Internal Architecture of Mitochondrial Complex I from Arabidopsis thaliana

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The NADH dehydrogenase complex (complex I) of the respiratory chain has unique features in plants. It is the main entrance site for electrons into the respiratory electron transfer chain, has a role in maintaining the redox balance of the entire plant cell and additionally comprises enzymatic side activities essential for other metabolic pathways. Here, we present a proteomic investigation to elucidate its internal structure. Arabidopsis thaliana complex I was purified by a gentle biochemical procedure that includes a cytochrome c–mediated depletion of other respiratory protein complexes. To examine its internal subunit arrangement, isolated complex I was dissected into subcomplexes. Controlled disassembly of the holo complex (1000 kD) by low-concentration SDS treatment produced 10 subcomplexes of 550, 450, 370, 270, 240, 210, 160, 140, 140, and 85 kD. Systematic analyses of subunit composition by mass spectrometry gave insights into subunit arrangement within complex I. Overall, Arabidopsis complex I includes at least 49 subunits, 17 of which are unique to plants. Subunits form subcomplexes analogous to the known functional modules of complex I from heterotrophic eukaryotes (the so-called N-, Q-, and P-modules), but also additional modules, most notably an 85-kD domain including γ-type carbonic anhydrases. Based on topological information for many of its subunits, we present a model of the internal architecture of plant complex I.

INTRODUCTION

Oxidative phosphorylation (OXPHOS) is the ATP formation driven by oxidative reactions of the respiratory chain. The process is based on the presence of a so-called OXPHOS system localized in specialized biomembranes (e.g., the plasma membrane in oxygenic prokaryotes and the inner mitochondrial membrane in eukaryotes). Complex I is the largest protein complex of the OXPHOS system (for reviews, see Friedrich and Böttcher, 2004; Brandt, 2006; Vogel et al., 2007; Remacle et al., 2008; Zickermann et al., 2008, 2009; Lazarou et al., 2009). It catalyzes NADH-quinone oxidoreduction and in many systems represents the main entrance site for electrons into the respiratory electron transfer chain. Coupled to this oxidoreduction, complex I translocates protons across the inner mitochondrial membrane or the plasma membrane of bacteria. The precise mechanism of complex I function is still not completely understood (reviewed in Zickermann et al., 2009).

Analyses by electron microscopy (EM) revealed that complex I is composed of two arms arranged in orthogonal configuration to form an L-shaped particle (Guénebaut et al., 1997, 1998; Grigorieff, 1998; Böttcher et al., 2002; Peng et al., 2003; Dudkina et al., 2005; Radermacher et al., 2006; Morgan and Sazanov, 2008; Clason et al., 2010). One arm is hydrophobic and embedded within the membrane (membrane arm); the other is hydrophilic and protrudes into the mitochondrial matrix or the lumen of a bacterial cell (peripheral arm). In Escherichia coli, complex I is composed of 14 subunits but in mitochondria complex I contains up to 31 additional subunits. The structure of the peripheral arm of the archaeabacterium Thermus thermophilus complex I was recently solved by x-ray crystallography. It includes eight subunits that bind one flavine mononucleotide and nine iron-sulfur clusters as redox prosthetic groups involved in electron transfer from NADH to quinone (Hinchliffe and Sazanov, 2005; Sazanov and Hinchliffe, 2006; Sazanov, 2007; Berrisford and Sazanov, 2009). By contrast, the inner architecture of the membrane arm, which is responsible for proton translocation, is less well understood. Locations of some subunits have been deduced by single particle electron microscopy using complex I subcomplexes or by immune-electron microscopy (Abdrakhmanova et al., 2004; Baranova et al., 2007a, 2007b; Clason et al., 2007). Overall, the subunits of the prokaryotic membrane arm are predicted to include >50 membrane-spanning helices. Even more membrane-spanning helices are predicted to be present within the membrane arm of eukaryotic complex I. Seven of the most hydrophobic subunits of the membrane arm in eukaryotes are encoded by the mitochondrial genome in most organisms investigated.

Nomenclature of complex I subunits unfortunately differs among the organisms investigated, although a large number of subunits likewise are present in different species. In this work,
the bovine nomenclature is used for all homologous complex I subunits of Arabidopsis because bovine complex I is particularly well-investigated (Carroll et al., 2003, 2006; Hirst et al., 2003). Subunits specific to complex I of plant mitochondria are named according to the corresponding genes annotated by The Arabidopsis Information Resource (TAIR; www.Arabidopsis.org).

The OXPHOS system of plant mitochondria is more complicated compared with its counterparts in heterotrophic eukaryotes. First of all, quite a large number of alternative oxidoreductases are present in the inner mitochondrial membrane; these participate in respiratory electron transport without contributing to the proton gradient across the inner mitochondrial membrane (reviewed in Rasmusson et al., 2008). Furthermore, the classical protein complexes of the OXPHOS system have several special features. Complex I of Arabidopsis, rice (Oryza sativa), and Chlamydomonas reinhardtii was reported to include plant-specific subunits (Heazlewood et al., 2003; Millar et al., 2003; Cardol et al., 2004; Sunderhaus et al., 2006; Meyer et al., 2008). So far, 13 complex I subunits of Arabidopsis that have no counterparts in complex I of mammals or fungi have been described. Some of these subunits introduce side activities into complex I, for example, subunits resembling γ-type carbonic anhydrases (CAs) and an L-galactone-1,4-lactone dehydrogenase (GLDH), which is associated with complex I in plants.

In plants, mitochondrial complex I has additional functions that are of great importance for the entire cell, e.g., in maintaining the redox balance during photosynthesis (Dutilleul et al., 2003). Also, plant complex I has a unique shape as revealed by single-particle EM (Dudkina et al., 2005; Sunderhaus et al., 2006; Peters et al., 2008; Bulterma et al., 2009). In contrast with all other investigated organisms, it comprises an additional peripheral domain that is attached to the membrane arm at a central position on its matrix-exposed side (Figure 1). The extra domain has a spherical shape, is estimated to have a molecular mass of 80 kD, and was shown to include CA subunits specific for plant complex I (Sunderhaus et al., 2006). By analogy with the prototype CA from the archaeabacterium Methanosarcina thermophila (CAMS) (Alber and Ferry, 1994), the spherical extra domain probably represents a CA trimer. Three distinct CAs have been found to be present within Arabidopsis complex I and are termed CA1, CA2, and CA3. Furthermore, two CA-like proteins named CAL1 and CAL2 have been described that have altered active sites compared with the one of CAM (reviewed in Braun and Zabaleta, 2007). The physiological role of the CA domain of plant complex I is currently a matter of debate.

To better understand structure and function of plant complex I, we analyzed its internal architecture. The study is based on a biochemical strategy that includes gentle complex I purification, controlled disassembly of the purified complex, separation of the generated subcomplexes by blue native (BN)-PAGE, separation of the subunits of the subcomplexes by SDS-PAGE, and, finally, subunit identification by tandem mass spectrometry. Integration of the obtained results is used to deduce the internal architecture of plant complex I.

RESULTS

Purification of Complex I from Arabidopsis

Biochemical analysis of the subunit arrangement within complex I first requires its gentle purification in the native conformation. A new three-step purification strategy was developed for complex I isolation, based on (1) purification of mitochondrial membranes, (2) separation of mitochondrial membrane protein complexes by sucrose gradient ultracentrifugation, and, finally, (3) cytochrome c affinity chromatography to remove complex III and complex III–containing supercomplexes from complex I–enriched fractions (Figure 2). The outcome of each purification step can be monitored by one-dimensional (1D) BN-PAGE.

The starting point for the complex I purification was mitochondria isolated from Arabidopsis cell suspension cultures. The organelle fraction included all five OXPHOS complexes, the I+III2 supercomplex and the F1 part of complex V (Figure 2A). Since complex I is the largest enzyme of the OXPHOS system, a size-based separation by sucrose gradient ultracentrifugation was employed to separate the solubilized complexes of the mitochondrial membranes. Under the conditions applied, complex I–enriched fractions were located close to the bottom of the centrifugation tube (Figure 2B, fractions 2 and 3). The only visible contaminations within these fractions were dimeric complex III and the I+III2 supercomplex.

Finally, cytochrome c affinity chromatography was applied to specifically remove complex III2 and the I+III2 supercomplex from complex I–enriched fractions. Originally, this procedure was developed to purify complex III2 by the binding to its natural substrate cytochrome c (Weiss and Juchs, 1978; Braun and Schmitz, 1992). Binding of complex III2 is most efficient if cytochrome c is in the oxidized state. Upon reduction of cytochrome c by ascorbate, affinity decreases and allows elution of complex III by a salt gradient of low ionic strength (Weiss and Juchs, 1978). No salt gradient is necessary to detach complex I from the cytochrome c sepharose because it simply passes through the column without interactions and therefore can be obtained in the flow-through fraction. Low salt conditions during purification ensure optimal integrity and native conformation of complex I (Figure 2C).

![Figure 1. EM Average Structure and Scheme of Mitochondrial Complex I from Arabidopsis.](image-url)

The EM average structure (left) was taken from Dudkina et al. (2005). The deduced scheme (right) shows the membrane arm of complex I in red and the peripheral arm in orange. The membrane arm is inserted into the inner mitochondrial membrane. M, matrix side; IMS, intermembrane space side.
Controlled Disassembly of Arabidopsis Complex I

To obtain information on subunit localizations within complex I, the isolated protein complex was destabilized using defined biochemical conditions to generate complex I subcomplexes and finally analyze their subunit composition. Several destabilization conditions were tested, such as temperature, urea, and detergent treatment or mild proteolysis. Results of experiments were monitored by 1D BN-PAGE. Many conditions allowed dissecting complex I (1000 kD) into a large membrane arm of 550 kD and a 370-kD subcomplex that represents the peripheral arm. Dissection of isolated complex I from Arabidopsis into subcomplexes of similar sizes was previously reported by the use of two-dimensional (2D) BN/BN-PAGE, if digitonin used for solubilization is replaced by dodecylmaltoside during the electrophoresis run of the second native gel dimension (Sunderhaus et al., 2006). However, slightly harsher conditions than these destabilization treatments consistently caused complete disassembly of complex I into its subunits.

We finally tested destabilization of isolated complex I by treatment with SDS at very low concentrations. Indeed, several additional complex I subcomplexes became visible upon analysis by 1D BN-PAGE and subsequent Coomassie blue or NADH activity staining (Figure 3). Treatment of complex I with only 0.01% SDS caused its partial dissociation into the 550- and 370-kD subcomplexes. Also, the molecular mass of the holocomplex is slightly reduced by ∼80 kD in the presence of 0.01% SDS, indicating loss of a few individual subunits (Figure 3). Increase of the SDS concentration to 0.04% and up to 0.12% generates further subcomplexes of 240, 210, and 140 kD, all of which can oxidize NADH as monitored by an in-gel activity assay. Therefore, they were considered to represent subcomplexes of the peripheral arm, which is known to include the NADH oxidation domain of complex I.

2D BN/SDS-PAGE analyses were performed to further examine SDS-induced subcomplex generation (Figure 4). In the absence of SDS, the subunits of the complex I holoenzyme form a vertical row of spots in the 1000-kD gel region (Figure 4, left gel). Treatment of intact complex I with 0.015% SDS leads to its partial dissection into the 550- and 370-kD subcomplexes, which clearly are composed of distinct subunits (Figure 4, middle gel). Further increase of the SDS concentration to 0.04% leads to the generation of several additional subcomplexes, which are separated into vertical rows of subunits upon analysis by 2D BN/SDS-PAGE. Besides the NADH oxidizing 240-, 210-, and 140-kD subcomplexes detected on 1D BN gels, further subcomplexes are visible at 450, 270, 160, and 85 kD.

Subunit Composition of Complex I Subcomplexes from Arabidopsis

Subunits of complex I subcomplexes resolved by 2D BN/SDS-PAGE were systematically identified by mass spectrometry (MS).
to determine the protein composition of the subcomplexes (Figure 5). Overall, 50 gel spots were analyzed, representing 40 different complex I subunits and a few contaminating proteins like prohibitin, the β-subunit of ATP synthase (complex V), Ser hydroxymethyltransferase, and lipoamide dehydrogenase (Table 1). Protein determinations revealed that the 550-, 450-, 270-, 160-, and 85-kD subcomplexes are related to the membrane arm (indicated in red on Figure 5) and the 370-, 240-, 210-, and two 140-kD subcomplexes to the peripheral arm (indicated in orange on Figure 5). MS-based protein identifications gave insights into the subunit compositions of 10 different complex I subcomplexes (first given for the peripheral arm and afterwards for the membrane arm).

1. The 370-kD subcomplex represents the entire peripheral arm. It includes at least 11 subunits: the so-called 75-kD, 51-kD, 49-kD (ND7), 30-kD (ND9), 24-kD, TYKY, B13, PSST, and B8 subunits (bovine nomenclature), the plant-specific subunit At1g67785 (~12 kD), and the previously not described Arabidopsis complex I subunit At3g03070 (~10 kD), which is homologous to the 13-kD subunit of the bovine enzyme (Figure 6).

2. The 240-kD subcomplex represents the so-called NADH oxidation-module (N-module) of the peripheral arm, which previously was identified after pH- and Triton X-100–induced destabilization of complex I isolated from E. coli (Leif et al., 1995). It is composed of at least four proteins: the 51-kD subunit, which includes the NADH oxidation domain, the 75-kD subunit, the 24-kD subunit, the B8 subunit, and/or the plant-specific At1g67785 protein.

3. The slightly smaller 210-kD subcomplex comprises only the three core subunits of the N-module, namely, the 75-, 51-, and 24-kD subunits.

4. Careful inspection of the 2D gel in Figure 5 revealed that the 140-kD subcomplex consists of two distinct comigrating subcomplexes termed 140a and 140b. Assignment of subunits to the two subcomplexes is based on spot shape (barbell shaped in the case of subcomplex 140a and round in the case of subcomplex 140b). Subcomplex 140b includes the 51- and 24-kD subunits. Due to the presence of the NADH binding 51-kD subunit, it becomes visible upon in-gel NADH staining like the 240- and 210-kD subcomplexes (Figure 3).
The 140a subcomplex includes subunits previously reported to form the so-called Q-reduction module (Q-module) of the peripheral arm in *E. coli* (Leif et al., 1995). It is composed of the 49-kD (ND7), 30-kD (ND9), TYKY, PSST, and B13 subunits.

(6) The 550-kD subcomplex represents the membrane arm of *Arabidopsis* complex I. It includes at least 27 subunits, 13 of which are homologous to known subunits of the membrane arm from bovine complex I (ND1, ND2, ND3, ND4, ND5, PDSW, B18, PGIV, 15 kD, B12, ASHI, AGGG, and MWFE). Additionally, the membrane arm of *Arabidopsis* complex I includes a bovine homolog known to be present in the peripheral arm, the so-called 16.6-kD subunit (GRIM19). Thirteen further subunits of the *Arabidopsis* membrane arm are plant-specific proteins, eight of which are rather small and hydrophobic (At4g16450, At2g27730, At2g42310/At3g57785, At4g00585, At4g20150, At2g31490, At1g67350, and the previously not described complex I subunit At5g14105). The five remaining plant-specific subunits of the membrane arm represent the CA subunits CA1, CA2, CA3, CAL1, and CAL2.

(7) The 450-kD subcomplex is a subcomplex of the membrane arm generated most efficiently at a slightly higher SDS concentration. The subunit composition resembles the one of the 550-kD complex, but it lacks all five CA subunits and some of the smaller subunits of the membrane arm.

(8) and (9) The 270- and 160-kD subcomplexes represent smaller forms of the 450-kD subcomplex that lack so far unknown subunits. The 160-kD subcomplex includes the ND5 subunit, the B18 subunit, and/or At2g27730 as well as the PGIV subunit and/or At1g67350.

(10) Finally, an 85-kD subcomplex includes the CA subunits present within plant complex I, the CA1, CA2, CAL1, and CAL2 proteins. Slightly differing horizontal positions of the CAL1 and CAL2 proteins with respect to the CA1 and CA2 subunits indicate that they might not be simultaneously present within individual CA subcomplexes.

The GLDH, which is associated to complex I in plants, and the so-called 39-kD subunit were identified as separate proteins on BN/SDS gels (Figure 5), which do not form part of complex I subcomplexes upon SDS treatment of any concentration and therefore are assumed to be either peripherally localized or at the interphase of the membrane and the peripheral arm.

Elucidation of subunit compositions of complex I subcomplexes allows us to deduce a model of internal subunit arrangement within plant complex I as discussed below.
Table 1. Proteins Identified by MS

Complex I–Related Proteins

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DISCUSSION

Biochemical Procedures to Characterize Complex I

A protocol for complex I isolation is presented that is based on mitochondrial preparation, sucrose gradient ultracentrifugation, and depletion of complex III–related contaminants by cytochrome c affinity chromatography. Protocols previously published for the isolation of complex I from plants include ion-exchange or immunoaffinity chromatography, which require salt gradients or acid treatment for complex I elution (Leterme and Boutry, 1993; Herz et al., 1994; Rasmusson et al., 1994; Trost et al., 1995; Combettes and Grienenberger, 1999). By contrast, the protocol presented here completely avoids high salt conditions since complex I passes the cytochrome c affinity column used for complex III depletion in the flow-through fraction. Complex I therefore can be expected to have optimal integrity for further structural analysis.

Insights into the internal subunit arrangement of complex I were obtained by its careful disassembly and biochemical analysis of the generated disassembly products. Incubation of complex I with low concentrations of SDS proved to be an ideal tool for careful subcomplex generation. It is textbook knowledge that SDS treatment of biochemical fractions is incompatible with native protein characterization. However, SDS concentrations usually employed in protein science often are in the range of 1% or higher (e.g., 5% for sample preparation prior to SDS-PAGE) (Laemmli, 1970; Schägger and von Jagow, 1987). Here, we report use of very low SDS concentrations in the range of 0.01 to 0.04%. Indeed, low SDS conditions cause very distinct
Arabidopsis (Sunderhaus et al., 2006; Meyer et al., 2008; this study). They are small and hydrophobic (Brandt, 2006). Sequence conservation often is low between related proteins if additionally present in fungal or mammalian complex I since part of the membrane arm. It currently cannot be excluded that and GLDH, most of them are small hydrophobic subunits forming 2006; Meyer et al., 2008; this study). Except for the CA subunits now been identified (Heazlewood et al., 2003; Sunderhaus et al., 2006; Meyer et al., 2008; this study). The novel plant-specific subunit At1g14105. Two further complex I subunits (B22 and At1g14450) were identified by another study the bovine 13-kD subunit (At4g03070; Figure 6), ND3, GLDH, and our study were not reported in Meyer et al. (2008): the homolog of the ND6, 18-kD, B17.2, B14.7, B14, and At1g68680 subunits). These subunits probably are lost during detection. On the other hand, four of the 40 subunits identified in the most extensive study on complex I subunits in plants (Meyer et al., 2008), which was based on the separation of complex I proteins by three-dimensional BN/SDS-PAGE, the enzyme complex from plants is slightly larger (Jänsch et al., 1995).

For several subunits, pairs of isoforms occur in Arabidopsis. MS analysis allowed us to detect isoforms for the PSDW subunit (encoded by At3g18410 and At1g49140), the PGIV subunit (At5g18800 and At3g06310), and a 12.6-kD subunit (At2g42310 and At3g77785; Table 1). For some other subunits, genes encoding isoforms are present in the Arabidopsis genome, but the encoded proteins are not distinguishable by the peptide sequences obtained.

The CA Subunits of Complex I from Arabidopsis

The CA1, CA2, CA3, CAL1, and CAL2 proteins also exhibit sequence similarity and might be considered to represent isoforms. However, biochemical evidence indicates the presence of at least three proteins of the CA/CAL family in individual complex I particles because the CA domain attached to complex I has a trimeric structure (Sunderhaus et al., 2006). Unfortunately, despite large efforts, the CA/CAL proteins so far could not be functionally characterized. The genes encoding CA1 and CA2 are downregulated if plants are cultivated in the presence of high CO2 concentrations (Perales et al., 2005), which is also known for enzymes involved in photorespiration. A role for the CA/CAL proteins in an intracellular CO2 transfer mechanism from mitochondria to chloroplasts analogous to the cyanobacterial carbon concentration mechanism has been proposed (Braun and Zabaleta, 2007). Recently, overexpressed CA2 was shown to bind CO2 and/or bicarbonate efficiently (Martin et al., 2009).

For reasons not understood, the five CA/CAL proteins are dissected of complex I into functional modules in a highly reproducible way. There are a few examples in the literature on usage of low SDS for native protein analyses, for example, the green native gel electrophoresis system developed for the analysis of protein complexes of the photosynthesis apparatus, which is based on a first gel dimension in the presence of low and a second gel dimension in the presence of high SDS (Thornber, 1986; Allen and Staehelin, 1991). We suggest the use of low SDS concentrations generally as a tool for the biochemical analysis of the internal architecture of protein complexes.

Subunits of Mitochondrial Complex I from Arabidopsis

Forty different complex I subunits were identified by MS analysis of complex I subcomplexes (Figure 5, Table 1). Compared with the most extensive study on complex I subunits in plants (Meyer et al., 2008), which was based on the separation of complex I proteins by three-dimensional BN/SDS/SDS-PAGE, six subunits were not detected (the ND6, 18-kD, B17.2, B14.7, B14, and At1g68680 subunits). These subunits probably are lost during low SDS treatment. Indeed, at least five subunits with apparent molecular masses of 39, 19, 16, 8, and 7 kD form part of intact complex I on BN gels but neither are part of the membrane nor the peripheral arm (see Supplemental Figure 1 online). The 39-kD subunit was identified by MS on the 2D BN/SDS gel shown in Figure 5 as a monomer, whereas the other subunits were not detected. On the other hand, four of the 40 subunits identified in our study were not reported in Meyer et al. (2008): the homolog of the bovine 13-kD subunit (At4g03070; Figure 6), ND3, GLDH, and the novel plant-specific subunit At1g14105. Two further complex I subunits (B22 and At1g14450) were identified by another study (Sunderhaus et al., 2006) but not found by Meyer et al. (2008) or within any of the complex I subcomplexes analyzed in this investigation. Overall, 17 plant-specific complex I subunits have now been identified (Heazlewood et al., 2003; Sunderhaus et al., 2006; Meyer et al., 2008; this study). Except for the CA subunits and GLDH, most of them are small hydrophobic subunits forming part of the membrane arm. It currently cannot be excluded that some of these proteins are structural homologs of small subunits additionally present in fungal or mammalian complex I since sequence conservation often is low between related proteins if they are small and hydrophobic (Brandt, 2006).

In addition to the overall 48 MS-identified complex I subunits of Arabidopsis (Sunderhaus et al., 2006; Meyer et al., 2008; this study), the mitochondrial genome of Arabidopsis encodes an ND4L homolog that is extremely hydrophobic and most likely difficult to detect by MS. The number of distinct complex I subunits in Arabidopsis therefore adds up to at least 49. Bovine complex I, which is the largest complex I particle biochemically characterized so far, has 45 distinct subunits (Carroll et al., 2006), and complex I of the fungus Yarrowia lipolytica has 40 subunits (Morgner et al., 2008). Indeed, upon direct size comparison of bovine and potato (Solanum tuberosum) complex I by 1D BN-PAGE, the enzyme complex from plants is slightly larger (Jänsch et al., 1995).

Figure 6. Alignment of At3g03070 with the 13-kD Subunit of Human and Beef Complex I. Amino acid positions identical in all three sequences are highlighted in dark blue and amino acid positions conserved in two sequences in light blue. The orange box indicates peptides identified by MS analyses.
kD (27.837 and 29.043 kD) due to a small insertion in At5g66510.2 at a central position. The functional relevance of this possible CA3 heterogeneity is so far unclear.

Mapping of MS-identified peptides for the CA/CAL proteins to the complete amino acid sequences deduced from the corresponding genes indicates the absence of a cleavable presequence for CA1, CA2, and CA3 (Figure 7). In the case of CA3, the N terminus of the primary translation product forms part of the mature protein. For CA1 and CA2, the most N-terminal peptides identified by MS start at amino acid 7 of the translation products. The six N-terminal amino acids of the translation products do not exhibit the typical properties of cleavable mitochondria targeting sequences (Glaser et al., 1998). Also, the apparent molecular masses of CA1 and CA2, which both migrate at 30 kD on 2D BN/SDS gels, exactly fit to the calculated molecular masses of the translation products (29.970 and 30.065 kD). By contrast, the CAL1 and CAL2 sequences have cleavable presequences. Removal of the targeting sequences was previously shown by an in vitro processing assay (Perales et al., 2004). The length of the presequences can be predicted to be 22 amino acids for CAL1 and 26 amino acids for CAL2 on the basis of a sequence comparison to the N-terminal region of a homologous 29-kD subunit of complex I from potato (Herz et al., 1994) (Figure 7). Also, N termini for the mature CAL2 and CAL1 proteins were recently directly determined by an elegant MS-based approach (Huang et al., 2009). The resulting masses of the mature CA1 and CAL2 subunits are 25.079 and 25.045 kD, which nicely corresponds to their apparent masses upon separation by BN/SDS-PAGE (Figure 5).

Internal Architecture of Complex I from Arabidopsis

Incubation of isolated complex I from Arabidopsis with SDS solutions of low concentration allows the dissection of the complex into 10 defined subcomplexes. At 0.01% SDS, the membrane arm (550 kD) and the peripheral arm (370 kD) are generated. Furthermore, approximately five subunits are detached as monomers during this first dissection step, including the 39-kD subunit, which was identified by MS within the dye front of the BN gel dimension. Increase of SDS to 0.04% further dissects the two arms into secondary subcomplexes of 450, 270, 240, 210, 160, 140, and 85 kD. The proposed disassembly process of complex I from Arabidopsis is summarized in Figure 8. Apparent molecular masses of the subcomplexes are in good accordance with calculated masses of the subcomplexes based on the sum of the masses of their subunits. However, in some cases, the calculated masses of the subcomplexes are slightly lower, most likely indicating that further subunits might additionally be present, which are not resolved on the Coomassie blue-stained gel (Figure 5).

Subfractionation procedures were previously used for the analysis of complex I particles from other organisms. In beef, treatment of isolated complex I with the nondenaturating detergent N,N-dimethyldodecylamine N-oxide dissociates the enzyme

Figure 7. Coverage of the CA Sequences by Peptides Identified by MS.

The five amino acid sequences of the CA/CA-like subunits of Arabidopsis complex I were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Identified peptides are highlighted in blue. The sequences correspond to the following accessions: At1g19580 (CA1), At1g47260 (CA2), At5g66510.1 (CA3), At5g36510 (CAL1), and At3g48680 (CAL2). The red arrow indicates the cleavage site for the removal of the presequences in CAL1 and CAL2.
into two parts, an $\alpha$-part including the subunits of the peripheral arm as well as adjacent subunits of the membrane arm and a $\beta$-part that represents the remaining membrane arm (Finel et al., 1992). Modified procedures allow the purification of a $\lambda$-subcomplex including the hydrophilic subunits and a $\gamma$-subcomplex that includes the more hydrophobic subunits of the $\alpha$-part of beef complex I (Arizmendi et al., 1992; Sazanov et al., 2000; Carroll et al., 2003). In *E. coli*, treatment of complex I with Triton X-100 leads to the dissociation of complex I into a membrane part, a water-soluble NADH dehydrogenase part, and an amphipathic part that connects the two other parts (Leif et al., 1995). The subunit compositions of the bovine and *E. coli* subcomplexes have been resolved (Leif et al., 1995; Carroll et al., 2003). Based on the protein composition of complex I subcomplexes, functional domains have been defined (reviewed in Brandt, 2006): an NADH oxidation (N) module, a quinone reduction (Q) module, and a proton translocating (P) module.

Systematic analysis of the subunit compositions of the 10 complex I subcomplexes generated by SDS treatment gave the following insights into the internal architecture of this protein.

Figure 8. Proposed Disassembly Process for Arabidopsis Complex I.

Top: 2D BN/SDS separations of complex I subcomplexes generated by 0.04% SDS. The membrane arm and its dissection products are indicated on the 2D gel on the left and the peripheral arm and its subcomplexes on the 2D gel to the right. Apparent molecular masses of the subcomplexes are given above the gels (in kD). Bottom: proposed disassembly pathway of Arabidopsis complex I. Membrane subcomplexes/subunits are given in red and the ones of the peripheral arm in orange. Numbers indicate apparent molecular masses (in kD). The table to the right of the scheme compares the apparent molecular masses of the generated complex I subcomplexes with the calculated molecular mass of the sum of their protein subunits identified by MS.

Since two subcomplexes deduced from the peripheral arm have apparent molecular masses of 140 kD, these are distinguished in the figure and in the table by (a) and (b). For unknown reasons, the calculated molecular mass of the 270 kD complex is too high.
complex in *Arabidopsis* (summarized in Figure 9). The 240-kDa subcomplex (75-kD, 51-kD, 24-kD, and B8 and/or At1g67785 subunits) represents the N-module and the 140-kD (140a subcomplex (49-kD/ND7, 30-kD/ND9, TYKY, PSST, and B13 subunits) the Q-module of complex I. Together with the 13-kD subunit (At3g03070), they form the peripheral arm (370 kD). The 240-kD subcomplex can be further dissected into a smaller 140-kD (140b) subcomplex including the 51- and 24-kD subunits and possibly some further subunits. This is the smallest subcomplex exhibiting NADH oxidation activity (Figure 3).

The membrane arm subcomplex (550 kD) includes at least five of the hydrophobic mitochondrially encoded subunits (ND1, ND2, ND3, ND4, and ND5), seven further mainly hydrophobic subunits homologous to nuclear-encoded complex I subunits from beef, eight additional plant-specific subunits, and the five CA/CAL proteins (Figure 9). Arrangement of all these subunits within the membrane arm still is largely unknown because the generation of secondary subcomplexes is difficult. However, based on single particle EM and cross-linking experiments, the location for some proteins is known. For instance, several lines of evidence suggest that the ND4 and ND5 proteins are in close proximity and form the tip of the membrane arm (Holt et al., 2003; Baranova et al., 2007a, 2007b). Analysis of a 210-kD subcomplex derived from the membrane arm of complex I from *Arabidopsis* indicates an association of the PDWS subunit and at least one of the three subunits included in spot 17 (Figure 5, Table 1) with ND5. The ND6 and possibly also the ND4L subunits, which could not be identified within the 550-kD membrane arm of *Arabidopsis* complex I, probably are detached from complex I during initial dissection into the membrane and the peripheral arm at 0.01% SDS and are speculated to represent the end of the membrane arm at the site of its connection to the peripheral arm. This is in line with detection of ND6 in subcomplex α of complex I from beef but its absence in subcomplex λ (Carroll et al., 2003). Most interestingly, the B16.6 subunit, which is identical to the GRIM-19 protein involved in apoptosis, forms part of the membrane arm of complex I in *Arabidopsis* but was reported to be part of the peripheral (λ) arm in beef (Carroll et al., 2003). This most likely points to a location of B16.6 at the intersection of the two complex I arms.

Some subunits were detached from *Arabidopsis* complex I during initial dissociation of the peripheral and the membrane arm (e.g., the 39-kD subunit). However, in a previous investigation (Sunderhaus et al., 2006), this protein was reported to be associated with the peripheral arm in *Arabidopsis*. Also, GLDH, which only in plants is part of complex I (Heazlewood et al., 2003), was not found to be associated with either of its two arms. Since this enzyme catalyzes the terminal step of ascorbic acid biosynthesis, it should be associated with the membrane arm of complex I on the side exposed to the intermembrane space. However, recent findings indicate that GLDH might represent an assembly factor important for complex I biogenesis (Pineau et al., 2008). Indeed, GLDH initially was described as a subunit of *Arabidopsis* complex I version of slightly reduced molecular mass (Heazlewood et al., 2003; Millar et al., 2003).

Finally, an 85-kD subcomplex is generated if the membrane arm is further destabilized using 0.04% SDS. This complex includes the 30-kD CA1 and CA2 proteins and the 25-kD CAL1 and CAL2 proteins. Based on previous findings that CAs are trimers, we conclude that probably three of the four proteins form a CA domain. The stoichiometry of the four proteins upon analysis by 2D BN/SDS-PAGE (Figure 5) differs: the amount of CA1+CA2 clearly exceeds the one of CAL1+CAL2. We speculate that the CA domain attached to complex I might include two CA proteins (CA1+CA2 or two copies of CA2 or two copies of CA1) and additionally either CAL1 or CAL2. Interestingly, interaction of CA2 with CAL1 and CAL2 was previously proved by a two-hybrid screen (Perales et al., 2004). Furthermore, using the same approach, it was shown that the N-terminal half of CA2 binds to the CAL subunits. By contrast, CA1–CA2 interaction has not been reported. For reasons currently not understood, CA3 was not found to interact with any of the other members of the CA/CAL protein family, neither by two-hybrid screening nor by MS analysis of complex I subcomplexes. Further investigations will be necessary to better understand the composition and function of the CA domain of plant complex I.

**Outlook**

The presented biochemical strategy, SDS-mediated dissection of isolated complex I, separation of subcomplexes by BN/SDS-PAGE, and protein identifications by MS, allowed us to achieve insights into the internal architecture of complex I. Since crystallization of the entire complex proved to be intractable, sophisticated biochemical procedures are required to better understand the structure and function of this key enzyme complex of the respiratory chain. Despite large efforts, knowledge on the subunit arrangements within the membrane arm of eukaryotic complex I still is fragmentary. New protocols to generate...
additional subcomplexes of this arm will be crucial for a deeper understanding of internal complex I structure. Further progress could also come from combining controlled complex I dissections and cross-link approaches in future experiments. Analysis of complex I certainly will remain one of the most fascinating challenges in biochemistry.

**METHODS**

**Arabidopsis thaliana Cultivation and Isolation of Mitochondria**

Cell cultures of *Arabidopsis* wild type (Columbia-0) were established as described by May and Leaver (1993). Cells were suspended in a suspension culture maintained as outlined previously (Sunderhaus et al., 2006). Mitochondria were isolated from *Arabidopsis* suspension culture as given by Werhahn et al. (2001).

**Purification of Complex I**

Purification of complex I from isolated *Arabidopsis* mitochondria followed a two-step procedure based on (1) sucrose gradient ultracentrifugation to prepare the ~1-1 MD protein fraction and (2) cytochrome c affinity chromatography to deplete the complex I–enriched 1-MD fraction from remaining contaminations of complex II and supercomplex I+II. Isolated mitochondria were pelleted by centrifugation and suspended in digitonin solubilization buffer (30 mM HEPES, 150 mM potassium acetate, 10% [v/v] glycerol, and 5% [w/v] digitonin) at a detergent/protein ratio of 5 mg/mg. Protein complexes were subsequently resolved by sucrose gradient ultracentrifugation. Solubilized mitochondria (~10 mg mitochondrial protein) were loaded onto a 12-M sucrose gradient (15 mM Tris, 20 mM KCl, 0.2% [w/v] digitonin, and 0.3 to 1.5 M sucrose). Ultracentrifugation was performed at 4°C using Beckmann 9/16 × 3 3/4 Ultra Clear Tubes and the Beckmann SW40Ti rotor (146,000 g for 20 h). Afterwards, the gradient was divided into 700-μL fractions, and the protein complex composition of each fraction was analyzed by 1D BN-PAGE (see below). Fractions containing complex I but devoid of complex V were used for a cytochrome c–mediated depletion of complex III contaminations.

For this approach, cytochrome c was coupled to CNBr-activated Sepharose as described by Weiss and Juchs (1978). Before usage, the cytochrome c sepharose was washed with 300 mL oxidation solution (5 mM K₃[Fe(CN)₆], 0.5% [v/v] Triton X-100, 0.1 M NaHCO₃, and 0.5 M NaCl) to oxidize the cytochrome c. Subsequently, this solution was removed by washing the Sepharose with 150 mL washing buffer (20 mM Tris-acetate, pH 7.0, 5% [w/v] sucrose, 0.04% [w/v] digitonin, and 0.2 mM PMSF). Six milliliters of Sepharose was filled into a column (1 cm × 10 cm) and washed with washing buffer another time. Afterwards, the complex I–containing sucrose gradient fractions were transferred on the top of the column, and the flow-through was collected in 1-ML fractions. Additional 2 mL of washing buffer was used to remove remaining complex I from the column. Finally, complex II and the I+II supercomplex were eluted from the column using a salt gradient (20 to 200 mM Tris-acetate, pH 7.0, 5.0% [w/v] sucrose, 0.04% [w/v] digitonin, 0.2 mM PMSF, and 2 mM Na-ascorbate). The eluate was collected in 1-ML fractions. For evaluation of the protein complex content of the fractions, 25 μL of each flow-through fraction and 75 μL of each eluate fraction were analyzed by 1D BN-PAGE (see below). Fractions containing pure complex I were concentrated using an ultra filtration cell (Millipore; polyethersulfone filter; exclusion limit, 100,000 kD) to a final protein concentration of ~5 mg/mL.

**Controlled Disassembly of Complex I**

For disassembly experiments, purified complex I (1 mg in 200 μL) was treated with low concentrations of SDS (0.01 to 0.12% [w/v]) for 5 min on ice. Afterwards, 1 μL BN loading buffer (750 mM aminocaproic acid and 5% [w/v] Coomassie Brilliant Blue G 250) was added.

**Gel Electrophoresis Procedures**

1D BN-PAGE was used to separate the generated complex I subcomplexes and 2D BN/SDS-PAGE to analyze their subunit composition. Procedures were performed as outlined previously (Wittig et al., 2006). Proteins were either visualized by Coomassie Brilliant Blue colloid staining (Neuhoff et al., 1988) or in-gel NADH dehydrogenase activity staining (Zerbetto et al., 1997).

**MS**

Tryptic digestion of proteins was performed as follows: Protein spots were cut from 2D BN/SDS gels and destained. Gel pieces were dehydrated using acetonitrile. For alkylation of cysteins, gel pieces were first incubated with 20 mM DTT for 30 min at 56°C, dehydrated, and incubated in 55 mM iodoacetamide at room temperature for the same time in the dark. The dehydrated gel pieces were incubated in 0.1 M NH₄HCO₃, and digestion was performed at 37°C overnight using trypsin (2 μg/mL resuspension buffer [Promega] in 0.1 M NH₄HCO₃). Tryptic peptides were extracted by incubation with acetonitrile for 15 min at 37°C. Supernatants were kept and basic peptides were extracted with 5% formic acid for 15 min at 37°C. The dehydration step was repeated, and the supernatant was pooled with the first one. Extracted peptides were finally dried via vacuum centrifugation and stored at −20°C.

Protein identification by MS was performed as follows: Tryptic peptides were resuspended in 20 μL of washing solution (2% [v/v] methanol and 0.5% [v/v] formic acid) and desalted using ZipTips (0.2 μL C₁₈; Millipore). For matrix-assisted laser desorption ionization (MALDI) analyses, 1 μL of Matrix solution (0.1% [v/v] α-Cyano-4-hydroxy-cinnamic acid, 25% [v/v] acetonitrile, and 0.1% [v/v] trifluoroacetic acid) was mixed with 1 μL of desalted peptide solution and spotted on a MALDI target. MALDI-time of flight (TOF)/TOF-MS analyses were performed with an Ultraflex II mass spectrometer (Bruker Daltonics). Offline electrospray ionization (ESI)-quadrupole (Q)-TOF-MS analyses were performed with a Q-TOF 2 mass spectrometer (Waters).

For liquid chromatography–ESI-Q-TOF-MS analyses, tryptic peptides were resuspended in 15 μL 0.1% [v/v] formic acid. The MS analyses were performed with the EASY-nLC System (Proxeon) coupled to a MicrOTOF-Q II mass spectrometer (Bruker Daltonics). Proteins were identified using the MASCOT search algorithm against (1) the *Arabidopsis* protein database (www.Arabidopsis.org; releaseTAIR 8), (2) a complex I database generated from the complex I subunits identified by Meyer et al. (2008), and (3) the NCBI nonredundant protein database (www.ncbi.nih.gov).

**Accession Numbers**

All peptides identified in our study match protein sequences deduced from the *Arabidopsis* genome sequence (www.Arabidopsis.org). The corresponding accession numbers are listed in Table 1 (column 2).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Subunits Detached from Complex I upon Treatment with Low Concentrations of SDS.

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