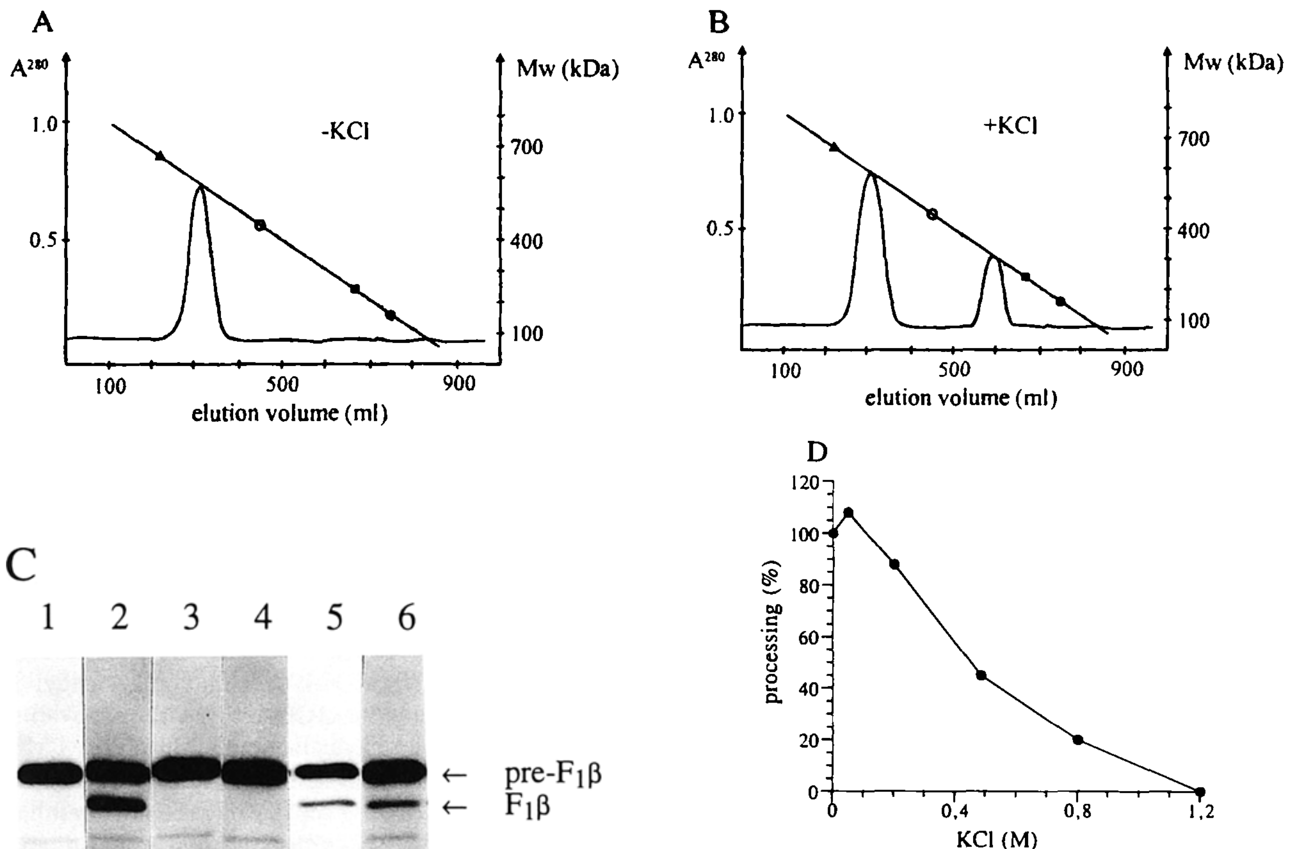

Fig. 1. Molecular mass determination of the MPP/bc_1 complex from spinach leaf mitochondria in the absence (A) and presence (B) of 0.5 M KCl. The graphs represents mobility of the MPP/bc_1 complex and a number of standard proteins: aldolase (158 kDa ●), catalase (232 kDa ■), ferritin (440 kDa ○), thyroglobulin (669 kDa ▲) during gel filtration chromatography using FPLC Superose 6 column. Experimental conditions as in Material and Methods. C. Processing of the *N. plumbaginifolia* $F_1\beta$ precursor with the MPP/bc_1 complex in the absence and presence of KCl. Lane 1, translation product; lane 2, processing of the precursor with MPP/bc_1 complex eluted in the absence of KCl; lane 3, lack of processing of the precursor with MPP/bc_1 complex eluted at 605 kDa in the presence of 0.5 M KCl; lane 4, lack of processing of the precursor with MPP/bc_1 complex eluted at 300 kDa in the presence of 0.5 M KCl; lanes 5 and 6, as lanes 3 and 4, respectively, but after dialysis of the MPP/bc_1 complex. Processing was detected as described in Materials and Methods. D. Inhibition of processing of the *N. plumbaginifolia* $F_1\beta$ precursor by KCl. Processing was catalyzed by the spinach MPP/bc_1 complex and performed as described in Materials and Methods. Graph represents calculation of the efficiency of processing as the ratio of the mature form of the precursor to the sum of precursor and mature form. The control containing no KCl was set as 100%.

cessing indicating that it is the porphyrin ring of hemin that exerts the inhibitory effect.

Integration of MPP into the bc_1 complex of the respiratory chain raises questions concerning dependence of the activity on the redox state and on turnover of the complex. Complete oxidation of the purified complex with ferricyanide did not influence the processing activity, whereas prolonged incubation (15 min) of the complex with the reducing agents DTT or dithionite prior to addition of the *N. plumbaginifolia* $F_1\beta$ precursor resulted in 59% and 42% inhibition of the processing activity, respectively (Table II). However, reduction of the redox centra of the bc_1 complex in submitochondrial particles by inhibition of respiration by addition of KCN, rotenone, antimycin A, or myxothiazol in the presence of respiratory chain substrates, succinate or NADH, had only slight or no effect on the processing activity (Table III). Processing can, however, be inhibited by higher concentrations of antimycin A and myxothiazol (5 μM). Control processing experiment was performed under conditions of ongoing respiration with continuous shaking in order to ascertain constant supply of oxygen. Our results show that the processing activity is neither dependent on the redox state of the bc_1 complex nor on the turnover of the complex.

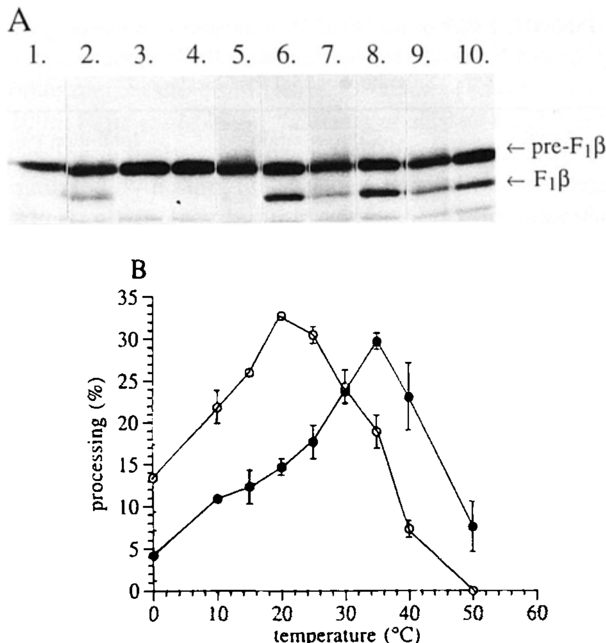

Fig. 2. A. Effect of various detergents on processing of the *N. plumbaginifolia* $F_1\beta$ precursor catalyzed by the spinach MPP/bc_1 complex. The isolated MPP/bc_1 complex was preincubated with different detergents at concentrations below and above CMC: lane 1, translation product; lane 2, processing with the MPP/bc_1 complex in the absence of detergent; lanes 3 and 4, processing in the presence of SDS at 2 and 10 mM; lanes 5 and 6, processing in the presence of Triton X-100 at 0.1 and 0.4 mM; lanes 7 and 8, processing in the presence of dodecyl-β-maltoside at 0. 1 and 0.3 mM; lanes 9 and 10, processing in the presence of CHAPS at 2 and 10 mM. Preincubation with detergent was performed for 10 min at 25°C prior to the processing reaction. Processing was detected as described in Materials and Methods. B. Effect of Triton X-100 on temperature optimum of processing of the *N. plumbaginifolia* $F_1\beta$ precursor catalyzed by the spinach MPP/bc_1 complex. The isolated MPP/bc_1 complex was preincubated at the indicated temperatures for 5 min prior to the processing reaction in the absence of Triton X-100 (●) and in the presence of 0.4 mM Triton X-100 (○).

Table I. Effect of Different Inhibitors on Processing of the *N. plumbaginifolia* $F_1\beta$ Precursor Catalyzed by the Isolated MPP/bc_1 Complex[a]

Inhibitor	Class	Concentration	Inhibition (%)
PMSF	Serine	1 mM	0
Pefabloc	Serine	1 mM	0
E-64	Cysteine	2 mM	5
Bestatin	Amino	0.1 mM	0
Pepstatin	Aspartic	1 mM	0
NEM	Thiol	10 mM	0
Hemin	—	1 μM	90
Vanadate	—	5 mM	0
EDTA	Metallo	0.5 mM	100
ortho-Phenanthroline	Metallo	0.5 mM	100

[a] The bc_1 complex was preincubated with the listed inhibitors for 10 min prior to the processing reaction. Processing was detected as described in Materials and Methods.

DISCUSSION

The mitochondrial general processing peptidase (MPP) in plants constitutes an integral part of the bc_1 complex of the respiratory chain. In order to investigate the biochemical consequence of the integration of the processing activity into a membrane-bound protein complex we have in this work characterized the processing activity catalyzed by the purified MPP/bc_1 complex.

The spinach MPP/bc_1 complex is isolated by solubilization in dodecyl-β-maltoside as a structural dimer. Raised ionic strength during gel filtration chromatography results in partial dissociation of the dimer into the monomeric form as well as loss of activity, which can be restored after removal of salt. MPP from *N. crassa* has been shown to be inactivated by both KCl and NaCl suggesting that the inhibitory effect of KCl on spinach MPP is due to increased ionic strength rather than specific ions (Hawlitschek *et al.*, 1988). The bc_1 complex from *N. crassa* has been shown to be a structural dimer where the contact between the two monomers involves cytochrome *b* and the Core proteins (Linke *et al.*, 1985; Linke and Weiss, 1986). Studies of the isolated Core complex from *N. crassa* bc_1, consisting of Core proteins 1 and 2, show that the Core complex is dimeric at low ionic strength and

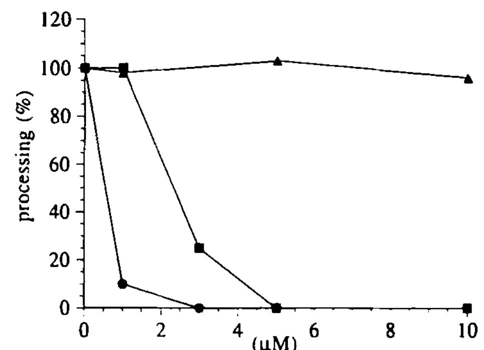

Fig. 3. Effect on processing of the *N. plumbaginifolia* $F_1\beta$ precursor of hemin (●), $FeCl_3$ (▲), and protoporphyrin IX (apo-heme) (■). Reagents were preincubated with the isolated MPP/bc_1 complex 10 min prior to the processing reaction. Processing was detected as described in Materials and Methods.

Table II. Effect of Oxidizing and Reducing Agents on Processing of the *N. plumbaginifolia* $F_1\beta$ Precursor Catalyzed by the Isolated MPP/bc$_1$ Complex[a]

Agent	Concentration (mM)	Inhibition (%)
Ferricyanide	5	14
	10	17
Dithionite	7.5	40
	15	42
DTT	1	8
	5	50
	10	59

[a] The isolated bc_1 complex was preincubated for 15 min prior to the processing reaction with the listed agents. Processing was detected as described in Materials and Methods.

Table III. Effect of Inhibition of Respiration on Processing of the *N. plumbaginifolia* $F_1\beta$ Precursor Catalyzed by Submitochondrial Particles[a]

Agent	Concentration	Inhibition (%)
KCN	5 mM	0
Rotenone	2 mM	0
Antimycin A	0.1 μM	20
	5 μM	42
Myxothiazol	0.1 μM	0
	5 μM	45

[a] Respiration was totally inhibited at all listed concentrations of the inhibitors. The inhibitors were added directly to the processing reaction. Processing was detected as described in Materials and Methods.

monomeric at 0.2 M NaCl (Perkins and Weiss, 1983). As the contact between the bc_1 monomers besides cytochrome *b* also involves the Core proteins, the decrease of processing activity catalyzed by the dimer at raised ionic strength may reflect dissociation between the Core proteins leading to loss of activity. Removal of KCl would then reestablish the contact between the Core proteins and subsequently catalytic function. The ability of the monomeric bc_1 complex to catalyze processing after removal of KCl may thus be due to dimerization, as no monomer is present in the absence of salt. It is not likely that the inhibitory effect of KCl is exerted on the structure of the precursor or on precursor/MPP interaction as the activity of potato MPP purified by affinity chromatography in the presence of Triton X-100 is stimulated by high salt when using the same precursor as substrate (Emmermann *et al.*, 1993). It has not been established whether the matrix located MPP in lower eukaryotes is monomeric or oligomeric, as the complex falls apart during the isolation procedure (Hawlitschek *et al.*, 1988). We interpret our results to indicate that the dimeric MPP/bc_1 complex is required for the processing activity.

When processing is measured in the presence of various detergents, the negatively charged SDS totally inhibits processing. The inhibition might be due to either interaction of the detergent with positively charged residues present in the presequence (Saavedra-Alanis *et al.*, 1994) or disturbance of subunit–subunit interactions within the MPP/bc_1 complex. Micellar concentrations of nonionic and zwitterionic detergents such as Triton X-100, dodecyl-β-maltoside, and CHAPS shift the entire temperature profile toward lower temperatures, changing the optimum for processing from 35°C to 20°C. Detergent micelles may interact with the MPP/bc_1 complex or with the precursor protein or both. The amphiphilic milieu of the micelles may affect the conformation of the complex by changes in border/surface interactions between the detergent phase and the enzyme phase. Detergent micelles may also affect the aggregational status of the enzyme (Helenius and Simons, 1975). Bacterial Sec A has been shown to be activated in the presence of phosphatidyl glycerol by exposure of the hydrophobic cleft of the enzyme to the membrane (Breukink *et al.*, 1993). Signal peptidases of yeast (YaDeau and Blobel, 1989) and bacteria (Tschantz *et al.*, 1995) have been shown to require phospholipids for optimal activity, indicating that phospholipids may play an important physiological role in the catalytic mechanism of the peptidases. It is, however, more likely that the stimulatory effect by detergent micelles is due to interactions with the precursor rather than with the enzyme. We have recently shown that an α-helical element of the C-terminal domain of the presequence of *N. plumbaginifolia* $F_1\beta$ subunit is important for the recognition by MPP (Sjöling *et al.*, 1994). The α-helical structure has previously been suggested to participate in helix/helix interaction with MPP (von Heijne *et al.*, 1989). Presence of detergent micelles may stabilize a favorable conformation of the presequence for interaction with MPP. This is also supported by the finding that many synthetic peptides derived from presequences form helical structures in the presence of detergent or phospholipid micelles but not in purely aqueous solutions (Endo *et al.*, 1989; Thornton *et al.*, 1993; Wang and Weiner, 1993).

MPP has been characterized as a metalloendopeptidase in all species. The processing activity is not dependent on ATP, which distinguishes the proteolytic

activity catalyzed by the MPP/bc_1 complex from the mitochondrial ATP-dependent proteolysis catalyzed by other membrane-bound serine proteases (Knorpp *et al.*, 1995; Pajic *et al.*, 1994; Yasuhara *et al.*, 1994). The processing activity of spinach MPP is, however, totally inhibited by hemin, the oxidized form of heme, which is known to inhibit ubiquitin-coupled cytosolic degradation and some other degradative proteases (Yasuhara *et al.*, 1994). Heme regulates a variety of metabolic systems in the cell that utilize oxygen (Padmanaban *et al.*, 1989). The fact that heme is involved in biogenesis of nuclear-encoded mitochondrial proteins involved in respiration indicates that the inhibitory effect by heme on processing may correspond to a regulatory role *in vivo*. Heme may act as a structural element either permitting or protecting precursors from the peptidase. Import of the mammalian mitochondrial δ-aminolevulinate synthase (ALAS), which regulates the level of intracellular heme in liver, has been shown to be inhibited by heme (Lathrop and Timko, 1993) as well as the catalytic activity of reticulocyte kinase (HRI) (Chen *et al.*, 1991; Lathrop and Timko, 1993). In the presence of hemin, inter-subunit disulfide-bond formation between HRI molecules is induced, resulting in an inactive dimer (Chen and London, 1995). The inhibitory effect on processing may also be due to the oxidation of free sulfydryl groups that are important for processing.

The bifunctionality of the bc_1 complex of plants, catalyzing both electron transfer and protein processing, raises questions concerning correlation between bioenergetics and biogenesis. We have previously reported that the processing activity can be inhibited to approximately 50% by antimycin A and myxothiazol. The inhibition was, however, not correlated to inhibition of electron transport (Eriksson *et al.*, 1994). In the present paper, we conclude that neither redox state nor turnover of the complex affect processing. Inhibition of the processing activity by reducing agents probably reflects reduction of ligands involved in metal chelation within the complex, leading to loss of the endogenous metal involved in catalysis and subsequent loss of processing activity. Inhibition may also be due to reduction of disulfide bonds which are important for a functional conformation of the complex.

In summary, our results suggest that the processing activity catalyzed by the MPP/bc_1 complex in plants requires a functional dimer and is stimulated by an amphiphilic milieu. We also conclude that respiration and protein processing are separate events in plants although the two functions have been integrated into the same oligomeric protein complex.

Determination of the native metal ion(s) involved in catalysis of processing as well as investigations aiming to understand the mechanism of the processing event are in progress in our laboratory.

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