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The Bifunctional Cytochrome *c* Reductase/Processing Peptidase Complex from Plant Mitochondria

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Cytochrome *c* reductase from potato has been extensively studied with respect to its catalytic activities, its subunit composition, and the biogenesis of individual subunits. Molecular characterization of all 10 subunits revealed that the high-molecular-weight subunits exhibit striking homologies with the components of the general mitochondrial processing peptidase (MPP) from fungi and mammals. Some of the other subunits show differences in the structure of their targeting signals or in their molecular composition when compared to their counterparts from heterotrophic organisms. The proteolytic activity of MPP was found in the cytochrome *c* reductase complexes from potato, spinach, and wheat, suggesting that the integration of the protease into this respiratory complex is a general feature of higher plants.

KEY WORDS: Cytochrome *c* reductase; bc_1 complex; respiratory chain; mitochondrial processing peptidase; protein import; mitochondria.

1. INTRODUCTION

The mitochondrial cytochrome *c* reductase, also called the bc_1 complex, is the middle segment of the respiratory chain and transfers electrons from ubiquinol to cytochrome *c*. In parallel to the oxidoreduction it contributes to the chemiosmotic gradient across the inner mitochondrial membrane in translocating protons from the matrix to the intermembrane space. The coupling between electron transfer and protonmotive force presumedly relies on a cycle that involves reduction of ubiquinone and re-oxidation of ubiquinol on opposite sites of the membrane. The precise mechanism is proposed by a model called the "Q cycle" (Mitchell, 1976; Trumpower, 1990a; Brandt and Trumpower, 1994).

The structural basis for the enzymatic activity of cytochrome *c* reductase is a membrane-bound oligomeric protein complex, which is best characterized in fungi and mammals. The bc_1 complex from yeast and beef was shown to comprise 10 subunits with molecular masses between 6 and 50 kDa (Schägger *et al.*, 1986; Brandt *et al.*, 1994): (i) the "respiratory subunits" cytochrome *b*, cytochrome c_1, and the "Rieske" iron-sulfur protein that are directly involved in electron transfer; (ii) two large subunits termed "core" protein I and II that were originally thought to form the center of the protein complex (meanwhile they were shown to be localized in the periphery on the matrix-exposed side of the respiratory enzyme); and (iii) five "small" subunits with molecular masses below 15 kDa. In beef the presequence of the "Rieske" iron-sulfur subunit is retained in the bc_1 complex after proteolytic cleavage of the precursor protein and is considered to be an eleventh subunit (Borchart *et al.*, 1985; Brandt *et al.*, 1993). In bacteria bc_1 complexes only comprise the three respiratory subunits and sometimes one additional small protein, but nonetheless exhibit a comparable enzymatic activity in respiration (Trumpower, 1990b). Hence the occurrence of the core proteins and the five small subunits seems to be an adaptation of the eukaryotic cell that is not entirely understood so far.

Cytochrome *c* reductase from plants was characterized in potato (Ducet and Diano, 1978; Berry *et al.*, 1991; Braun and Schmitz, 1992; Braun *et al.*, 1994a),

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sweet potato (Nakajima *et al.*, 1984; Berry *et al.*, 1991), Jerusalem artichoke (Degli Esposti *et al.*, 1985; Spinelli and Zannoni, 1987), maize (Hawkesford and Leaver, 1987; Hawkesford *et al.*, 1989), red beet (Berry *et al.*, 1991), spinach (Eriksson *et al.*, 1994), and wheat (Pfeiffer *et al.*, 1990; Braun *et al.*, 1995). However, most information on the plant bc_1 complex comes from analysis of the enzyme from potato, which was the first to be isolated in pure and active form (Berry *et al.*, 1991; Braun and Schmitz, 1992) and from which the sequences of all 10 subunits have been determined (Jänsch *et al.*, 1995 and references within). Interestingly the cytochrome *c* reductase complex from potato not only transfers electrons, but also contains the activity of the mitochondrial processing peptidase (MPP; see Braun *et al.*, 1992a, 1993; Emmermann *et al.*, 1993a). The proteolytic properties of the potato bc_1 complex are very similar to the MPP activity from fungi and mammals, which is localized in the mitochondrial matrix (Hawlitschek *et al.*, 1988; Yang *et al.*, 1988; Ou *et al.*, 1989). Two subunits of cytochrome *c* reductase from potato, which seem to substitute for the two core proteins, structurally resemble the two components of MPP from other organisms (termed α-MPP and β-MPP; see Kalousek *et al.*, 1993). In yeast and mammals the core proteins and the subunits of MPP also exhibit sequence similarity, but are distinct proteins.

There is an enormous amount of data on the bc_1 complex from fungi, mammals, and prokaryotes, which are summarized in excellent overview articles (Weiss, 1987; Trumpower, 1990b; Bechmann *et al.*, 1992). This review will concentrate on the bc_1 complex from higher plants and only cover the corresponding complexes from other organisms where helpful for the understanding of the plant enzymes. In the first parts of this article we focus on cytochrome *c* reductase from potato, on its enzymatic activities, and on the structure, function, and mitochondrial targeting of its 10 subunits. In the last part we briefly go through the data on the bc_1 complexes from other higher plants.

2. CYTOCHROME *c* REDUCTASE FROM POTATO

Two isolation procedures proved to be suitable for the preparation of highly pure and active cytochrome *c* reductase from potato (Berry *et al.*, 1991; Braun and Schmitz, 1992, 1995a). Starting point for both methods are purified mitochondria. The first procedure involves solubilization of mitochondrial protein by dodecyl maltoside and preparation of the respiratory enzyme by ion exchange and hydroxyapatite chromatography. The second method is based on the binding of cytochrome *c* reductase to cytochrome *c*. It involves solubilization of mitochondrial membrane proteins by Triton X-100 and isolation of the enzyme complex by affinity chromatography, ultrafiltration, and gel filtration. Cytochrome *c* reductase from potato prepared by both procedures is very stable.

2.1. Catalytic Activities of Cytochrome *c* Reductase from Potato

The turnover number of isolated ubiquinol:cytochrome *c* oxidoreductase from potato tubers varies between 50–200 s^{-1} (Berry *et al.*, 1991; Braun and Schmitz, 1992). As pointed out by Berry *et al.* (1991), this variability in turnover of the purified enzyme is related to phospholipid content. Preparations of the complex containing 5–6 mol of phospholipid per mole of complex are three times more active than preparations with an equimolar lipid content. The elevated activity level of the more lipid-rich preparations is only observed when optimal detergent concentrations are chosen. Without detergent the isolated complex is inactive probably because a hydrophobic phase is needed around the quinone binding site. Antimycin A and myxothiazol inhibit the enzymatic activity of the potato complex stoichiometrically presumedly by binding to one of the redox centers of cytochrome *b*. In the context of proton translocation a quinol/quinone transhydrogenation reaction was characterized in *Neurospora* (Zweck *et al.*, 1989). This activity was also monitored in the cytochrome bc_1 complex from potato. Using a molar absorbance coefficient of 9.1 mM^{-1} cm^{-1} the turnover number of the reaction is 16 s^{-1} for the potato enzyme (Braun and Schmitz, 1992).

The proteolytic activity of cytochrome *c* reductase from potato cleaves off the mitochondrial targeting signals of precursor proteins upon their import into the organelle (Fig. 1). As detailed below, both subunits of the mitochondrial processing peptidase form part of the bc_1 complex from potato. Conditions to separate the proteolytic subunits from the enzyme complex cause loss of processing activity (Emmermann *et al.*, 1993a). The complex integrated processing activity tolerates high salt conditions. While the mitochondrial processing peptidase from fungi and mammals is inac-

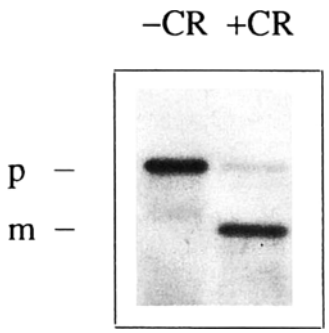

Fig. 1. Processing activity of isolated cytochrome *c* reductase from potato. The precursor of the β-subunit of F_1-ATPase from tobacco was synthesized *in vitro* in the presence of ^{35}S-methionine and treated with or without cytochrome *c* reductase (+CR/−CR) from potato. The radiolabelled protein was subsequently resolved by SDS/PAGE and visualized by fluorography. In the presence of cytochrome *c* reductase the precursor (p) is converted to its mature form (m).

tivated by 150 mM NaCl, the potato complex exhibits full processing activity in the presence of 1 M NaCl (Emmermann and Schmitz, 1993). Inhibitors of cysteine, serine, or aspartate proteases like NEM, PMSF, or leupeptin do not reduce the proteolytic activity of the potato bc_1 complex. However, the potato processing peptidase is very sensitive toward chelators of divalent cations like EDTA and *o*-phenanthroline and therefore is probably a metalloprotease. While the processing activity of the soluble mitochondrial protease from fungi and mammals depends on the addition of Mn^{2+} or Zn^{2+} ions, the potato bc_1 complex depleted from loosely bound ions still exhibits processing activity toward mitochondrial precursors. It is conceivable that a tightly bound metal ion is associated with the proteolytically active subunits of the complex. Unlike most metalloproteases that show maximal processing activity at neutral pH, the processing peptidase from potato has a pH optimum between 8 and 9 (Emmermann and Schmitz, 1993).

An interesting question concerns the physiological consequences of the physical relation between respiratory and proteolytic polypeptides. Inhibition of electron flow with antimycin A or myxothiazol does not affect the proteolytic activity of the potato enzyme (Emmermann and Schmitz, 1993). On the other hand, it has been reported that high levels of these inhibitors reduce processing activity of the spinach complex (Eriksson *et al.*, 1994). However, there is obviously no physiologically significant interrelation between both activities as complexes depleted of the iron-sulfur subunit still retain full processing activity (Emmermann, Braun, and Schmitz, unpublished).

2.2. Composition of Cytochrome *c* Reductase from Potato

Cytochrome *c* reductase from potato is composed of several subunits, which can be separated by SDS/PAGE (Fig. 2). As the number of visible subunits depends on the gel system applied, the composition of the protein complex was not elucidated until recently (Jänsch *et al.*, 1995). Glycine-SDS/PAGE according to Laemmli (1970) allows the resolution of 10 major components: three large polypeptides (55, 53, and 51 kDa) that correspond to the core proteins from fungi and mammals, three bands between 20 and 40 kDa that are the counterparts of the respiratory proteins from other organisms, and four small proteins with molecular masses below 20 kDa. As the 55- and 53-kDa proteins turned out to be alternative isoforms of the same subunit (Jänsch *et al.*, 1995), glycine-SDS/PAGE reveals a cytochrome *c* reductase complex with nine distinct proteins. Tricine-SDS/PAGE according to Schägger and von Jagow (1987) is not capable of separating the 53- and 55-kDa isoforms but allows one to resolve a very small subunit of only 6.7 kDa that is not separated by the Laemmli system (Jänsch *et al.*, 1995). Hence cytochrome *c* reductase from potato is composed of 10 different subunits. In addition, both gel systems reveal some substoichiometric protein bands (Fig. 2). A faint band at 50 kDa, which is only resolved on glycine-SDS/PAGE, was identified as an isoform of the 51-kDa subunit (Emmermann *et al.*, 1994a). The band at 40 kDa represents a degradation product of the 53-kDa subunit and the 5.7-kDa band on Tricine gels is a fragment of the 6.7-kDa protein (Braun and Schmitz, unpublished).

2.3. Subunits of Cytochrome *c* Reductase from Potato

The structure and composition of cytochrome *c* reductase from beef and yeast was extensively studied, and sequencing of the subunits of the protein complexes was completed recently (Gencic *et al.*, 1991; Brandt *et al.*, 1994). Meanwhile, all 10 subunits of cytochrome *c* reductase from potato were characterized and their primary structures were determined by direct sequence analysis and sequencing of corresponding

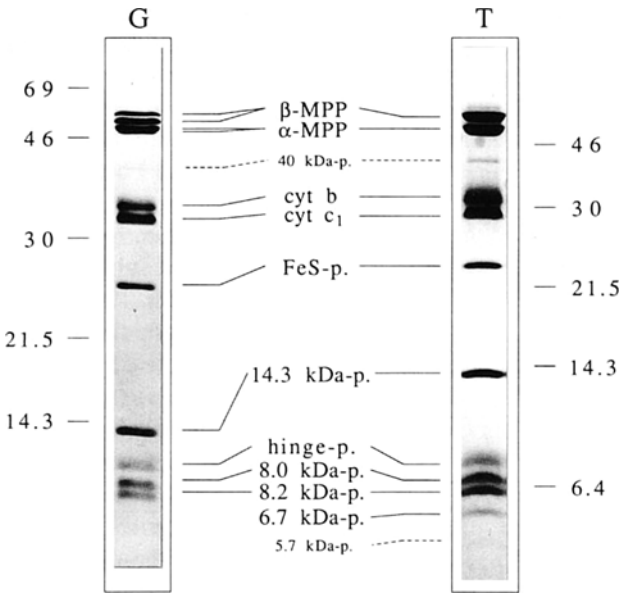

Fig. 2. Separation of the subunits of isolated cytochrome *c* reductase from potato by SDS/PAGE using the glycine (G) and the Tricine (T) buffer system according to Laemmli (1970) and to Schägger and von Jagow (1987), respectively. The gels were stained with Coomassie blue. The numbers on the right and left indicate the sizes of standard proteins and the designations in the middle refer to the identities of the ten subunits (p. = protein, cyt = cytochrome). The bands at 40 and 5.7 kDa represent degradation products of β-MPP and the 6.7-kDa protein, respectively.

cDNA clones (Braun *et al.*, 1994a; Jänsch *et al.*, 1995). In the following paragraphs we review the data on the 10 subunits from potato and provide a comparative analysis with the corresponding proteins from yeast and beef. Since no common nomenclature for the subunits of cytochrome *c* reductase from potato, yeast, and beef has been established so far, their designations and their calculated molecular weights are summarized in Table I. Some if not all subunits of cytochrome *c* reductase from potato are present in isoforms, which also will be discussed. However, until now there are no data showing that any of these isoforms occur in a tissue- or organ-specific manner and no differential activities can be attributed to them. Possibly these isoforms simply reflect the tetraploidy of potato.

2.3.1. The β-MPP Subunit from Potato

The largest subunit of cytochrome *c* reductase from potato is present in two isoforms, which have calculated molecular weights of 56,204 and 56,190 Da (Emmermann *et al.*, 1993a; Emmermann and Schmitz, 1995). If analyzed by electrophoresis on 14% acrylamide gels according to the protocol of Laemmli, the two proteins can be separated and exhibit apparent molecular weights of 55 and 53 kDa (Braun and Schmitz, 1992). Both isoforms are present in potato tubers, stems, and roots, the 55-kDa protein being always less abundant (Braun and Schmitz, unpublished results). The two forms of this subunit exclude each other in individual protein complexes (Jänsch *et al.*, 1995). They exhibit more than 70% sequence conservation and differ by only two amino acids in length (Emmermann *et al.*, 1993a). Nevertheless the two proteins can be distinguished by an antiserum raised against the 53-kDa band (Braun and Schmitz, 1992). Both polypeptides strongly crossreact with antibodies against the core proteins from *Neurospora* or against the β-MPP subunit from yeast (Braun and Schmitz, 1992; Emmermann *et al.*, 1993b). Sequence comparison between the proteins from potato and the core I proteins from beef and yeast reveals 36 and 27% identity, respectively (Table II). The highest degree of

Table I. Composition of the Cytochrome *c* Reductase Complexes from Potato (p), Beef (b), and Yeast (y)[a]

Subunit	Calculated molecular weight		
	Potato	Beef	Yeast
Core protein I (b,y), β-MPP (p)	56.2	49.2*	47.4
Core protein II (b,y) α-MPP (p)	51.9	46.5	38.7
Cytochrome *b*	43.9	42.6	43.7
Cytochrome c_1	27.2	27.3	27.8
Iron-sulfur protein	23.1	21.6	20.1
SU VI (b), SU VII (y), 14 kDa (p)	14.3	13.3	14.4
"Hinge"-protein (b,p), SU VI (y)	7.8	9.2	14.5
9.5 kDa (b), SU VIII (y), 8.2 kDa (p)	8.2	9.5	10.8
7.2 kDa (b), SU IX (y), 8.0 kDa (p)	8.0	7.2	7.3
6.5 kDa (b), SU X (y), 6.7 kDa (p)	6.7	6.5	8.5
Presequence of the FeS-protein (b)	—	8.0	—
Σ	247.3	240.9	233.2

[a] If the nomenclature of a subunit varies between the three organisms, several designations are given (SU = subunit). The calculated molecular weights are based on the sequences of the mature proteins. All sequences are available in the Swissprot or Genbank sequence databanks. The accession numbers (from top to bottom) are: (i) potato: X80235, P29677, P29757, P25076, P37841, X79276, X79273, X79275, X79274, X82325; (ii) beef: P31800, P23004, P00157, P00125, P13272, P00129, P00126, P13271, P00130, P07552, P07588; (iii) yeast: P07256, P07257, P00163, P07143, P08067, P00128, P00127, P08525, P22289, P37299. The molecular weight of the core protein I from beef (*) is taken from electrospray ionization mass spectrometry (Musatov and Robinson, 1994) as the sequence of a corresponding cDNA clone presumedly does not encode the entire protein.

Table II. Percent Sequence Identity between the Subunits of Cytochrome *c* Reductase from Potato (p), Beef (b), and Yeast (y)[a]

Subunit	Percent identity			
	Potato/ Beef	Potato/ Yeast	Beef/ Yeast	Ø
Core protein I (b,y), β-MPP (p)	36	27	30	31
Core protein II (b,y) α-MPP (p)	30	26	26	27
Cytochrome *b*	53	51	51	52
Cytochrome c_1	59	55	58	57
Iron-sulfur protein	47	52	55	51
SU VI (b), SU VII (y), 14 kDa (p)	37	22	36	32
"Hinge"-protein (b,p), SU VI (y)	38	31	40	36
9.5 kDa (b), SU VIII (y), 8.2 kDa (p)	18	23	23	21
7.2 kDa (b), SU IX (y), 8.0 kDa (p)	29	23	36	29
6.5 kDa (b), SU X (y), 6.7 kDa (p)	25	28	23	25
Ø	37.2	33.8	37.8	

[a] The calculations are based on pairwise alignments of corresponding sequences by the GAP program of the Genetics Computer Group software package using a gap weight of 3.0 and a length weight of 0.1. The average identity for each subunit is given in the right column and the overall identity between the cytochrome *c* reductase complexes from the three organisms is given at the bottom of the table (SU = subunit).

sequence conservation is found between the potato proteins and β-MPP from fungi and mammals (>40%; Emmermann *et al.*, 1993a). The two proteins from potato comprise the inverse zinc binding site His-Xxx-Xxx-Glu-His, which is thought to be the active site of β-MPP and which is absent in the core I proteins from yeast and mammals (Braun and Schmitz, 1995b). Hence the two forms of the largest subunit of cytochrome *c* reductase from potato are considered to have proteolytic activity and were named βI-MPP and βII-MPP. The function of the core I proteins from yeast and mammals is not entirely understood, but they seem to be essential for the assembly of the respiratory protein complex (Tzagoloff *et al.*, 1986; Oudshoorn *et al.*, 1987, Crivellone *et al.*, 1988; Gatti and Tzagoloff, 1990). Recently it was proposed that the core proteins are relics of a processing peptidase that was integrated into the bc_1 complex during early stages of endosymbiosis (Braun and Schmitz, 1995b).

The β-subunits of MPP from potato are hydrophilic proteins that are presumedly localized on the matrix side of the enzyme complex, as shown for the core proteins from other organisms (Karlsson *et al.*, 1983; Gonzalez-Halphen *et al.*, 1988). However, they are tightly bound to cytochrome *c* reductase and cannot be cleaved off from the complex by various detergents and strongly denaturing salts (Emmermann *et al.*, 1993a).

2.3.2. *The α-MPP Subunit from Potato*

The second largest subunit of cytochrome *c* reductase from potato, which is the third largest protein band (51 kDa) of the enzyme complex upon analysis by glycine-SDS/PAGE, has a calculated molecular weight of 51.9 kDa (Braun *et al.*, 1992a). Sequence comparison between the potato subunit and proteins from fungi and mammals reveals 26–30% identity with core II proteins and 30–35% identity with α-MPPs. The protein from potato comprises a sequence motif of 20 amino acids, which is completely conserved between α-MPP from *Neurospora*, yeast, and rat and which has been implicated in the function of the α-subunit of

MPP (Schneider *et al.*, 1990; Kleiber *et al.*, 1990). Therefore the second largest subunit of cytochrome *c* reductase from potato is probably involved in the proteolytic activity of the enzyme complex and was named α-MPP. Together with β-MPP it forms the complex integrated heterodimeric processing peptidase from plant mitochondria. Recently an isoform of α-MPP was characterized, which is only weakly expressed in all potato tissues analyzed (Emmermann *et al.*, 1994a). It is termed αII-MPP, has a calculated molecular weight of 51.7 kDa, and exhibits 85% identity to α(I)-MPP. Upon analysis of isolated cytochrome *c* reductase from potato by glycine-SDS/PAGE, αII-MPP can be detected as a faint band at 50 kDa just below the 51-kDa band of αI-MPP. Like the β subunits of MPP, the potato α-MPP proteins are hydrophilic and assumed to be peripherally localized on the matrix side of the enzyme complex. Treatment of isolated cytochrome *c* reductase with 1 M guanidinium hydrochloride or 2 M NaBr dissects α-MPP and two of the small subunits from the protein complex (Emmermann *et al.*, 1993a; Braun and Schmitz, 1995a).

2.3.3. *Cytochrome b from Potato*

Cytochrome *b* is a very hydrophobic protein that forms the center of cytochrome *c* reductase (reviewed in Degli Esposti *et al.*, 1993). Two heme groups are attached noncovalently to this protein, a "high-potential" heme b_H and a "low-potential" heme b_L. Difference spectra for cytochrome *b* from potato reveal an α-band for heme b_H at 559.8 nm and a split α-band for heme b_L at 557.6 and 565.6 nm (Berry *et al.*, 1991). Potato apocytochrome *b* has a calculated molecular mass of 43.9 kDa (Zanlungo *et al.*, 1991; Braun and Schmitz, 1993). The apparent molecular mass of the subunit is significantly smaller—as often reported for hydrophobic proteins—and depends on the acrylamide concentration of the gel: on 18% gels it migrates at 37 kDa, on 9% gels only at 30 kDa and thereby even faster than cytochrome c_1 (Berry *et al.*, 1991). Cytochrome *b* from potato is mitochondrially encoded and its transcripts are edited at several sites (Zanlungo *et al.*, 1993). The sequence of potato cytochrome *b* is more than 50% identical to those from beef and yeast (Table II). Cytochrome *b* and the "Rieske" iron-sulfur protein (see below) are the only subunits of cytochrome *c* reductase for which the primary structure is known in higher plants besides potato. The identity between cytochrome *b* from different plants is generally higher than 90% (Hauska *et al.*, 1988).

2.3.4. *Cytochrome c_1 from Potato*

Cytochrome c_1 is located on the intermembrane space side of the inner mitochondrial membrane and passes electrons to cytochrome *c*. It is a hydrophilic protein that is anchored in the membrane by a short stretch of hydrophobic amino acids close to the carboxy terminus. Cytochrome c_1 from potato occurs in two isoforms with calculated molecular masses of 27.1 and 27.2 kDa (Braun *et al.*, 1992b). The sequences of the two proteins are 96% conserved and also exhibit a high degree of identity to cytochrome c_1 from fungi and mammals (Table II). Several domains characterized for cytochrome c_1 from other organisms are also present in potato: the heme-binding structure Cys-Xxx-Xxx-Cys-His close to the amino terminus, two domains that potentially interact with cytochrome *c* (Broger *et al.*, 1983; Stonehuerner *et al.*, 1985), and the C-terminal anchor domain. The gene for one of the two forms of cytochrome c_1 from potato was isolated and sequenced (Wegener and Schmitz, 1993). The coding region spans 5.1 kb and contains eight introns, the largest comprising 2.8 kb (Wegener and Schmitz, 1993). Upon analysis by SDS/PAGE both isoforms of potato cytochrome c_1 have apparent molecular masses of 33 kDa and cannot be resolved. As the heme group is covalently attached the subunit can be identified as a reddish band on gels without staining. Difference spectra of potato cytochrome c_1 exhibit an α-band with maximal absorbance at 552.4 nm (Berry *et al.*, 1991).

2.3.5. *The "Rieske" Iron-Sulfur Protein from Potato*

The "Rieske" iron-sulfur protein carries a binuclear FeS cluster which is involved in electron transport. It is an amphiphilic protein and localized on the intermembrane space exposed side of cytochrome *c* reductase. The primary structure of the FeS subunit from potato exhibits sequence identity to the corresponding protein from fungi and mammals in the range of 50% (Table II) and to the protein from maize and tobacco of 85 and 93% (Huang *et al.*, 1991, 1994; Emmermann *et al.*, 1994b). Highest sequence conservation is found in the caboxy-terminal third which is

believed to bind the FeS cluster via two histidine and two cysteine residues. The N-terminal third of the protein contains a hydrophobic region which probably anchors the subunit in the membrane. In contrast to maize, where several different forms for the FeS protein are described (Huang *et al.*, 1994), no isoforms were found in potato. The calculated molecular mass of the mature potato FeS protein is 23.1 kDa, which is slightly smaller than the apparent molecular mass determined by SDS/PAGE. Upon delipidation of isolated cytochrome *c* reductase from potato by Triton X-100, the FeS protein easily becomes detached from the protein complex (Braun *et al.*, 1992a). Electron spin resonance spectroscopy of the reduced FeS protein from potato reveals a characteristic signal with *g* values of 1.76 (*x*), 1.90 (*y*) and 2.03 (*z*) (Berry *et al.*, 1991).

2.3.6. The 14.3-kDa Subunit from Potato

Cytochrome *c* reductase from potato contains a 14.3-kDa protein that corresponds to the 13.4-kDa subunit VI of the enzyme complex from beef and to the 14.4-kDa subunit VII from yeast (de Haan *et al.*, 1984; Wakabayashi *et al.*, 1985; Braun and Schmitz, 1995c). The beef protein was originally called "ubiquinol binding protein" as it was thought to bind ubiquinone derivatives upon photoaffinity labelling (Yu and Yu, 1982), but meanwhile a slightly smaller 9.5-kDa subunit was identified as the labelled protein (Usui *et al.*, 1991). The specific function of the 14.3-kDa protein has not been elucidated. Disruption of the gene for the yeast subunit seems to affect the assembly of the protein complex (de Haan *et al.*, 1984; Crivellone *et al.*, 1988; Schoppink *et al.*, 1989a; Hemrika *et al.*, 1994a). Subfractionation and crosslink experiments of isolated cytochrome *c* reductase from beef reveal a localization of the 13.4-kDa protein next to cytochrome *b* and the core protein II (Schägger *et al.*, 1986; Gonzalez-Halphen *et al.*, 1988). A matrix-exposed localization in the neighborhood of cytochrome *b* was confirmed for the yeast subunit (Japa *et al.*, 1987; Hemrika and Berden, 1990). The 14.3-kDa protein from potato most likely also has a peripheral localization as it can be separated from the isolated enzyme complex by treatment with 6 M urea or 1 M guanidinium hydrochloride (Braun and Schmitz, 1995c; Emmermann *et al.*, 1993a). The potato protein exhibits about 30% sequence identity to the 13.4- and 14.4-kDa proteins from beef and yeast (Table II) and 75% identity to the equivalent subunit from wheat, which was sequenced partially (Braun and Schmitz, 1995c). The homology of the four proteins is also reflected by a conserved polarity profile.

2.3.7. The "hinge" Protein from Potato

The 9.2-kDa protein of cytochrome *c* reductase from beef was reported to be essential for complex formation between the isolated cytochromes *c* and c_1 (Kim and King, 1981, 1983) and to increase electron transfer between these two proteins (Kim *et al.*, 1987). Hence the protein was named the "hinge" protein (Wakabayashi *et al.*, 1982). The "hinge" protein can be crosslinked with cytochrome c_1 and forms part of a cytochrome c_1 subcomplex upon cleavage of isolated cytochrome *c* reductase (Schägger *et al.*, 1986; Gonzalez-Halphen *et al.*, 1988). The equivalent subunit from yeast has a calculated molecular mass of 14.5 kDa (van Loon *et al.*, 1984). Its specific function is not quite understood, as deletions of the gene for the 14.5-kDa protein do not have much effect on function and assembly of the enzyme complex (Schoppink *et al.*, 1988; Crivellone *et al.*, 1988; Kim and Zitomer, 1990; Schmitt and Trumpower, 1990). Recently analysis of mutants revealed a role of the 14.5-kDa protein in the maturation of cytochrome c_1 (Yang and Trumpower, 1994). Cytochrome *c* reductase from potato contains a 7.8-kDa subunit that resembles the 9.5-kDa protein from beef and the 14.5-kDa protein from yeast (Braun *et al.*, 1994b). However, although the overall sequence identity between the three polypeptides lies in the range of 40%, the polarity profile of the potato protein exhibits a striking difference in comparison to the "hinge" proteins from yeast and beef: it lacks the characteristic acidic domain close to the N-terminus that was thought to be essential for the function of this protein (Braun *et al.*, 1994b). The apparent molecular mass of the potato "hinge" protein very much depends on the presence of β-mercaptoethanol during SDS/PAGE, indicating the occurrence of disulfide bridges (Braun *et al.*, 1994b).

2.3.8. The 8.2-kDa Subunit from Potato

The 8.2-kDa subunit of cytochrome *c* reductase from potato exhibits low but significant sequence identity to the 9.5-kDa subunit of the enzyme complex from beef and the 11-kDa subunit from yeast (Borchart *et al.*, 1986; Braun *et al.*, 1994c; Maarse and Grivell,

1987). The beef 9.5-kDa protein can be labelled with ubiquinone derivatives (Usui *et al.*, 1990) and hence might play a supportive role in electron transport. Truncations of the gene encoding the 11-kDa protein from yeast cause disturbance of the assembly of the enzyme complex (Maarse *et al.*, 1988; Schoppink *et al.*, 1989b; Hemrika *et al.*, 1993, 1994b). The subunit possibly spans the inner membrane, as immunological and crosslink experiments with cytochrome *c* reductase from beef indicate an exposure of the protein toward the matrix and the mitochondrial intermembrane space (Gonzalez-Halphen *et al.*, 1988; Usui *et al.*, 1990). The polarity profiles of the potato 8.2- the beef 9.5- and the yeast 11-kDa protein are very similar. All three proteins have a large number of basic residues and have an isoelectric point above 10. Upon analysis of isolated cytochrome *c* reductase from potato by SDS/PAGE the 8.2-kDa subunit migrates faster than the 7.8-kDa "hinge" protein and the 8.0-kDa subunit (see below). It represents the smallest polypeptide on glycine-SDS/PAGE, as the 6.7-kDa protein (see below) is only resolved by Tricine-SDS/PAGE (Fig. 2).

2.3.9. The 8.0-kDa Subunit from Potato

The potato 8.0-kDa protein of cytochrome *c* reductase comprises 72 amino acids (Braun and Schmitz, 1995d). The subunit seems to have a segmental structure as its sequence can be devided into four parts, each containing a central Arg-$(Xaa)_5$-Val motif. It is homologous to the 7.2-kDa protein of cytochrome *c* reductase from beef, to the 7.3-kDa protein from yeast (Table II), and also to a 7.8-kDa protein of the enzyme complex from Euglena (Schägger and von Jagow, 1983; Phillips *et al.*, 1990; Cui *et al.*, 1994; Braun and Schmitz, 1995d). Like the proteins from beef and yeast the potato 8.0-kDa protein has a tripartite polarity profile with a hydrophilic C- and N-terminus and a central uncharged/hydrophobic region. The function of this subunit from potato is not known. The equivalent 7.2-kDa protein from beef is part of a cytochrome c_1 subcomplex of cytochrome *c* reductase together with cytochrome c_1 and the "hinge" protein (Schägger *et al.*, 1986). As it can be crosslinked not only with cytochrome c_1, but also with cytochrome *c*, a supportive role of the protein in the interaction of the two cytochromes was postulated (Gonzalez-Halphen *et al.*, 1988). Yeast cells with a deletion of the gene encoding the 7.3-kDa subunit have no cytochrome *c* reductase activity and an altered spectrum of cytochrome *b* (Phillips *et al.*, 1990). Recently a role of the 7.3-kDa subunit in insertion of the iron-sulfur cluster into the iron-sulfur protein was demonstrated (Phillips *et al.*, 1993).

2.3.10. The 6.7-kDa Subunit from Potato

The 6.7-kDa protein is the smallest subunit of cytochrome *c* reductase from potato (Jänsch *et al.*, 1995). The protein is only resolved by Tricine-SDS/PAGE and has an apparent molecular mass of 6 kDa (Fig. 2). Upon delipidation of cytochrome *c* reductase it dissociates from the protein complex together with the iron-sulfur protein. The same result was reported for a 6.5-kDa protein (Schägger *et al.*, 1985, 1990), which represents the smallest subunit of cytochrome *c* reductase from beef [the protein was therefore called the "ISP (iron-sulfur protein)-binding factor"]. The beef 6.5-kDa protein and the potato 6.7-kDa protein exhibit sequence homology and resemble the 8.5-kDa protein of cytochrome *c* reductase from yeast, which was characterized recently (Brandt *et al.*, 1994). Like the 8.0-kDa subunit of the enzyme complex from potato the 6.7-kDa protein has a tripartite structure with hydrophilic regions flanking a hydrophobic core. The function of the 6.7-kDa subunit is unknown. Cytochrome *c* reductase isolated from a yeast strain carrying a deletion for the gene encoding the equivalent 8.5-kDa protein does not only lack this subunit, but also the iron-sulfur protein (Brandt *et al.*, 1994). Also a point mutation of yeast cytochrome *b* was reported to cause loss of both, the iron-sulfur protein and the 8.5-kDa subunit (Geier *et al.*, 1992; Giessler *et al.*, 1994). Hence it was speculated that the 8.5-kDa protein plays a role in the assembly of the cytochrome *c* reductase complex in contributing to a proper association of the iron-sulfur protein with cytochrome *b* (Brandt *et al.*, 1994).

In summary, the composition of cytochrome *c* reductase from three very different organisms—potato, beef, and yeast—is remarkably similar. The sum of the calculated molecular masses of the individual subunits of the bc_1 complexes from these organisms is 240 kDa ± 3% (Table I). All three enzyme complexes comprise 10 subunits: two core proteins, three respiratory subunits, and five proteins with molecular weights below 15 kDa. In addition, the presequence of the iron-sulfur protein remains in the complex from beef. The core and small proteins, which are absent in the simple bc_1 complexes from bacteria, are likewise part of the

eukaryotic enzyme. Hence the mitochondrial cytochrome *c* reductase must be an old enzyme, which presumedly was already present in the common progenitor of potato, beef, and yeast—the early eukaryotic cell. Although the function of the core and the small subunits is not quite understood, all of them seem to be essential components, as none got lost during evolution in any of the three organisms analyzed. However, sequence conservation between the respiratory subunits from beef, yeast, and potato is higher than the identity between the core and the small subunits from these organisms (Table II), possibly reflecting an involvement of the "supernumerary" subunits rather in assembly and stability of the enzyme complex than in enzymatic activity.

2.4. Targeting of the Subunits of Cytochrome *c* Reductase from Potato

An important issue of modern cell biology is the question of how proteins synthesized in different subcellular localizations find the right destination within the cell and assemble there into multisubunit protein complexes. The genes encoding the subunits of cytochrome *c* reductase and other complexes of the respiratory chain are localized in the nucleus and on the mitochondrial genome, thus requiring the coordinated expression of both genetic systems. Except cytochrome *b*, which is synthesized in the mitochondrial matrix and subsequently targeted to the inner mitochondrial membrane, all genes for the subunits of cytochrome *c* reductase are localized in the nuclear genome, synthesized on cytoplasmic ribosomes, and imported into the organelle (Teintze *et al.*, 1982; van Loon *et al.*, 1983). For proper targeting to the mitochondrial matrix, the inner membrane, or the intermembrane space, the proteins carry topological signals. In beef and yeast five of the nine nuclear encoded subunits of cytochrome *c* reductase have transient N-terminal signals while in potato only four subunits have a cleavable presequence.

The presequences of the two largest subunits of the potato complex, which represent the α- and β-subunit of MPP, exhibit typical features of mitochondrial matrix targeting signals (Fig. 3). They have a preponderance of positively charged and hydroxylated residues and the potential to form an amphipathic α-helix. The presequence of αI-MPP is 24 amino acids long (Emmermann *et al.*, 1994a) and represents one of the shortest signals for protein import into plant mitochondria. Although the isoforms of β-MPP share high sequence similarity, their presequences differ profoundly in amino acid composition and length: the one of βI-MPP is 32 amino acids long and contains cysteine and phenylalanine but lacks glutamine and tyrosine; vice versa the latter two amino acids are present in the 26 amino acid long prepiece of βII-MPP which is devoid of cysteine and phenylalanine (Emmermann *et al.*, 1993a). The precursors of α- and β-MPP are processed by the purified cytochrome bc_1 complex from potato, indicating that the complex integrated processing enzyme removes the targeting signals of its own subunits (Emmermann *et al.* 1993a; 1994a).

Two of the respiratory subunits of cytochrome *c* reductase are exposed to the intermembrane space and carry long N-terminal presequences. The presequence of cytochrome c_1 from potato is 77 amino acids long and has a bipartite polarity profile characteristic of import signals for targeting to the intermembrane space of fungal mitochondria (Braun *et al.*, 1992b). It consists of a positively charged N-terminal part which resembles "matrix targeting domains" and a hydrophobic "intramitochondrial sorting domain" (Hurt and van Loon, 1986). In fungi the matrix targeting domain is removed by MPP while the intramitochondrial sorting domain is cleaved off by the inner membrane protease I (Schneider *et al.*, 1991; Nunnari *et al.*, 1993). The cleavage site of the latter protease differs between the two isoforms of potato cytochrome c_1 (Fig. 3). This finding is unexpected as the intramitochondrial sorting domain is conserved in cytochrome c_1 from bacteria, potato (isoform 1), fungi, and mammals; an alanine residue is always the last amino acid of the presequence. The high conservation of the C-terminal part of the presequence supports the theory that during early endosymbiosis the bacterial cytochrome c_1 gene was transfered to the nucleus together with its prokaryotic sorting signal. Also the fact that there is no intron between the DNA sequence encoding the presequence and the mature protein suggests that the intramitochondrial sorting domain represents the ancestral bacterial secretion signal (Wegener and Schmitz, 1993). The relevancy of this argument is corroborated by the finding that the presequence of cytochrome c_1 from potato is encoded on four exons (Wegener and Schmitz, 1993).

The other respiratory subunit of cytochrome *c* reductase which faces the intermembrane space and is cytoplasmically synthesized with a transient targeting signal is the iron-sulfur protein. In fungi its presequence exhibits two-step processing with the second

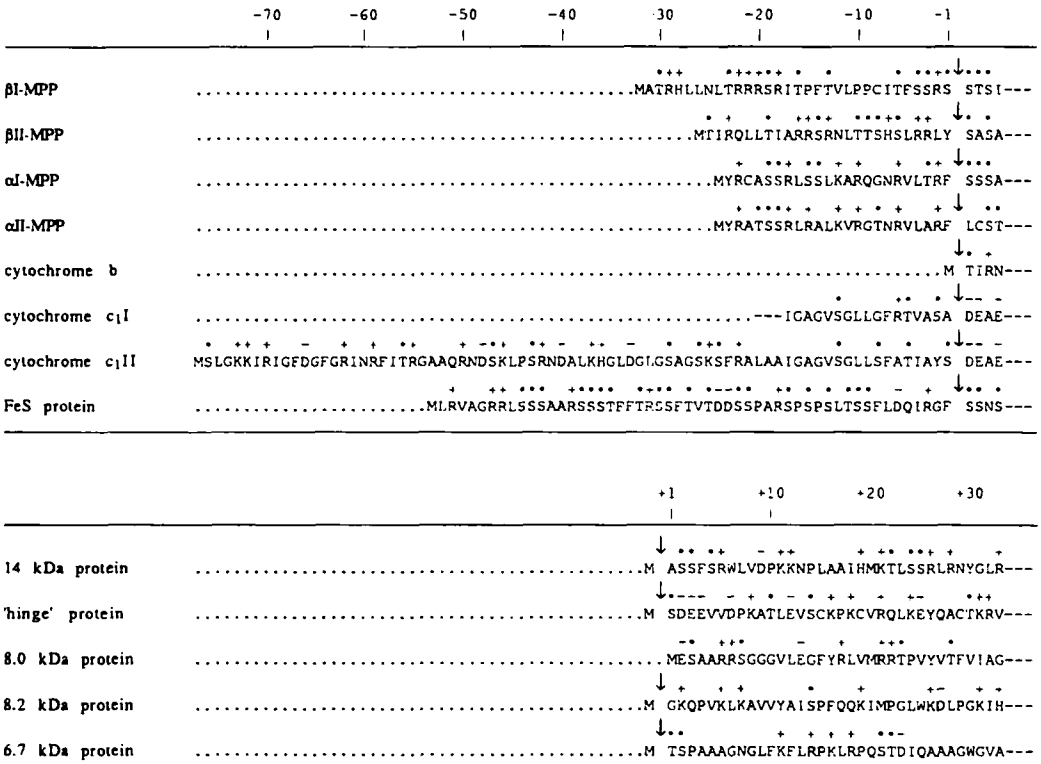

Fig. 3. Amino terminal sequences of the 10 precursor proteins of cytochrome *c* reductase from potato. For β-MPP, α-MPP, and cytochrome c_1 two isoforms are shown. Basic or acidic residues are marked by + or −, respectively, · stands for hydroxylated amino acids, ↓ points to the cleavage site of precursor proteins. The numbers on top of the two parts of the figure indicate the position of the residues (the first amino acid of the mature protein is defined as "+1"). All respiratory and core proteins (upper part) comprise presequences, except for cytochrome *b*, which is mitochondrially encoded. The presequences exhibit a preponderance for basic and hydroxylated residues and may form amphiphilic helices. The small proteins (lower part) lack presequences, but comprise amino-terminal primary structures, which resemble import signals. The "hinge" protein in exceptional in having an acidic N-terminus.

step being performed by the "mitochondrial intermediate peptidase" (MIP) which was characterized recently (Kalousek *et al.*, 1992). Surprisingly, in mammals the presequence of the iron-sulfur protein is cleaved off in a single step and retained in the complex as an individual subunit (Brandt *et al.*, 1993). While it was initially speculated that the iron-sulfur protein from plant mitochondria might be processed in two steps like those from yeast and Neurospora (Huang *et al.*, 1991) studies on this protein from potato mitochondria unequivocally showed that the presequence is removed in a single step (Emmermann *et al.*, 1994b). *In vitro* import and *in vitro* processing experiments revealed that the imported FeS protein from potato has exactly the same size as the one generated by the purified cytochrome *c* reductase/processing peptidase complex, suggesting that no second protease acts on the precursor. Determination of the cleavage site by protein and cDNA sequencing showed that the common sequence motif of proteins processed by MIP (Hendrik *et al.*, 1989) does not occur in the iron-sulfur protein from potato. Assuming that the cleavage site of MPP in the iron-sulfur protein from maize and tobacco is located in a similar area as in the protein from potato, neither of these plants has the common sequence motif of MIP in the correct position. Additionally the length of the presequence of the iron-sulfur protein from potato (53 amino acids) is similar to the one from beef while those which are processed in two steps are about 20 amino acids shorter. In summary these data make it likely that the iron-sulfur protein from plants is generally processed in a single step. Whether the presequence of the plant mitochondrial iron-sulfur protein is retained as a subunit in the cytochrome *c* reductase complex still has to be determined.

Apart from the iron-sulfur protein and cytochrome c_1 the mitochondrially encoded cytochrome *b* represents the third redox-center containing subunit. After synthesis of the apocytochrome *b* protein in the mitochondrial matrix two heme groups have to be attached and the protein has to find its way to the inner mitochondrial membrane. While the N-terminus of cytochrome *b* from yeast, *Neurospora,* and beef is blocked by a formyl group (Mannhaupt *et al.,* 1985; Weiss, 1976; von Jagow *et al.* 1981), cytochrome *b* from potato turned out to be accessible to Edman degradation (Braun and Schmitz, 1993). So far only three different mitochondrially encoded proteins, subunit I of cytochrome *c* oxidase from *Neurospora,* subunit II of the same complex from yeast, and *Neurospora* and subunit 6 of ATPase from yeast, were found not to be blocked at the N-terminus due to the presence of a transient presequence. Analysis of potato cytochrome *b* revealed that only the initiator methionine is removed, providing the first evidence for the presence of a deformylase and a methionine aminopeptidase in mitochondria (Braun and Schmitz, 1993). Cytochrome *b* is one of the few proteins which is without known exception always encoded on the mitochondrial genome (Degli Esposti *et al.,* 1993). Different approaches have been used to target an allotopically expressed cytochrome *b* from the cytosol into the mitochondria. Import experiments with fusions between apocytochrome *b* and the "reporter" proteins β-glucuronidase (Mozo and Schmitz, submitted) or the maturase encoded by intron IV of cytochrome *b* from yeast (Claros *et al.,* 1995) revealed that no more than three to four transmembrane helices of apocytochrome *b* can be imported into mitochondria. Similarly, cytoplasmically synthesized fusion proteins consisting of apocytochrome *b* and different mitochondrial import signals, did not complement a cytochrome *b*-deficient yeast strain. The results suggest that the hydrophobicity and structure of the apocytochrome *b* protein inhibit its import into mitochondria, thus preventing a stable nuclear localization of the corresponding gene during evolution.

Figure 3 shows that only the nuclear encoded subunits of the cytochrome *c* reductase complex from potato which are larger than 15 kDa are synthesized with a transient presequence. All five smaller subunits contain internal targeting information which in the case of the 14-, 8.2-, 8.0-, and 6.7-, kDa proteins is potentially localized within the positively charged N-terminal part of the proteins. Proteolytic posttranslational modifications are confined to the removal of the initiator methionine. This contrasts with the situation in fungi and mammals where at least one of the small subunits, the "hinge" protein, is made with a presequence (Ohta *et al.,* 1987). On the other hand, it has not been demonstrated yet that the atypical acidic presequence of the "hinge" protein from yeast and human is involved in mitochondrial targeting. As mentioned above, the "hinge" protein from potato lacks the acidic domain that is characteristic for "hinge" proteins from fungi and mammals. However, among the small subunits of the potato cytochrome bc_1 complex the "hinge" protein is the only polypeptide with several consecutive negatively charged amino acids at its N-terminus, raising the possibility that it may follow a similar import pathway as the "hinge" protein from hetrotrophic organisms.

There is a single protein in the cytochrome *c* reductase complex from potato which retains its N-terminal methionine: the 8.0-kDa subunit. Although being localized on the intermembrane side of the complex it needs a membrane potential ($\Delta\Psi$) across the inner membrane for mitochondrial import (Braun and Schmitz, 1995d). Interestingly the biophysical properties of the N-terminus of the 8.0-kDa protein resemble those of presequences for targeting to the mitochondrial intermembrane space: a positively charged hydrophilic segment is followed by a stretch of hydrophobic amino acids. Import experiments with fusions between this bipartite N-terminal part of the 8.0-kDa subunit and a reporter protein may clarify whether the putative import signal is sufficient for targeting polypeptides to the intermembrane space. Mitochondrial import in a $\Delta\Psi$-dependent fashion is not confined to the 8.0-kD subunit but is a general feature of the small subunits of the cytochrome *c* reductase/processing peptidase complex from potato (Braun *et al.* 1994c; Braun and Schmitz, 1995c,d).

3. CYTOCHROME *c* REDUCTASE FROM OTHER PLANTS

Besides cytochrome *c* reductase from potato the bc_1 complexes from two other plant species—spinach and wheat—have been analyzed in detail. Cytochrome *c* reductase from spinach was purified to homogeneity by anion-exchange chromatography and gel filtration (Eriksson *et al.,* 1993, 1994; Glazer *et al.,* 1994). Like the enzyme complex from potato, cytochrome *c* reductase from spinach is involved in both respiration and protein processing. Analysis of the spinach enzyme by

glycine-SDS/PAGE allows the separation of 10 protein bands with apparent molecular masses of 61, 54, 52, 34, 32, 26, 15, 12, 11, and 10 kDa. The identity of some subunits was determined by immunoblotting using heterologous antibodies: cytochrome *b* (34 kDa), the "Rieske" iron-sulfur protein (26 kDa), α-MPP (54 and 52 kDa), and β-MPP (61 kDa). The 32-kDa protein band most likely represents spinach cytochrome c_1. The occurrence of the two forms of α-MPP was not analyzed, but it can be speculated that individual cytochrome *c* reductase complexes from spinach also comprise only one form of α-MPP. In comparison to potato the cytochrome *c* reductase complex from spinach has a very similar subunit composition, except that one of the five small subunits seems to be absent upon analysis by glycine-SDS/PAGE. However, the smallest subunit of the enzyme complex from potato is also not resolved with the Laemmli system but only becomes visible upon analysis by Tricine-SDS/PAGE (Fig. 2).

Cytochrome *c* reductase from wheat was prepared by anion exchange chromatography (Pfeiffer *et al.*, 1990) and affinity chromatography (Braun *et al.*, 1995) using either isolated mitochondria or wheat germ extract as starting material. The enzyme complex contains the three respiratory subunits, at least three small subunits, and probably two distinct proteins corresponding to α-MPP and β-MPP as shown by N-terminal and internal protein sequencing. Analysis by SDS/PAGE allows resolution of two pairs of bands in the range of the MPP subunits. Protein sequencing and characterization of cDNA clones encoding the high-molecular-weight subunits revealed that the two polypeptides of each pair are structurally very similar (Braun *et al.*, 1995; Emmermann and Schmitz, unpublished) and most likely represent isoforms. Cytochrome *c* reductase from wheat transfers electrons from ubiquinol to cytochrome *c* and also processes mitochondrial precursor proteins (Braun *et al.*, 1995). Hence the integration of the mitochondrial processing protease into the bc_1 complex is a general feature of higher plants. We speculate that the occurrence of a bifunctional cytochrome *c* reductase/processing peptidase complex represents the original situation in eukaryotes and that the subunits of MPP became detached from the bc_1 complex in mammals and fungi after some gene duplications had occurred (Braun and Schmitz, 1995b). From this perspective the core subunits of other organisms represent degenerated protease subunits that are important for assembly of the respiratory protein complex. Characterization of cytochrome *c* reductase from further organisms might give interesting insights into the co-evolution of the bc_1 complex and the mitochondrial processing peptidase.

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