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Integration of the Mitochondrial-Processing Peptidase into the Cytochrome bc_1 Complex in Plants[1]

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The plant mitochondrial cytochrome bc_1 complex, like nonplant mitochondrial complexes, consists of cytochromes *b* and c_1, the Rieske iron–sulfur protein, two Core proteins, and five low-molecular mass subunits. However, in contrast to nonplant sources, the two Core proteins are identical to subunits of the general mitochondrial processing peptidase (MPP). The MPP is a fascinating enzyme that catalyzes the specific cleavage of the diverse presequence peptides from hundreds of the nuclear-encoded mitochondrial precursor proteins that are synthesized in the cytosol and imported into the mitochondrion. Integration of the MPP into the bc_1 complex renders the bc_1 complex in plants bifunctional, being involved both in electron transport and in protein processing. Despite the integration of MPP into the bc_1 complex, electron transfer as well as translocation of the precursor through the import channel are independent of the protein-processing activity. Recognition of the processing site by MPP occurs via the recognition of higher-order structural elements in combination with charge and cleavage-site properties. Elucidation of the three-dimensional (3-D) structure of the mammalian cytochrome bc_1 complex is highly useful for understanding of the mechanism of action of MPP.

KEY WORDS: bc_1 complex; ubiquinol:cytochrome *c* oxidoreductase; core proteins; mitochondrial-processing peptidase; plant mitochondria; protein import; presequence; mitochondrial biogenesis.

INTRODUCTION

Cytochrome bc_1 complex (also known as ubiquinol:cytochrome *c* oxidoreductase, E.C.1.10.2.2) is an oligomeric protein complex of the mitochondrial and bacterial electron transfer chain, localized in the inner membrane of mitochondria and in the plasma membrane of bacteria, for reviews see, (Brandt and Trumpower, 1994; Crofts and Berry, 1998). The bc_1 complex catalyzes a central step of the respiratory electron transfer, i.e., the step between the reduced form of ubiquinone, UQH_2, and cytochrome *c*, in a reaction that is coupled to transfer of protons across the membrane and the generation of a transmembrane proton gradient (Mitchell, 1976; Crofts, 1985). The coupling between electron and proton transfer occurs via a protonmotive Q-cycle mechanism (Mitchell, 1976) involving reduction of ubiquinone and uptake of protons, on one side, and the reoxidation of ubiquinol and dissociation of protons, on the opposite side of the membrane.

The bc_1 complex in all species contains three common subunits retaining four active redox centers, cytochrome *b* (with two heme centers, b_H and b_L), cytochrome c_1, and the Rieske iron–sulfur protein. The bacterial system consists of only the three respiratory subunits and, in some cases, one low-molecular mass noncatalytic subunit (Trumpower, 1990), whereas the mitochondrial bc_1 complex comprises ten subunits. Besides the subunits with redox centers, there are several additional subunits that are not involved in the electron transfer. The noncatalytic subunits include two relatively high molecular mass subunits named Core

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proteins and five low molecular mass subunits, for reviews see (Schagger *et al.*, 1986; Bechmann *et al.*, 1992; Braun and Schmitz, 1995b). The beef heart cytochrome bc_1 complex contains an additional eleventh subunit (Brandt *et al.*, 1993). The presequence of the Rieske iron–sulfur protein when cleaved off in mammalian mitochondria, is incorporated as an integral noncatalytic subunit of this complex. The role of the noncatalytic subunits is not entirely understood; they seem to be involved as assembly and stability factors in the cytochrome bc_1 complex (Crivellone *et al.*, 1988; Boumans *et al.*, 1997; Malaney *et al.*, 1997; Nett and Trumpower, 1999). The photosynthetic electron transfer chain also comprises a counterpart of the cytochrome bc_1 complex, the b_6f complex, with comparable structural, redox, and proton-motive properties (Cramer *et al.*, 1994).

During the past 3 years, we have seen exciting developments toward the goal of a complete atomic structure of the cytochrome bc_1 complex. In 1996–1997, the three-dimensional (3-D) structure of the cytochrome bc_1 complex from beef heart mitochondria was resolved to 2.9 A resolution (Yu *et al.*, 1996; Xia *et al.*, 1997). The crystalline cytochrome bc_1 complex was shown to be a dimer containing 13 transmembrane helixes in each monomer. The cytochrome *b* protein was located mainly within the membrane, whereas Core proteins 1 and 2 protruded from the matrix side of the membrane and cytochrome c_1 and the iron–sulfur protein, including their redox centers, were located on the cytoplasmic side of the membrane. The distances between these redox centers have been determined and several electron transfer inhibitor-binding sites in the complex have been located (Xia *et al.*, 1997). In 1998, crystals from another source, the chicken heart bc_1 complex, were obtained that diffracted to 3.0 A. This work included determination of the structure of the Rieske iron–sulfur protein, cytochrome c_1, as well as three additional low-molecular mass subunits that were not previously assigned (Zhang *et al.*, 1998). The authors suggested that the extrinsic domain of the iron–sulfur protein moves during the catalytic cycle of the cytochrome bc_1 complex, shuttling electrons from ubiquinol to cytochrome *c*. Also, in 1998, the complete structure of the beef heart mitochondrial cytochrome bc_1 complex containing all eleven subunits was reported (Iwata *et al.*, 1998). This work also showed that the Rieske iron–sulfur protein exhibits significant conformational changes in different crystal forms, suggesting a new electron-transport mechanism of the enzyme. Furthermore, it shows that the mitochondrial targeting presequence of the Rieske iron–sulfur protein is lodged between the two Core subunits (Iwata *et al.*, 1998).

In recent years, there has also been an extensive increase in our knowledge of the plant mitochondrial cytochrome bc_1 complex, both with respect to its organization as well as novel findings concerning its function. These studies have revealed an exciting and striking property: the cytochrome bc_1 complex in plants is bifunctional, involved both in electron transport and in the general proteolytic processing of nuclear-encoded mitochondrial precursor proteins. The Core proteins of the cytochrome bc_1 complex in plants are identical to the subunits of the general mitochondrial processing peptidase (MPP) (Braun *et al.*, 1992; Emmermann *et al.*, 1993; Eriksson *et al.*, 1993, 1994). The MPP recognizes and processes the N-terminal targeting presequence that functions as a recognition signal, from precursor proteins, after protein import into mitochondria, for review see (Schatz, 1996; Neupert, 1997; Glaser *et al.*, 1998). MPP is essential for mitochondrial growth and cell viability (Yaffe *et al.*, 1985; Witte *et al.*, 1988). A striking feature of MPP is that it is a general peptidase, acting on several hundred mitochondrial precursor proteins, yet it is highly specific as it recognizes a distinct cleavage site on presequences that do not show obvious sequence similarity. The consequences and implications of MPP integration into the plant cytochrome bc_1 complex and the recognition of mitochondrial presequences by the Core–MPP subunits of the cytochrome bc_1 complex, discussed in the light of recent structural data, will be the main themes of this review.

ISOLATION AND COMPOSITION OF CYTOCHROME bc_1 COMPLEX IN PLANTS

During the 1980s, partial purification of the cytochrome bc_1 complex was reported from different plant species: potato tubers (Ducet and Diano, 1978), sweet potato (Nakajima *et al.*, 1984), Jerusalem artichoke (Degli Espositi *et al.*, 1985; Spinelli and Zannoni, 1987), maize (Hawkesford and Leaver, 1987; Hawkesford *et al.*, 1989), and wheat (Pfeiffer *et al.*, 1990). However, most of our knowledge has come, in the last few years, from extensive studies including purification, organization, characterization, function, and evolution of the cytochrome bc_1 complex from potato (Berry *et al.*, 1991; Braun *et al.*, 1992, 1994),

spinach (Eriksson *et al.*, 1993, 1994, 1996), wheat mitochondria (Braun *et al.*, 1995), and also to some extent from mitochondria of lower plants (Nurani *et al.*, 1997; Brumme *et. al.*, 1998), (see section on Evolutionary Models for Integration of MPP into Cytochrome bc_1 Complex).

The cytochrome bc_1 complex from potato (*Solanum tuberosum* L.) mitochondria has been isolated using two procedures: (1) separation of the dodecylmaltoside-solubilized complex by ion-exchange and hydroxyapatite chromatography (Berry *et al.*, 1991); (2) separation of the Triton X-100-solubilized complex using cytochrome *c* affinity and gel-filtration chromatography (Braun *et al.*, 1992, 1994). Both procedures resulted in a highly pure and active protein complex. Upon reconstitution into phospholipid vesicles, it catalyzed electron transfer from a synthetic ubiquinol to cytochrome *c* in a reaction that was sensitive to antimycin A and myxothiazol. The cytochrome bc_1 complex of *Spinacia oleracea* has been isolated starting from the total membrane-processing extract obtained after treatment of the membranes with dodecylmaltoside followed by FPLC anion-exchange and gel-filtration chromatography (Eriksson *et al.*, 1994). The bc_1 complex from *Triticum aestivum* L. was purified by cytochrome *c* affinity chromatography and gel filtration using either etiolated seedlings or wheat-germ extract as starting material (Braun *et al.*, 1995). The protein subunit composition for the plant cytochrome bc_1 complex from different sources is shown in Table I. Analysis of the components, including sequence determination, immunological methods, and sequence analysis of the genes showed that the plant cytochrome bc_1 complex contains three subunits with redox centers, cytochrome *b*, cytochrome c_1, and the Rieske iron–sulfur protein, two Core proteins, which occur as multiple isoforms, and five small subunits below 15 kDa (for review see Braun and Schmitz, 1995b).

Table I. Polypeptide Composition of the Cytochrome bc_1 Complex in Potato, Spinach, and Wheat

Polypeptide	Potato		Spinach	Wheat
Core 1/β-MPP	55	56.2[a]	61	55.5
	53			55.0
Core 2/α-MPP	51	51.9[a]	54	51.5
			52	51.0
Cytochrome *b*	35	43.9[a]	34	35
Cytochrome c_1	33	27.2[a]	32	33
Rieske Fe-S protein	25	23.1[a]	26	25
14 kDa	14	14.3[a]	15	+[b]
"Hinge" protein	12	7.8[a]	12	+[b]
8.2 kDa	11	8.2[a]	11	+[b]
8 kDa	10	8.0[a]	10	nd[b]
6.7 kDa	6.7	6.7[a]	nd	nd[b]

[a] Molecular mass determined from sequence; all other values are the apparent molecular mass.
[b] +, Indicates subunits identified, but molecular mass not designated. nd, Indicates not detected.

The enzyme from potato tubers and spinach leaves has been reported to contain three Core proteins, 55, 53, and 51 kDa (Braun *et al.*, 1992) and 61, 54, and 52 kDa (Eriksson *et al.*, 1994), respectively, and four Core proteins in wheat and spinach roots, 55.5, 55.0, 51.5, and 51.0 kDa (Braun *et al.*, 1995) and 59, 57, 52, and 50 kDa (Sjoling, 1998), respectively. Occurrence of these multiple forms is unique for plants and differs from the situation reported in fungi and mammals, where only two Core proteins were reported (Bechmann *et al.*, 1992). In yeast, it was suggested that extra polypeptides in the region of the Core proteins were due to the existence of incompletely processed precursors of the Core proteins (Trumpower, 1990). The multiple forms of the Core proteins in plants were, however, shown to represent isoforms of the two Core proteins (Emmermann *et al.*, 1994a; Eriksson *et al.*, 1994; Jansch *et al.*, 1995). The isoforms were shown to exclude each other in individual protein complexes resulting in a simultaneous occurrence of only two Core proteins per monomer of the cytochrome bc_1 complex, as revealed by blue native–gel electrophoresis and immunoprecipitation. Studies with gene-specific oligonucleotides revealed that the genes encoding the isoforms of the Core proteins of potato are differently expressed in separate tissues, but transcript levels do not vary between tissues (Jansch *et al.*, 1995). The biological significance of the occurrence of these isoforms remains to be determined.

Most interestingly, the multiple forms of Core proteins in potato, spinach, and wheat were shown by sequencing (Braun *et al.*, 1992, 1995; Emmermann *et al.*, 1993) and immunological methods (Braun *et al.*, 1992; Emmermann *et al.*, 1993; Eriksson *et al.*, 1993, 1994) to be related to the general mitochondrial-processing peptidase (MPP) subunits from other sources. The striking homologies that the Core subunits exhibit with components of the MPP from fungi and mammals will be discussed in the next section.

Small subunits of the plant cytochrome bc_1 complex were shown to correspond to their nonplant counterparts but some of the subunits show differences in the structure of their targeting signals or in their molecular composition (for review see Braun and

Schmitz, 1995b). The functions of these subunits are not clear in plant or other systems, with the exception of the hinge protein, which enhances electron transfer between cytochromes *c* and c_1 by forming a complex between them. While the homology between the plant subunits is high (e.g., about 75% between potato and wheat for the 14.3 kDa subunit), the homology with the equivalent yeast and beef subunits is low (about 30%), but is evidenced through similar polarity profiles. The exception is the hinge protein in potato, which, in plants, lacks an acidic N-terminal domain considered to be functionally essential in other organisms (Braun and Schmitz, 1995b).

MPP–bc_1 COMPLEX IN PLANTS

Integration of MPP into the bc_1 Complex

MPP purified from *Saccharomyces cerevisiae* (Yang, 1988), *Neurospora crassa* (Hawlitschek *et al.*, 1988) and rat liver (Ou *et al.*, 1989) has been shown to consist of two structurally related subunits, α-MPP and β-MPP, which cooperate in processing. In mammals and yeast, both MPP subunits are soluble in the matrix, whereas in *N. crassa*, 70% of β-MPP can be found as a Core 1 protein of the cytochrome bc_1 complex (Schulte *et al.*, 1989; for review see Neupert, 1997). The original studies of processing activity in plants using crude mitochondrial extracts from *Vicia faba*, cauliflower (Whelan *et al.*, 1988), and spinach mitochondria (Whelan *et al.*, 1991) showed that the plant enzyme revealed antigenic cross-reactivity with the *N. crassa* β-MPP and that the enzyme was specific for mitochondrial precursor proteins.

However, in contrast to nonplant sources, fractionation studies of spinach and potato mitochondria in our laboratory showed that the MPP activity resides in the mitochondrial inner membrane and that the activity could not be dissociated from the membrane using routine procedures for dissociating loosely attached membrane components (Eriksson and Glaser, 1992). The soluble matrix fraction did not stimulate the membrane-located processing activity (Eriksson and Glaser, 1992). Two independent approaches were used to identify the membrane-associated MPP in plants. The first report came from studies with the potato cytochrome bc_1 complex purified by affinity chromatography (Braun *et al.*, 1992; Emmermann *et al.*, 1993), which showed that the complex contained MPP activity and that MPP constituted an integral part of the bc_1 complex of the respiratory chain. The genes encoding the 55, 53, and 51 kDa proteins have been cloned (Braun *et al.*, 1992; Emmermann *et al.*, 1993, 1994a) and shown to be identical to the MPP subunits. The approach in our laboratory, through efforts to isolate the spinach membrane-bound MPP activity by fractionation of mitochondrial membrane components using anion-exchange and gel-filtration chromatography, revealed that the total mitochondrial MPP activity was integrated into the cytochrome bc_1 complex (Eriksson and Glaser, 1992; Eriksson *et al.*, 1993, 1994). In potato, the 55 and 53 kDa proteins are identical to β-MPP and the 51 kDa protein to α-MPP (Braun *et al.*, 1992; Emmermann *et al.*, 1993). In spinach leaves, the 61 kDa protein corresponds to β-MPP and the 54 and 52 kDa proteins to α-MPP (Eriksson *et al.*, 1994) and in spinach roots, the 59 and the 57 kDa to β-MPP and the 52 and 50 kDa to α-MPP (Sjoling, 1998). The cytochrome bc_1 complex in wheat mitochondria was also shown to contain the MPP activity (Braun *et al.*, 1995). The 55.5 and 55.0 kDa proteins in wheat represent the β-MPP and the 51.5 and 51.0 kDa proteins the α-MPP (Braun *et al.*, 1995). Comparing the occurrence of MPP isoforms in different plant mitochondria indicates species-dependent variations in the appearance of the processing components (Emmermann *et al.*, 1994a, 1994; Glaser *et al.*, 1998). More recently, also the cytochrome bc_1 complexes from lower plants, the staghorn fern *Platycerium bifurcatum*, and the horsetail *Equisetum arvense* were shown to process mitochondrial precursor proteins (Brumme *et al.*, 1998). Therefore, it was concluded that integration of MPP into the bc_1 complex of the respiratory chain is a general feature for plants. Figure 1 shows a schematic representation of the localization of MPP in relation to the cytochrome bc_1 complex in different organisms.

The isolated spinach cytochrome bc_1 complex was shown to process three precursor proteins with a different intramitochondrial localization: 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), as well as chimeric precursors and nonplant mitochondrial precursors (see section on Localization of bc_1 Complex in Relation to Import Sites). The processing activity was totally inhibited by EDTA and orthophenanthroline (Eriksson *et al.*, 1994). There was no effect on the processing activity by inhibitors of the serine-,cysteine-, amino-, aspartic-, or thiol-type proteases (Eriksson *et al.*, 1996), or by ATP (Glaser *et al.*, 1996).

The genes encoding the potato MPP have been cloned and shown to be homologous to each other and

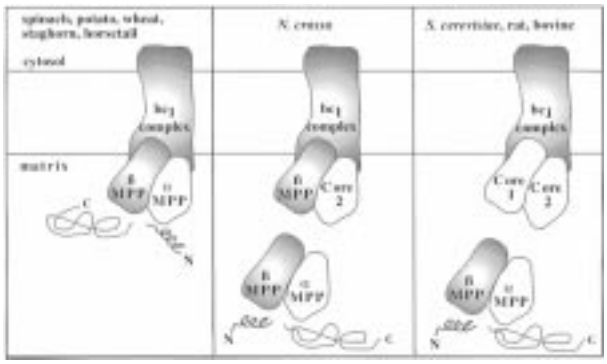

Fig. 1. Localization of the MPP subunits in different organisms. α-MPP and β-MPP subunits are integrated into the cytochrome bc_1 complex in higher and in some lower plants. In *N. crassa*, 70% of β-MPP is found as a Core 1 protein of the cytochrome bc_1 complex, whereas α-MPP is localized in the matrix. In mammals and yeast, both MPP subunits are soluble in the matrix. Subunits of MPP and Core proteins belong to the same family of bifunctional proteins involved in both electron transfer and protein processing. N stands for amino-terminal, C for carboxy-terminal.

to Core proteins of the bc_1 complex of mammals, *N. crassa*, and yeast (Braun *et al.*, 1992; Emmermann *et al.*, 1993). Sequence comparison between the potato MPP subunits and Core proteins and MPP subunits from fungi and mammals revealed the following: the potato α-MPP shares 30–35% sequence identity with α-MPPs and 26–30% with the corresponding Core proteins, whereas, the potato β-MPP shares >40% sequence identity with β-MPPs and 27–36% with the corresponding Core proteins (Braun and Schmitz, 1995b). In *S. cerevisiae* and mammals, both the α-MPP and β-MPP proteins show sequence similarity, but not identity to the respective Core proteins of the bc_1 complex. It is not known whether the plant MPP subunits incorporated into the bc_1 complex of the respiratory chain exhibit the functions ascribed to the Core proteins as assembly and stability factors. It is, however, believed that subunits of MPP and Core proteins belong to the same family of bifunctional proteins involved in both electron transfer and protein processing (for reviews see Glaser *et al.*, 1994; Braun and Schmitz, 1995b).

Properties of MPP as a Consequence of the Integration into the bc_1 Complex

The purified spinach MPP–bc_1 complex has a molecular mass of 550 kDa, corresponding to a dimer (Eriksson *et al.*, 1994, 1996). Increased ionic strength results in partial dissociation of the dimer as well as loss of processing activity, however, it is not known whether the dimeric form is essential for peptidase activity in plants. The *N. crassa*, MPP is also inactivated by both KCl and NaCl (Hawlitschek *et al.*, 1988), whereas the activity of potato MPP has been shown to be stimulated by 1.2 *M* NaCl (Emmermann *et al.*, 1993). The processing activity of the plant MPP–bc_1 complex is remarkably stable. It can be detected over a broad temperature range, 10–50°C, with maximum activity at 35°C. Unlike most metalloproteases that show maximal processing activity at neutral pH, the spinach and potato MPP–bc_1 complex is active over a broad pH range, pH 6–11, and has a pH optimum between 8 and 9 (Eriksson *et al.*, 1996). For the spinach MPP–bc_1 complex, micellar concentrations of nonionic and zwitterionic detergents were shown to stimulate the activity, whereas anionic detergents totally suppressed the activity (Eriksson *et al.*, 1994, 1996). Interestingly, hemin, a potent regulator of mitochondrial and cytosolic biogenesis and an inhibitor of proteosomal degradation, inhibits the processing activity of the spinach MPP–bc_1 complex (Eriksson *et al.*, 1996), indicating that hemin may be involved in the regulation of the processing activity *in vivo*.

The maturation of nuclear-encoded precursor proteins catalyzed by MPP is totally inhibited by metal chelators, EDTA and orthophenanthroline, classifying the enzyme as a metalloendopeptidase. None of the other protease inhibitors, such as PMSF, pefabloc, NEM, E64, pepstatin, and bestatin inhibiting the serine-, cysteine-, amino-, aspartic-, or thiol-type proteases reduced the processing activity catalyzed by the spinach MPP–bc_1 complex (Eriksson *et al.*, 1996). The processing activity of the isolated MPP–bc_1 complex is not dependent on the addition of metals, but the activity of the spinach enzyme is slightly stimulated by Mn^{2+}, Ca^{2+}, or Mg^{2+}. Processing activity is inhibited by 1 μ*M* Zn^{2+} and Cu^{2+} (Eriksson, 1996). Preparation of the apo-MPP followed by reconstitution experiments revealed that the native metal can be replaced by a number of other metals rendering the processing peptidase active (Eriksson, 1996). The requirement of a metal for processing activity implies that, in plants, the bc_1 complex contains an additional metal besides Fe, which is present in respiratory subunits. Direct measurements of the metal content of the spinach MPP–bc_1 complex using particle-induced X-ray emission (PIXE), inductively coupled plasma–atomic emission spectroscopy (ICP–AES), and total reflection X-ray fluorescence (TRXE) show that the spinach MPP–bc_1 complex contains iron, zinc, copper, and calcium (Eriksson, 1996). Data with the yeast

soluble MPP suggest that the enzyme is a Zn^{2+} rather than a Mn^{2+} metallopeptidase (Luciano *et al.*, 1998). The yeast MPP and β-MPP overexpressed in *Escherichia coli* contained 0.86 and 1.05 atoms of Zn^{2+} per molecule, respectively, whereas an enzymatically inactive MPP mutant retained less than 0.2 atom of Zn^{2+} per molecule.

Requirement of the metal for processing activity in plants means that the plant bc_1 complex contains a metal ion bound to the Core proteins. In the 3-D structural data from the bovine bc_1 complex, no metal binding site can be conclusively identified, which could be due to EDTA treatment of the mitochondrial membranes prior to enzyme purification. Metal ion density was not observed in the region near the incomplete zinc binding consensus sequence on the Core subunits (Iwata *et al.*, 1998) (see also the section entitled "What Can We Learn about MPP Specificity from the Structure of the Mammalian bc_1 Complex).

Protein Processing and Electron Transfer Are Independent Events

The bifunctionality of the bc_1 complex of plants, catalyzing both electron transfer and protein processing, raises questions concerning the correlation between bioenergetics and biogenesis. Eriksson *et al.* (1994) reported that the processing activity of the purified spinach MPP–bc_1 complex can be inhibited by 5 μ*M* antimycin A or myxothiazol, however, this inhibition was not correlated to the inhibition of electron transport. Direct measurements of the processing activity at different respiratory states of the submitochondrial particles showed that neither redox state nor turnover of the complex affected processing (Eriksson *et al.*, 1994, 1996). Lack of inhibition of the potato MPP–bc_1 complex by antimycin A and myxothiazol was reported in the presence of Triton X-100 (Emmermann *et al.*, 1993). Complete reduction of the bc_1 complex by reducing agents partially inhibits the processing activity. This effect probably reflects reduction of ligands involved in metal chelation within the complex, leading to loss of the endogenous metal involved in catalysis and a subsequent decrease and loss of processing activity (Eriksson *et al.*, 1994). Inhibition may also be due to reduction of disulfide bridges, which are important for an active conformation of the complex. On the basis of the results discussed above, it can thus be concluded that, despite the bifunctionality of the MPP–bc_1 complex in plants, there appears to be no direct correlation between electron transfer and protein processing.

In fact, the published crystal structure of the beef bc_1 complex revealed the binding sites of both inhibitors: myxothiazol and antimycin A (Xia *et al.*, 1997; Iwata *et al.*, 1998; Zhang *et al.*, 1998). The sites are far removed from the Core subunits making it unlikely that the binding of these inhibitors could effectively induce large conformational changes upon electron transfer that could affect the efficiency of the MPP activity.

Localization of bc_1 Complex in Relation to Import Sites

The presence and nature of the bifunctional MPP–bc_1 complex in plant mitochondria raises another intriguing question. What is the relationship, if any, between the translocation event across the inner mitochondrial membranes and the processing event? Are the presequences cleaved off upon import into mitochondria or does the processing occur after translocation. Definite answers have not been forthcoming, but the question has been addressed using different approaches.

Mitochondria import the majority of their proteins from the cytosol, as they are nuclear encoded and translated in the cytosol. A series of receptor and channel proteins span both the outer and inner membranes, forming the translocase of the outer membrane (TOM) and the inner membrane (TIM). These complexes catalyze recognition and translocation of precursors into the organelle. Figure 2 shows the arrangement of these complexes in plant mitochondria. The precursor is passed presequence-first through the membranes and is translocated into the matrix by the TIM complex in a membrane potential, $\Delta\Psi$, and ATP-dependent manner (for reviews see Schatz, 1996; Neupert, 1997; Glaser *et al.*, 1998).

In an attempt to separate the translocation and processing events, specific inhibitors of MPP activity were used (Whelan *et al.*, 1996). The addition of 1,10-phenanthroline, a metal chelator that can cross both mitochondrial membranes, abolished import of mitochondrial precursors. Other metal chelators such as EDTA, 1,7-phenanthroline, and 4,7-phenanthroline, which cannot cross the mitochondrial membranes, had no effect on import. When processing, a known metal-dependent step inside mitochondria, was inhibited using a mutagenesis approach (changing a -2 arginine

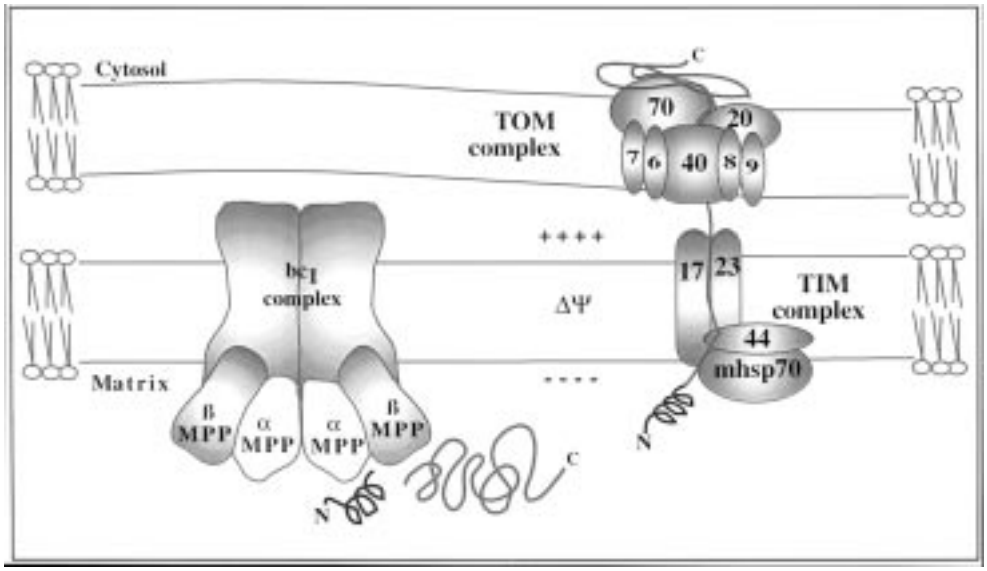

Fig. 2. The MPP/bc_1 complex in relation to import sites in plant mitochondria. The location of the plant MPP–bc_1 complex in the inner mitochondrial membrane is shown in relation to the plant mitochondrial import channel. Processing of precursor proteins to remove the N-terminal presequence occurs after translocation to the matrix has been completed (see section on MPP–bc_1 Complex in Plants). TOM, translocase of the outer membrane; TIM, translocase of the inner membrane.

to a -2 glycine in the presequence of the precursor), so was import. Thus, it would appear that in soybean translocation of proteins across the mitochondrial membrane, as well as processing, relies on a metal-dependent step, which might be the processing of the precursor. The data was interpreted to suggest that import and processing may be directly connected in soybean mitochondria (Whelan *et al.*, 1996).

Recent data from our laboratory using import translocation intermediates (Dessi *et al.*, 1999) provide some new insight. Elegant work in yeast, using specially designed chimeric precursors that could be immobilized in the import channel, showed that the presequence may be cleaved by the matrix soluble MPP when a precursor has been only partially translocated to the matrix (Rassow *et al.*, 1990). Processing occurred when the processing site was exposed to the matrix, implying that processing may occur during translocation. The chimeric constructs consist of the presequence attached to different lengths of the mature N-terminal region of yeast cytochrome b_2 (ranging from 25 to 136 residues), fused to mouse dihydrofolate reductase (DHFR), a cytosolic protein. The C-terminal domain of the chimeric construct, the DHFR domain, can be tightly folded by a specific inhibitor, methotrexate (MTX). Upon import, the folded DHFR domain is trapped at the surface of the outer membrane, blocking the import channel and generating a translocation intermediate. The length of cytochrome b_2 that can project into the matrix depends upon the length of mature protein behind the presequence. Cytochrome b_2 has a presequence 30 residues long. A construct comprising the presequence plus the first 50 residues of the mature protein had the minimum length needed for processing by isolated yeast mitochondria. The 50 residues of mature sequence spanned the double membrane, exposing the presequence processing site to the matrix. Shorter precursors with 35 residues of mature sequence were not processed by yeast, but all constructs containing about 50 residues or more of mature sequence were processed (Rassow *et al.*, 1990).

We have determined that translocation intermediates with mature cytochrome b_2 sequences ranging from 25 to 130 residues, that are immobilized in the membrane, are not processed by isolated plant (spinach and potato) mitochondria (Dessi *et al.*, 1999). In the absence of MTX, the precursors are imported and processed to the expected size, incidentally demonstrating, once again, the universality of mitochondrial targeting sequences and their ability to direct non mitochondrial proteins to the organelle (Roise and Schatz, 1988). Control experiments with a precursor lacking the DHFR domain show that import and processing are unaffected by MTX. Processing of cytochrome b_2–

DHFR precursors with mitochondrial membrane fractions or the purified MPP–bc_1 complex is not inhibited by methotrexate. The inability to process translocation intermediates implies that the MPP–bc_1 complex in plants is distant from the translocation channel and cannot be directly involved in the translocation mechanism.

We conclude, therefore, that presequence removal occurs after translocation and that the translocation of the precursor and processing catalyzed by the cytochrome bc_1 complex in plants are independent events (Fig. 2). This conclusion inevitably raises further questions: is the MPP–bc_1 complex really located so far from the TIM channel or does the preprotein require complete translocation to the matrix to adopt the correct conformation required for processing? Hence, is the processing event dependent not only on the proximity of MPP–bc_1 complex to the TIM channel, but also on the conformational state of the processing site? Further studies into this aspect are underway in our laboratory.

OCCURRENCE OF MATRIX PEPTIDASE IN PLANT MITOCHONDRIA

In spinach root mitochondria, part of the processing activity was located in the soluble fraction and the membrane-bound activity could be disassociated from the membrane by salt treatment (Knorpp *et al.*, 1994). In potato mitochondria fractionated with octylpolyoxyethylene, the processing activity was found only in the membrane fraction (Braun and Schmitz, 1992). However, upon sonication in the presence of salts, part of the activity was found in the soluble fraction (Eriksson and Glaser, 1992).

As a collaborative project between four laboratories, we investigated the occurrence of an additional, matrix-located processing activity (Szigyarto *et al.*, 1998). A matrix-located peptidase specifically processed the precursors to the predicted mature form in a reaction which was sensitive to orthophenanthroline, a characteristic inhibitor of mitochondrial-processing peptidase (MPP). The activity was also inhibited by NEM, an inhibitor of the soluble fungal MPP. The specificity of the matrix peptidase was illustrated by the inhibition of processing of the alternative oxidase precursor in both soybean and spinach matrix extracts upon altering a single amino acid residue in the targeting presequence (-2 Arg to Gly). These results demonstrated that there is an additional specific processing activity in the matrix of soybean and spinach, in addition to the previously well-characterized membrane-bound MPP integrated into the cytochrome bc_1 complex of the respiratory chain (Szigyarto *et al.*, 1998). The activity in soybean corresponds to about 50% of the total processing activity and in spinach it is lower than 20%. The relation of this activity to the activity catalyzed by the Core–MPP subunits of the cytochrome bc_1 complex is not known at present. Occurrence of the matrix-located processing activity is independent of the age of the plant in contrast to protein import efficiency (Huang *et al.*, 1998) that has been shown to decrease during the maturation of pea plants (Wood *et al.*, 1998).

SUBSTRATE SPECIFICITY OF MPP

The integration of the MPP into the cytochrome bc_1 complex in plants requires that several hundred nuclear-encoded mitochondrial precursors have to be in contact with the MPP–bc_1 complex during their biogenesis. A striking feature of MPP is that it is a general peptidase, as it acts on so many mitochondrial precursor proteins, yet MPP is specific as it recognizes a distinct cleavage site on presequences that show no sequence similarity. A great majority of the nuclear-encoded mitochondrial proteins contain presequences that are proteolytically cleaved off after import into mitochondria. Only proteins of the outer mitochondrial membrane and a few small subunits of the oligomeric protein complexes of the inner mitochondrial membrane contain noncleavable signal peptides (for recent reviews see Braun and Schmitz, 1995b; Whelan and Glaser, 1997; Glaser *et al.*, 1998). In nonplant systems, some precursors are cleaved in a two-step process. First, an intermediate-size product is produced by MPP and, subsequently, a matrix-located mitochondrial intermediate peptidase, MIP (Kalousek *et al.*, 1992) or inner membrane peptidase, IMP (Pratje *et al.*, 1994) catalyzes cleavage to the mature size product. MPP catalyzes cleavage of the mitochondrial presequences in a single proteolytic step.

MPP Belongs to the Pitrilysin Family of Proteases

The MPP subunits are encoded by nuclear genes and are synthesized in the cytosol with cleavable presequences, which are proteolytically processed by

already preexisting MPP peptidase inside the mitochondria. Both MPP subunits were shown to be required for catalysis.

Sequence alignments revealed that the MPP–Core proteins belong to a new family of metalloendopeptidases, the Pitrilysin family named after pitrilysin (or protease III), which is an oligopeptidase from the periplasm of *Escherichia coli* (Rawlings and Barrett, 1991). Pitrilysins are highly specific metalloendopeptidases, which recognize their substrates without defined amino acid residues around the scissile bond, indicating that recognition is on the basis of higher-order structure rather than of the amino acid sequence (Anastasi *et al.*, 1993; Becker and Roth, 1993). The $HXXEHX_{74-76}E$ zinc-binding motif, which constitutes the catalytic site in pitrilysins, is conserved in all β-MPPs but degenerate in α-MPPs and Core proteins (Braun and Schmitz, 1995a). Studies with rat (Kitada *et al.*, 1995, 1998) and yeast (Luciano *et al.*, 1997, 1998) β-MPP show that mutations of the histidines and the glutamate in the inverted motif abolished processing, indicating that β-MPP is the catalytic subunit and that the discussed residues are important for activity. The mutagenesis studies of yeast MPP (Luciano *et al.*, 1997) indicate that the whole $HXXEHX_{76}H$ region of β-MPP is important for the proper conformation of the active site and may be in contact with α-MPP. The nonconserved central region of β-MPP surrounding Lys215 involved in the interaction with α-MPP and the C-terminal region of β-MPP surrounding Ser314 are also of importance for the catalysis. However, β-MPP has been shown in fungi to be unable to catalyze processing in the absence of α-MPP, indicating an essential function of α-MPP in processing (Geli, 1993). Cross-linking studies indicate that purified α-MPP bound a precursor protein in the absence of any β-MPP. Furthermore, the interaction of MPP and its subunits with a peptide substrate, as analyzed by surface plasmon resonance, showed that α-MPP bound a peptide substrate as efficiently as MPP, suggesting that the α-MPP is responsible for the binding of mitochondrial presequences (Luciano *et al.*, 1997). It was shown (Shimokata *et al.*, 1998) that truncation of the C-terminal 41 amino acids of α-MPP led to a loss of binding and processing activity. Glu353 of α-MPP is required for processing of all tested precursors, whereas Glu377 and Asp378 are needed only for the processing of precursors with longer presequences. Recent studies showed that MPP bound the substrate peptides with high affinity only in the dimeric complex and each subunit monomer had about a 30-fold less affinity than the complex. The individual subunit required arginines at different positions in the peptide for binding, although their affinities were much lower than that of MPP. Fluorescence quenching analysis showed that the peptide bound to MPP was buried in the enzyme. Thus, both subunits of MPP might be required for the formation of a substrate-binding pocket with multiple subsites lying across them (Kojima *et al.*, 1998).

Recognition of the Processing Site by MPP–bc_1 Complex

How does the MPP recognize such a diversity of mitochondrial precursor proteins? What features determine the MPP cleavage? What are the structural features of the precursor protein that are recognized by the MPP? Is the sequence of a few amino acids around the scissile bond sufficient or do other structural elements contribute to the recognition of the cleavage site? Are there any particular restrictions in plants due to the integration of the MPP subunits into the cytochrome bc_1 complex? These questions have been addressed using several approaches, including statistical analysis of plant presequences for common features, *in vitro* studies of affinity of chemically synthesized targeting peptides for MPP, structural analysis of presequence peptides, and site-directed mutagenesis of precursor proteins.

General Characteristics of the Mitochondrial Presequences

Mitochondrial precursors or, more specifically, their presequences, together with the N-terminal portions of the mature proteins, function as substrates for MPP. The presequences display common features. A collection of all available presequences of nuclear-encoded mitochondrial proteins from the databases contains, at present, over 1000 sequences, including about 100 plant presequences (Glaser *et al.*, 1998). We have studied characteristic features of the presequences using statistical analysis, sequence alignment, and secondary structure predictions (Schneider *et al.*, 1998; Sjoling and Glaser, 1998; Zhang *et al.*, 1999). The plant presequences display common, but also some unique, features when compared with presequences from other sources.

The length of the known presequences varies from 8 (13 in plants) to 121 (85 in plants) amino acid resi-

dues with an average length calculated to be 32 amino acid residues (Schneider *et al.*, 1998). Compared to mammalian, yeast, and *N. crassa* presequences, the plant presequences are on the average 7–9 residues longer. The presequences exhibit a high content of basic and hydroxylated residues (with the exception of histidine), in particular, arginine and serine, but also alanine and leucine and a low content of acidic and aromatic residues. A unique feature of plant presequences is that they have a higher content of serine (17%) compared to yeast (7%), mammals (3%), or *N. crassa* (10%) (Sjoling and Glaser, 1998). Analysis of presequences corresponding to the same protein from different plant species revealed that presequences have higher amino acid identity in the N- and C-terminal regions, than in the central parts of the presequences (Sjoling and Glaser, 1998). The more conserved N- and C-terminal regions of these presequences may correspond to the functional importance of these domains. The N-terminal "import domain" (8–20 amino acids) shows a rather regular alternation between basic and hydrophobic residues and has the potential to form amphiphilic α-helix (von Heijne, 1986). The C-terminal "processing domain," most often contains a cleavage motif for MPP and a secondary structure, which is compatible for processing (Klaus *et al.*, 1996; Sjoling and Glaser, 1998) (see the following sections). Both domains may overlap (for review Sjoling and Glaser, 1998).

Cleavage Motifs

Analysis of the amino acid composition in the region around the cleavage site, the C-terminal part of the presequence, shows a high arginine content, about 40%, at position −2 and −3 from the scissile bond (von Heijne *et al.*, 1989; Gavel and von Heijne, 1990). Based on the classification of the Kohonen network and statistical analysis, the pattern around the R-2 and R-3 can be classified as MPP cleavage motifs R-2 (V/A/S-R-X*A/S) and R-3 (V/A/S-R-X-Y/L*A/S), where * denotes the cleavage site (Schneider *et al.*, 1998). The R-10 motif (V/A/S-R-X*F/L-S/A), typical for MIP cleavage, has also been found in fungal and mammalian, but not in plant presequences (von Heijne *et al.*, 1989; Gavel and von Heijne, 1990; Schneider *et al.*, 1998). The Kohonen networks were unable to extract significant differences between the mitochondrial presequences from the different taxonomic groups, suggesting that the features important for recognition of the cleavage site have been conserved in evolution (Schneider *et al.*, 1998). Although the local arginine motifs, R-2 and R-3, represent parts of the features enhancing precursor processing, the motifs are highly degenerate and can even be found elsewhere in the precursor protein, since arginine residues are present at multiple positions in all proteins. MPP, however, does not cleave at these other sites, making it obvious that the R-3 or R-2 motifs are not sufficient for specific cleavage, but that additional common features are required.

Proximal and Distal Basic Residues and Bend-Inducing Residues N-Terminal to the Cleavage Site

The importance of the arginine residues proximal to the cleavage site has been confirmed experimentally using site-directed mutagenesis in mammalian presequences of malate dehydrogenase (Niidome *et al.*, 1994; Ogishima *et al.*, 1995), adrenodoxin (Ou *et al.*, 1994), ornithine aminotransferase (Song *et al.*, 1996), in yeast presequence of cytochrome b_2 (Klaus *et al.*, 1996), and in the plant presequences of soybean alternative oxidase (Tanudji *et al.*, 1999) and of the tobacco $F_1\beta$ (Sjoling *et al.*, 1999). The arginine residue at position −2 from the cleavage site of the alternative oxidase presequence could not be substituted by a glycine, leucine, alanine, glutamine, threonine, phenylalanine, or by other basic residues such as lysine or histidine without inhibition of processing or processing at an incorrect cleavage site (Tanudji *et al.*, 1999). How important is the position of the proximal arginine residue? The proximal arginine of alternative oxidase does not have to be at position −2 but can also be at position −4 of the cleavage site in order for the precursor to interact correctly with the active site of MPP. The optimal efficiency of processing though, is achieved with a −2Arg. There are plant precursors that are processed, although they lack a proximal arginine residue: the presequence of tobacco $F_1\beta$ does not contain an arginine residue at position −2, but at position −5. The −5Arg could, however, be substituted by a leucine or alanine residue without inhibition of processing.

Beside the proximal arginines, also distal arginines of the cleavage site in the mammalian malate dehydrogenase (Niidome *et al.*, 1994) and preadrenodoxin (Ou *et al.*, 1994) presequence have been shown to be necessary for effective processing. Studies of

plant precursor proteins (Tanudji *et al.*, 1999) show that in soybean alternative oxidase −10 Arg is not important for processing whereas the other basic residues as distant 30 and 35 residues from the cleavage site, affected processing of the soybean alternative oxidase with purified spinach MPP. However, as a triple mutant of the three distal positive residues was processed efficiently, as was a deletion mutant with two of the distal positive residues removed, this suggests that the distal residues play a structural role essential for processing (Tanudji *et al.*, 1999). We concluded from the mutational analysis of the plant presequence that the most proximal arginine is important, but not essential, for processing (Sjoling *et al.*, 1999; Tanudji *et al.*, 1999). In addition, basic residues distant to the cleavage site, even those located at the N-terminus of the presequence, are important for processing. The distal arginine is probably recognized by the MPP as any basic amino acid and promotes the processing, while the proximal amino acid must be arginine and this interacts with the catalytic site on β-MPP and could be the primary determinant for substrate recognition and position for cleavage (Sjoling *et al.*, 1999; Tanudji *et al.*, 1999).

In addition, there is often a proline residue between distal and proximal arginines that may serve as a flexible linker (Thornton *et al.*, 1993; Hammen *et al.*, 1994, 1996b; Niidome *et al.*, 1994). It was shown that when a flexible linker Arg–Gly–Pro was deleted in the presequence of rat liver aldehyde dehydrogenase, the processing activity was inhibited (Thornton *et al.*, 1993). Using intramolecularly quenched fluorescent substrates, it was (Ogishima *et al.*, 1995) demonstrated that at least one proline and glycine between the distal and proximal arginine residues in mammalian malate dehydrogenase is important, while other connecting sequences were dispensable. This would make it possible for the distal basic residues to vary in distance from the cleavage site for interaction with negative charges on MPP. This could facilitate the formation of a specific structure so that the scissile bond can be correctly presented in the enzyme pocket to an active water molecule on the catalytic metal (probably zinc). This has been suggested to be an "induced fitting" (Ogishima *et al.*, 1995) as the substrate peptides lack secondary structure in an aqueous environment.

Residues C-Terminal to the Cleavage Site

Studies with mammalian malate dehydrogenase, showed that MPP had a considerable preference for aromatic and, to a lesser extent, hydrophobic amino acids in the +1 position, indicating that the +1 amino acid could be one of the critical determinants for specific cleavage beside the proximal and distal arginine residues and proline and/or glycine between them (Ogishima *et al.*, 1995). Synthetic peptides that possessed only the distal and proximal arginine residues and phenylalanine at the +1 site in a polyalanine sequence were not cleaved by the peptidase, although they inhibited the peptidase activity. However, when serine was introduced into the C-terminal portions of the sequence, processing was observed (Song *et al.*, 1998). The efficiency of the resultant peptides improved as the number of serine residues was increased. A peptide with serine or histidine at +2 and threonine at +3 was processed most efficiently (Song *et al.*, 1998). Replacement of Trp5 with proline, or of Trp5 and Trp6 at the N-terminus of the mature aspartate aminotransferase with either alanine (Trp5Ala/Trp6Ala mutant) or valine and alanine (Trp5Val/Trp6Ala mutant), allowed import, but interfered with correct processing of the imported protein despite the presence of an intact cleavage site for the processing peptidase (Lain *et al.*, 1998). Tanudji *et al.*, (1999) showed that replacement of serine in +2 position from the cleavage site to glycine or deletion of glutamate at +1, drastically inhibited processing of soybean alternative oxidase. Taken together these results indicate that the processing reaction catalyzed by the MPP depends not only on the N-terminal portion but also on the C-terminal portion from the cleavage site.

Higher Order Structural Elements

Despite the fact that sequences around the MPP cleavage sites are highly degenerate and that the conserved R-2 and R-3 motifs are not present in all presequences, processing by MPP is highly specific. This indicates that the efficiency of processing is determined by additional structural factors. The overall conformation of a precursor protein, or at least the conformation, of the C-terminal region may be important for processing. The MPP belongs to the pitrilysin metal-dependent protease family. Proteases from the pitrilysin family recognize a three-dimensional structure rather than an amino acid sequence around the scissile bond (Anastasi *et al.*, 1993).

Structures of the mammalian signal sequence from mitochondrial proteins that are not processed by the MPP have been determined by two-dimensional

NMR and circular dichroism and were shown to form a long continuous helix through the signal sequence (Hammen *et al.*, 1994). It was suggested that this helix prevented the protein from adopting a conformation that would be necessary for interaction with MPP and processing. Mutational insertions, disrupting the helix and making the signal more flexible, plus insertion of a typical R-3 cleavage site, made two of the mammalian, nonprocessable proteins, processable by rat MPP (Waltner and Weiner, 1995) and spinach MPP (Sjoling *et al.*, 1996). However, the same mutations of thiolase did not make it processable, indicating that a typical cleavage motif, R-2 or R-3, is not the only criterion for efficient processing.

Circular dichroism measurements show that synthetic plant presequence peptides have no detectable structure in aqueous solution, but develop a secondary structure in the presence of detergent micelles or charged lipid vesicles (Hammen *et al.*, 1996a). In addition, we have shown that a peptide derived from the C-terminal part of *N. plumbaginifolia* $F_1\beta$ protein that had high affinity for spinach MPP and efficiently inhibited processing was shown to contain a helical region (Sjoling *et al.*, 1994, 1996). Circular dichroism studies and secondary predictions showed that other synthetic peptides derived from the C-terminal part of the precursor that had much lower affinity for spinach MPP did not have this helical region (Sjoling *et al.*, 1996), indicating that the helical region in the C-terminal part could be important for specificity or processing efficiency. To reduce the ability of the $F_1\beta$ peptide to form a helix, we synthesized a peptide replacing the serine residue with a proline in the helical region. The helical content of this peptide was 45% less compared with the wild-type peptide (Sjoling *et al.*, 1999). The mutant peptide did not inhibit processing, showing that it had lower affinity for MPP. Furthermore, mutants were designed to affect the secondary helical structure of the $F_1\beta$ presequence. An exchange of proline at position -12 for a leucine was predicted to result in an extended helix. This proline may serve as a flexible linker between the basic residues (Niidome *et al.*, 1994) positioning the presequence on MPP. Substitution of arginine at position -19 by a glycine (R-19G) was predicted to abolish this helical region. Both constructs were processed less efficiently (Sjoling *et al.*, 1999).

Secondary structure predictions of plant presequences from the same collection used for analysis of amino acid distribution (Schneider *et al.*, 1998; Sjoling and Glaser, 1998) using nnPredict software, show that there is a common secondary structure around the scissile bond. This structure consists of a helix, or a helix followed by an extended stretch, located in the C-terminal region of the presequence in front of the known cleavage site. This predicted motif can be found in at least 50% of the plant presequences (Sjoling, 1998).

These results show that higher-order structural elements upstream of the cleavage site are important for processing by MPP. The α-MPP, which probably recognizes a three-dimensional motif adopted by the presequence, presents the presequence to β-MPP (Luciano and Geli, 1996). The structural element helix-turn-helix, adopted by cleavable presequences in a membrane-mimetic environment, may be required for processing, but is not sufficient for proteolysis. Presequence binding by α-MPP tolerates a high degree of mutations in the presequence. α-MPP may present a degenerated cleavage site motif to β-MPP in an accessible conformation for processing (Luciano and Geli, 1996), however, the conformation of mitochondrial presequences bound to MPP remains, at present, largely unknown.

In summary, at present the known determinants for recognition of the processing site include proximal arginines, distal positively charged residues, flexible linkers, and secondary structure elements, such as a helix, followed by an extended conformation on the N-terminal side from the cleavage site and also residues on the C-terminal side from the cleavage site. All these factors have potential to facilitate the recognition event.

WHAT CAN WE LEARN ABOUT MPP SPECIFICITY FROM THE STRUCTURE OF THE MAMMALIAN bc_1 COMPLEX?

The recently solved 3-D structure of the mammalian bc_1 complex at 2.8 A resolution opens possibilities to model the interaction between the plant MPP and precursor given that there exists structural similarity between Core proteins of the beef cytochrome bc_1 complex and the Core–MPP subunits of the plant cytochrome bc_1 complex. Alignment of the bovine Core 1 protein and potato β-MPP reveals that there exists a 43% sequence identity. Furthermore, a degenerate version of the potentially active site of MPP, the inverted zinc-binding motif, can be found in the Core 1 protein of the bovine bc_1 complex, (Xia *et al.*, 1997) with the first histidine replaced by tyrosine. Glu137

may interact with Tyr57 and His61 (Deng *et al.*, 1998). This site may well be the active site of MPP.

No MPP activity is detected in bovine heart cytochrome bc_1 complex (Deng *et al.*, 1998), neither in the chicken bc_1 complex (E. Glaser, unpublished). However, when the bovine complex is treated with increasing concentrations of Triton X-100, the peptidase activity increases (Deng *et al.*, 1998). Activated MPP is completely inhibited by metal ion chelators, such as EDTA and *o*-phenanthroline and partially inhibited by myxothiazol (58%), ferricyanide (28%), and dithiothreitol (81%) in accordance with the situation in spinach (Eriksson *et al.*, 1996). The metal ion chelator-inhibited activity can be partially restored by the addition of divalent cations, indicating that a metal ion is required for MPP activity. The cleavage site specificity of activated MPP depends more on the length of amino acid sequence from the mature protein portion and less on the presequence portion, when a synthetic peptide composed of NH_2-terminal residues of a mature protein and the COOH-terminal residues of its presequence is used as a substrate (Deng *et al.*, 1998). These results show that the bovine bc_1 complex has a latent MPP activity. On the basis of the 3-D structure, it was concluded that the lack of MPP activity may be due to binding of an inhibitor polypeptide to the active site of MPP located at the interface of Core subunits 1 and 2 (Deng *et al.*, 1998).

Indeed in the complete structure of the bovine cytochrome bc_1 complex of Iwata *et al.* (1998), subunit 9 corresponding to the 78 amino acid-long presequence of the Rieske iron–sulfur protein is found between Core 1 and Core 2 subunits. The location of subunit 9 close to the possible catalytic site suggests that it is cleaved from the Rieske iron–sulfur protein by the Core proteins directly. The structural data show that the binding site of subunit 9 is predominantly on Core protein 2. The C-terminal 15 amino acid residues of subunit 9 form an extended β-sheet together with two β-strands of the N-terminal domain of the Core 2 subunit. Specific interactions are observed for all three consensus residues Arg 62, Leu64, and Ser 67 of subunit 9 with MPP (Brandt *et al.*, 1993). The location of the potential metal binding motif is not clear. Tyr57, Glu60, His61, and Glu137 are found in close vicinity and may be involved in this interaction. Crystallography data indicates that large structural rearrangements must occur after cleavage of the presequence as the N-terminus of the Rieske iron–sulfur protein and the C-terminus of subunit 9 are distant (Iwata *et al.*, 1998). The Rieske iron–sulfur protein from potato, a 25 kDa protein, is made as a larger precursor of 30 kDa, which is processed by the MPP–cytochrome bc_1 complex. The 53 amino acid-long presequence is removed in one processing step, as in mammals, but in contrast to two-step processing in fungi (Emmermann *et al.*, 1994b; Braun and Schmitz, 1995b), however, there is no evidence that it remains as a distinct subunit in the plant MPP–bc_1 complex.

Assuming that the structure of the bovine Core proteins correlates with the plant MPP–Core protein structure, we can anticipate that the plant presequence has to be flexible enough to reach into the cavity of the Core–MPP subunits to the active site. Indeed, it has been shown that presequences have to be flexible in order to be processed (see section on Proximal and Distal Basic Residues and Bend-Inducing Residues N-Terminal to the Cleavage Site; see also Higher-Order Structural Elements). We have also shown that the secondary structure of the presequence is important for affinity to MPP and, thereby, also for cleavage. Why is the predicted helix motif, or helix extended motif, more pronounced in plant presequences than in other species? The Core 1 and Core 2 proteins have a bowllike structures, that together form a ball enclosing a cavity, with a crack leading into the cavity. It is possible that the presequence of the plant precursor protein preferably has a C-terminal part that is flexible enough to reach into this cavity. The predicted helix-extended structure found in most plant presequences would facilitate binding to the MPP. Together with basic residues, the secondary structure could induce a fitting of the scissile bond to the active center. An electrostatic interaction between negatively and positively charged faces could contribute to the specific recognition of the presequence by MPP.

EVOLUTIONARY MODELS FOR INTEGRATION OF MPP INTO THE CYTOCHROME bc_1 COMPLEX

Why is plant mitochondrial MPP attached to the bc_1 complex? What is the origin of MPP? Is incorporation of the MPP into the cytochrome bc_1 complex in plants an ancestral event in relation to the matrix localization of MPP in yeast and mammals? Bacteria have a bc_1 complex that lacks Core proteins. Braun and Schmitz (1995a) suggested that the different location of MPP in various organisms may represent divergence from a single original evolutionary event. The evolution of the MPP and Core subunits could have

started with an ancestral prokaryotic protease that was hydrophilic and located in the cytosol of bacteria. During endosymbiosis, the processing peptidase might have become attached to the membrane as it was advantageous for the function of the early MPP to be located close to the protein import sites. Alternatively, the bc_1 complex might have been dependent on new subunits exposed to the matrix for protection from the matrix proteases (Boumans *et al.*, 1997). The detachment of MPP from the bc_1 complex in yeast and mammals could reflect the necessity for independent regulation of respiration and mitochondrial import. The extra subunits of the bc_1 complex could have become necessary for protection against proteolytic degradation and for assembly of the complex (Schoppink, 1989) and, therefore, another gene duplication resulted in the Core proteins without catalytic activity and soluble MPP in the matrix. The Core proteins in yeast and mammals would, in this situation, be evolutionary relics of the processing peptidase (Braun and Schmitz, 1995a).

However, processing activity is independent of respiration (Eriksson *et al.*, 1994, 1996) and, therefore, there was no necessity for the MPP subunits to become detached from the bc_1 complex in yeast and mammals. Therefore, it cannot be eliminated that evolution of the MPP subunits could have started with a prokaryotic ancestor where a proteolytic activity was integrated into the primitive bc_1 complex before the acquisition of the Core proteins (Eriksson, 1996).

To elucidate the evolution of MPP, processing activity was investigated in lower eukaryotic organisms. In a lower eukaryotic photosynthetic organism *C. reinhardtii*, *in vitro* processing studies revealed that, in contrast to the situation in higher plants, and in accordance with studies in yeast and mammals, the processing of the precursors was catalyzed by a matrix-located peptidase and not by a peptidase integrated into the cytochrome bc_1 complex of the respiratory chain (Nurani *et al.*, 1997).

The cytochrome bc_1 complexes from lower plants, the staghorn fern *P. bifurcatum*, the horsetail *E. arvense*, and from the colorless algae *Polytomella* were purified by a microisolation procedure based on blue-native polyacrylamide gel electrophoresis and electroelution and tested for processing activity (Brumme *et al.*, 1998). The cytochrome bc_1 complexes from *P. bifurcatum* and *E. arvense* were shown to efficiently process mitochondrial precursor proteins, whereas the enzyme complex from *Polytomella* lacked proteolytic activity. Authors suggested that there might be a correlation between the presence of an active MPP within the cytochrome bc_1 complex and the occurrence of chloroplasts in lower eukaryotes (Brumme *et al.*, 1998).

It has recently been shown that integration of MPP into the bc_1 complex is perhaps not unique for plants. The Core proteins of the beef heart cytochrome bc_1 complex can process precursor peptides *in vitro* if an inhibitory subunit is removed by washing (as discussed in earlier in the section on "What Can We Learn About MPP Specificity from the Structure of the Mammalian bc_1 Complex"). It is not known whether the beef bc_1 complex actually has processing activity *in vivo* during different developmental or physiological states. Nonetheless, integration of processing activity within the bc_1 complex may be common among eukaryotes and may reflect an evolutionary importance of the cytochrome bc_1 complex.

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