“Respirasome”-like Supercomplexes in Green Leaf Mitochondria of Spinach*

Frank Krause‡, Nicole H. Reifschneider, Dirk Vocke, Holger Seelert, Sascha Rexroth, and Norbert A. Dencher

From the Physical Biochemistry, Department of Chemistry, Darmstadt University of Technology, Petersenstrasse 22, D-64287 Darmstadt, Germany

Higher plant mitochondria have many unique features compared with their animal and fungal counterparts. This is to a large extent related to the close functional interdependence of mitochondria and chloroplasts, in which the two ATP-generating processes of oxidative phosphorylation and photosynthesis, respectively, take place. We show that digitonin treatment of mitochondria contaminated with chloroplasts from spinach (Spinacia oleracea) green leaves at two different buffer conditions, performed to solubilize oxidative phosphorylation supercomplexes, selectively extracts the mitochondrial membrane protein complexes and only low amounts of stroma thylakoid membrane proteins. By analysis of digitonin extracts from partially purified mitochondria of green leaves from spinach using blue and colorless native electrophoresis, we demonstrate for the first time that in green plant tissue a substantial proportion of the respiratory complex IV is assembled with complexes I and III into “respirasome”-like supercomplexes, previously observed in mammalian, fungal, and non-green plant mitochondria only. Thus, fundamental features of the supramolecular organization of the standard respiratory complexes I, III, and IV as a respirasome are conserved in all higher eukaryotes. Because the plant respiratory chain is highly branched possessing additional alternative enzymes, the functional implications of the occurrence of respiratory supercomplexes in plant mitochondria are discussed.

The inner mitochondrial membrane contains the four major respiratory complexes I–IV of the standard respiratory chain and the MFOF1-ATP synthase (complex V), designated as oxidative phosphorylation (OXPHOS) complexes, found in almost all eukaryotes (1). The respiratory complexes I (NADH:ubiquinone:oxidoreductase), III (ubiquinol:cytochrome c oxidoreductase), and IV (cytochrome c oxidase) transduce the energy of organic compounds ultimately leading to the generation of ATP by the MFOF1-ATP synthase. In contrast to the textbook view (2), it was recently proposed that the OXPHOS complexes of mammals are organized as large assemblies constituting a supramolecular network, the so-called “respirasome,” consisting of two supercomplexes (I,III,IVc and III,IVc) as building blocks in a 2:1 ratio (3–6). This model was based on results by blue native PAGE (BN-PAGE) after mild solubilization of mitochondria with digitonin by which most of complexes I, III, and a smaller but significant proportion of complex IV were resolved as stoichiometric supercomplexes. Recently, it could be demonstrated that the complex IV-dependent respiratory chain of the filamentous fungus Podospora anserina is organized according to the respirasome model as well (7). In mitochondria of the yeast Saccharomyces cerevisiae, which possesses no complex I, the complexes III and IV are assembled into the supercomplexes III,IVc and III,IVc (3, 8). Enzymatic analyses suggested that substrate channeling between the complexes is one major functional significance of the respirasome architecture in mammals and S. cerevisiae (3, 9–11). Another crucial function of respiratory supercomplexes turned up because compelling evidence suggested that assembled complex III is essential for the stability of complex I (5–7, 12–14).

Higher plant mitochondria are unique in many aspects compared with those of animals and fungi (for review, see Ref. 15). One of the most outstanding features of plant mitochondria is the functional cooperation with chloroplasts in photosynthetically active tissues (16, 17). As part of the photosynthetic pathway, mitochondria decarboxylate large quantities of glycerol by reduction of NAD+ to NADH (16, 18). The plant respiratory chain is branched due to the occurrence of additional alternative respiratory enzymes besides the respiratory complexes I–IV (for review, see Refs. 19–21). Specifically, there are at least four alternative NAD(P)H-dehydrogenases (19, 20) and several isoforms of the alternative oxidase (AOX) (22). The latter transfers electrons directly from ubiquinol to oxygen, thereby bypassing complexes III and IV. All these alternative enzymes are non-proton-pumping and consequently do not contribute to energy conservation. Until now, their functional significance for respiration in plants is enigmatic in many aspects (19–21). Interestingly, an important link to photosynthesis emerged recently due to the discovery of light-dependent gene expression of some alternative respiratory enzymes (23, 24).

To date, the only two studies addressing the supramolecular organization of the plant respiratory chain by BN-PAGE analysis found that in non-green tissue mitochondria of the four plant species Arabidopsis thaliana, potato, bean, and barley, 50–90% of complex I is assembled with complex III into the

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‡ To whom correspondence should be addressed. Tel.: 49-6151-184474; Fax: 49-6151-184474; E-mail: f.krause@pop.tu-darmstadt.de.
* The abbreviations used are: OXPHOS, oxidative phosphorylation; 6-ACA, 6-aminoacaproic acid; AOX, alternative oxidase; BN-PAGE, blue-native PAGE; CN-PAGE, colorless-native PAGE; DDM, n-dodecymaltoside; DM, n-decylmaltoside; DS, n-dodecanolsucrose; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MALDI, matrix-assisted laser desorption ionization; PSI, photosystem I; Rubisco, ribulose biphosphate carboxylase/oxygenase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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stable supercomplex I_1III_2 (25), whereas stable interactions of complex IV with complex I and complex III could only be demonstrated in potato mitochondria (26). Importantly, these authors reported without further details that they detected only very minor amounts of complex IV-containing supercomplexes in Arabidopsis leaves (26).

To address the involvement of complex IV in any supramolecular organization of the standard respiratory complexes in photosynthetically active tissues of higher plant mitochondria, we analyzed digitonin extracts of partially purified mitochondria from spinach green leaves using BN-PAGE and a more gentle colorless native (CN)-PAGE. Our results show that under these conditions in HEPES/potassium acetate buffer (pH 7.4) or in Tricine/6-aminocaproic acid (6-ACA) buffer (pH 8.0), the mitochondrial membranes were selectively solubilized with only minor amounts of extracted stroma thylakoid membrane proteins. Significantly, the latter did not disturb the analysis of OXPHOS complexes. We demonstrate that most of complexes I, III, and to a lesser extent complex IV form common supercomplexes and reveal direct complex I-III as well as complex III-IV interactions, resembling the respirasome architecture of the mammalian and fungal standard respiratory chains. Additionally, a small proportion of the MF_d-F_o-ATP synthase was observed in the dimeric state.

EXPERIMENTAL PROCEDURES

Electrophoretic Techniques—Digitonin, n-decylmaltoside (DM), n-dodecylmaltoside (DDM), and n-dodecanoylsucrose (DS) were of high purity grade from Calbiochem Merck, as well as Triton X-100 of high purity grade from Roche Diagnostics. Commercially purchased spinach green leaves were used as starting material. Mitochondria were partially purified using a standard procedure by differential centrifugation and Percoll density gradient centrifugation (27). Pure chloroplasts were isolated as previously described (28–30). The membranes were solubilized with digitonin using detergent/protein ratios of 1–10 g/g in 1% (w/v) potassium acetate, 30 mM HEPES, 10% (w/v) glycerol, 0.5 mM Pefabloc™ SC (pH 7.4) at a constant detergent concentration of 1% (w/v) unless otherwise indicated essentially as described (3, 7, 31). Alternatively, the digitonin solubilization was performed according to Rechroth et al. (32, 33) using detergent/protein ratios of 1–10 g/g in 200 mM 6-ACA, 5 mM MgCl_2, 5 mM MnCl_2, 5 mM dithiothreitol, 10% (w/v) glycerol (pH 8.0) at a constant detergent concentration of 1% (w/v). As a third approach, membranes were solubilized with DM or DS, respectively, in 1 M 6-ACA, 50 mM Bis-Tris (pH 7.0) using a detergent/protein ratio of 1 g/g at a detergent concentration of 1% (w/v). The samples were incubated for 30 min on ice unless otherwise indicated and subsequently centrifuged at 21,500 × g for 10 min. For BN-PAGE linear 4–13% gradient gels overlaid with a 3.5% stacking gel were used (3, 7, 34). CN-PAGE was performed as described (7, 34) with complete omission of Coomassie Blue G-250 dye using linear 3–16% gradient gels respectively, in 1M 6-ACA, 50 mM Bis-Tris (pH 7.0) using a detergent/protein ratio of 1 g/g, 5% (w/v) glycerol, and the subunit IV of NADH dehydrogenase of complex I was probed by in-gel formazan precipitation, and the cytochrome c oxidase activity of complex IV was probed by precipitation of 33'-diaminobenzidine oxides and indamine polymers as described in Krause et al. (7). In-gel staining of succinate dehydrogenase activity was performed according to Zerbetto et al. (35).

RESULTS

Separation of Respirasome-like Supercomplexes from Partially Purified Spinach Green Leaf Mitochondria after Selective Digitonin Solubilization—Mitochondria from green tissue have to be investigated to unravel the unique functions of plant mitochondria, especially of respiration, during photosynthesis. Using a standard procedure we prepared enriched mitochondrial fractions from spinach green leaves, which yet displayed green color, indicating a substantial contamination with chloroplasts. Over a period of 19 months nine independent mitochondria-rich fractions from spinach green leaves purchased from various sources have been prepared, each with a varying mitochondrial content.

To examine whether a respirasome organization is present in mitochondria of higher plant green leaves, we solubilized such mitochondria-rich fractions with 1% (w/v) digitonin at a detergent/protein ratio of 1 g/g in HEPES/potassium acetate (pH 7.4) under approximately the same conditions as previously described, leading to the separation of respiratory supercomplexes from mammalian (3–6), fungal (3–7), and non-green plant tissue mitochondria (25, 26). Remarkably, after centrifugation the digitonin extracts always had only a very slight green color, in contrast to the dark green pellet, indicating that the thylakoid membranes of the contaminating chloroplasts were largely resistant against the digitonin treatment.

To investigate this effect in more detail we compared one of the nine partially purified mitochondrial fractions possessing a particular high and clearly predominant chloroplast content (named M1) with pure chloroplasts both solubilized in the same manner with digitonin as well as with fraction M1 quantitatively solubilized by DS or DM. In fact, BN-PAGE analysis of the digitonin extract (Fig. 1, A and B, lane 2) revealed a protein band pattern very similar to that of individual OXPHOS complexes (molecular mass 130–1,000 kDa) and supercomplexes (molecular mass 1,000–20,000 kDa) as well as dimeric ATP synthase (V_2) were assigned according to their subunit compositions and apparent molecular masses. An attempt was made to determine the stoichiometries of the larger supercomplexes with apparent molecular masses of 1,800–3,000 kDa resolved by first dimension BN- and CN-PAGE, which were assigned as I_1III_2IV_1 and as I_1III_3IV_1,

Enzymatic Analyses—The NADH dehydrogenase activity of complex I was probed by in-gel formazan precipitation, and the cytochrome c oxidase activity of complex IV was probed by precipitation of 33'-diaminobenzidine oxides and indamine polymers as described in Krause et al. (7). In-gel staining of succinate dehydrogenase activity was performed according to Zerbetto et al. (35).

The increase of the digitonin/protein ratio up to 10 resulted in a very different pattern with one additional non-green protein complex at ~600 kDa discernible on the BN gel (Fig. 1, A and B, lane 1). Besides one dominant chloroplast protein band at ~450 kDa (Rubisco, see below) only one green protein complex could be recognized as a rather weak band at ~650 kDa.

The increase of the digitonin/protein ratio up to 10 resulted in a very different pattern with one additional non-green protein complex at ~600 kDa discernible on the BN gel (Fig. 1, A and B, lane 3) despite the predominant content of chloroplasts in this mitochondria-rich fraction. This became recognizable upon approximately quantitative solubilization with DM or DS, leading to a very thin colorless pellet after centrifugation and resulting in the large amounts of chlorophyll-containing green protein bands (photosystems I and II, light-harvesting complexes) (Fig. 1, A and B, lanes 6 and 7). In line with this observation, the digitonin treatment of pure spinach chloroplasts in HEPES/potassium acetate (pH 7.4) even at 10 g of digitonin/g of protein (Fig. 1, A and B, lanes 4 and 5) or using...
Increased digitonin concentrations up to 7.5% (w/v) (not shown) was far less efficient than the solubilization with DM or DS. Most striking was the comparably minor yield of extracted green chlorophyll-containing proteins after digitonin treatment. Furthermore, it became apparent that some digitonin-solubilized proteins of pure chloroplasts were not or hardly were extracted from the mitochondria-rich fraction (Fig. 1, A and B, compare lanes 2 and 3 with 4 and 5). Interestingly, digitonin extraction of pure chloroplasts yielded minor amounts of heavy protein complexes (≥1,500 kDa); yields increased at higher digitonin/protein ratios. If these would be extracted from mitochondria-rich fractions, the identification of OXPHOS supercomplexes could be potentially complicated; this, however, was not the case (see below). The overall result was surprising at first glance because digitonin has been long since used as a very suitable detergent in chloroplast research (e.g. 32, 33, 36–39). In particular, digitonin solubilization of thylakoids from the green alga *Chlamydomonas reinhardtii* in Tricine/6-ACA buffer (pH 8.0) proved to be efficient in analyzing the entire photophosphorylation system in quantitative yield (32). Therefore, a further test in examining the rather selective extraction of mitochondrial proteins with digitonin in chloroplast-contaminated spinach mitochondria, the mitochondria-rich fraction M1 as well as pure chloroplasts were analyzed by BN-PAGE after digitonin solubilization (10 g of digitonin/g of protein) in Tricine/6-ACA buffer (pH 8.0) essentially as recently described by us (32, 33) and additionally at a low digitonin/protein ratio of 1 g/g. Overall, in all cases the quantities of extracted proteins as well as the patterns of separated proteins were very similar to solubilization in HEPES/potassium acetate buffer (pH 7.4) (Fig. 1, A and B, compare lanes 2–5 with 9–12). Likewise, the yields of extracted proteins were the same if the samples were solubilized at 4 °C under continuous agitation according to the original protocol (not shown). Interestingly, solubilization of bovine heart mitochondria under these conditions revealed no substantial differences to digitonin solubilization in the standard HEPES/potassium acetate buffer because nearly the same yield of OXPHOS supercomplexes was obtained (Fig. 1, A and B, compare lanes 1 and 8).
FIG. 2. Identification of OXPHOS supercomplexes from spinach green leaf mitochondria. A and B, BN-PAGE/second-dimension SDS-PAGE of mitochondria-rich fraction M1 with a very high chloroplast content upon solubilization in Tricine/6-ACA (pH 8.0) with digitonin/protein ratios of 1 g/g (A) or 10 g/g (B). The first-dimension gels were Coomassie Blue-stained (upper panels), and the second dimension gels were silver-stained (lower panels). The characteristic subunit patterns of OXPHOS complexes I, III, IV, and V, as well as supercomplexes III,IV, a (I,III,IV), b (I,III,IV), and the larger ones (I,III,IV, see D and E) are recognizable. The assignment of subunits is as follows: complexes I (band 1, ND7; band 2, ND9); V (band 3, α; band 4, β), III (band 5, core I; band 6, core II), and IV (band 7, Cox II). The subunits of the contaminating chloroplast protein complexes cytochrome b6f, PSI, and chloroplast ATP synthase (CF,FO1) are indicated by green continuous vertical lines, and the large and small subunits of Rubisco are indicated by green circles.

C–E, characterization of OXPHOS supercomplexes by analysis of a representative mitochondria-rich fraction (M2), with lower chloroplast content than M1, solubilized in HEPES/potassium acetate (pH 7.4) with digitonin. C, first-dimension BN-PAGE of M2 solubilized with the indicated digitonin/protein ratios (lanes 1–4) as well as in lane 5 of digitonin-solubilized bovine heart mitochondria (BHM) as the mass standard (individual complexes I-V (130–1,000 kDa) and supercomplexes a–e (I,III,IV, 1,500–2,300 kDa)). The contaminating chloroplast complexes Rubisco (green bar) as well as PSI and CF,FO1 (green box) are marked. D, first-dimension CN-PAGE (upper panel) of M2 (digitonin/protein, 1 g/g) and second-dimension resolution by SDS-PAGE (middle panel) or BN-PAGE with DDM in the cathode buffer to dissociate the OXPHOS supercomplexes into their individual complexes (lower panel). E, analysis of M2 (digitonin/protein, 3 g/g) by a more gentle first-dimension CN-PAGE (upper panel) does not preserve higher amounts of OXPHOS supercomplexes. Second-dimension (2D) resolution by SDS-PAGE (middle panel) or BN-PAGE with DDM in the cathode buffer (lower panel) is shown.
The identities of two OXPHOS complexes were verified by identification of the ND7 and ND9 subunits of monomeric complex I and of the supercomplex a (I1III2) as well as the α- and β-subunits of monomeric complex V using MALDI time-of-flight mass spectrometry of trypsin-digested spots from second-dimension SDS gels. The identity of complex IV was confirmed by cytochrome c oxidase in-gel activity staining (not shown). Besides the individual complexes I1 (|1,000 kDa), III2 (|500 kDa), and IV0 (|200 kDa), additional supercomplexes were observed. Most of the complex I was assembled together with complex III into a group of supercomplexes with molecular masses of 1,500–3,000 kDa. The supercomplexes a (I1III2, |1,500 kDa) and b (I1III2IV1, |1,700 kDa) were assigned according to their subunit compositions and their apparent molecular masses. Apart from these high molecular weight complexes, a substantial fraction of dimeric complex III was found assembled together with complex IV (predominantly III2IV1), which demonstrated a direct complex III-IV interaction in accordance with the respirasome model. The major contaminant from chloroplasts in the digitonin extract of the mitochondria-rich fraction was the highly abundant stroma protein Rubisco (|650 kDa) which became apparent. Only very minor amounts of the major chloroplast contaminant (Fig. 2D, middle panel). The identities of complexes II and IV were confirmed by succinate dehydrogenase or cytochrome c oxidase in-gel activity staining, respectively (not shown). Besides the individual complexes I1 (|1,000 kDa), III2 (|500 kDa), and IV0 (|400 kDa), the above-described supercomplexes were observed. The occurrence of complex IV as a component of supercomplex b and of the larger supercomplexes (I1III2IVx) was unambiguously confirmed by second-dimension BN-PAGE with the addition of 0.02% DDM to the cathode buffer, dissociating the supercomplexes into their constituting, individual OXPHOS complexes. Only low amounts of undissociated I1III2 from supercomplexes a, b, and I1III2IV2 remained (Fig. 2D, lower panel). Silver staining was required to detect the dissociated complex IV monomers of the large supercomplexes I1III2IVx (not shown). Complexes from the first-dimension BN-PAGE that retained their masses after second-dimension BN-PAGE migrated on a diagonal. Apparently, some of the slow migrating species I1III2 were the respirasome-like supercomplexes I1III2IVx as well as larger ones. Additionally, the presence of complexes I and/or IV as constituents of all the respective supercomplexes was verified by in-gel staining of NADH dehydrogenase or cytochrome c oxidase activities, respectively (not shown). We assign the individual complex IV resolved by first dimension BN-PAGE as a dimer (IV2) because its apparent mass of |400 kDa fitted rather well to two monomers (|200 kDa). Furthermore, the dissociated complex IV monomers migrated well below the corresponding position of individual complex IV on the diagonal in second-dimension BN-PAGE (Fig. 2D, lower panel). A small fraction of the total MF2F1-ATP synthase (complex V) was observed in the dimeric state (Fig. 2D), which was recently found in four other plant species as well (25) and was also demonstrated to occur in mammals (3), the green alga C. reinhardtii (33, 40), yeast (3, 31) and the filamentous fungus P. anserina (7).

**CN-PAGE Analysis of Digitonin-solubilized Spinach Mitochondria Does Not Lead to Higher Yields of Preserved OXPHOS Supercomplexes**—The dissociative properties of anionic Coomassie Blue G-250 dye employed during BN-PAGE are known, which is able to disrupt labile subunits or weak interactions between protein complexes (7, 30, 41). This becomes particularly apparent in the cases of mammalian and fungal OXPHOS supercomplexes (7). Therefore, it is mandatory to complement the analysis of mitochondrial protein-protein interactions by the particularly gentle CN-PAGE. This electrophoresis is based on the same gel system as BN-PAGE; however, in CN-PAGE the use of Coomassie Blue G-250 dye is completely omitted, and mild detergents are present in a constant concentration throughout the gel to avoid unspecific aggregation of hydrophobic proteins (34). We added 0.01% (w/v) Triton X-100 or 0.01% (w/v) digitonin, respectively, to the gels (7). During CN-PAGE the protein-lipid detergent complexes solubilized from membranes migrate due to their intrinsic charge, whereby the separating principle is the molecular sieving effect of the polyacrylamide gradient gel as in BN-PAGE. On the other hand, the resolving power of CN-PAGE is usually inferior to that of BN-PAGE but was previously found satisfactory for mammalian and fungal mitochondrial OXPHOS complexes (7, 34, 41). Overall, analysis by CN-PAGE with 0.01%

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2 N. H. Reischneider, H. J. Schwassmann, H. Seelert, N. A. Dencher, and F. Krause, manuscript in preparation.

3 F. Krause, N. H. Reischneider, S. Goto, and N. A. Dencher, submitted for publication.
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Triton X-100 present in the gel of the mitochondria-rich fraction M2 (3 g of digitonin/g of protein) analyzed also by BN-PAGE did not result in significantly higher yields of preserved supercomplexes of the respiratory chain (Fig. 2E), in particular of dimeric ATP synthase (V$_2$) compared with BN-PAGE (Fig. 2, C and D). Essentially the same results were obtained using CN gels with 0.01% digitonin present in the gel as well as by analysis of the other independently prepared mitochondria-rich samples (not shown). Interestingly, the second-dimension BN-PAGE (Fig. 2E, lower panel) revealed that the electrophoretic mobilities of the complexes I, III, IV, and V as well as of Rubisco were approximately the same or similar, respectively, under the conditions of BN-PAGE and CN-PAGE.

DISCUSSION

Selective Extractability of Mitochondrial and Stroma Thylakoid Membrane Protein Complexes by Digitonin Treatment—An important finding of this study is the nearly selective extraction of spinach mitochondrial membrane proteins with digitonin in the presence of chloroplasts, observed for two different buffer systems, which enables the solubilization of OXPHOS supercomplexes. This quite selective effect of digitonin treatment is in clear contrast to that in the case of corresponding mixed fractions of mitochondria and chloroplasts from the green alga C. reinhardtii, leading to efficient solubilization of thylakoid membrane proteins as well, in line with the results of our recent reports on digitonin-solubilized C. reinhardtii thylakoids (32, 33). Specifically, the major extracted chloroplast proteins by the digitonin treatment of mixed fractions of spinach organelles were the most abundant stroma protein Rubisco as well as low amounts of PSI, cytochrome $b_{6, f}$, and the chloroplast ATP synthase CF$_{0}$F$_{1}$ (Figs. 1 and 2, A and B), which in higher plants are located exclusively or in large proportion, respectively, in the non-apoplastic stroma thylakoids (42, 43). Otherwise, there was no significant extraction, if any, of the membrane proteins segregated in higher plants in the tightly appressed grana thylakoids, particularly of photosystem II. These results indicate that digitonin treatment under the conditions applied is able to significantly solubilize only the better-accessible stroma thylakoid membrane proteins, whereas the tightly appressed grana thylakoids remain largely resistant. In agreement with this conclusion, in the green alga C. reinhardtii the thylakoids are organized in a widely irregular manner, which is clearly distinct to that of higher plant thylakoids (43). Obviously, the easily accessible thylakoid membrane architecture, which is not given in higher plant thylakoids, is the crucial underlying molecular basis to explain the complete solubilization of C. reinhardtii thylakoids with digitonin. During the revision of this paper we studied many of the numerous pioneering reports describing the employment of digitonin fragmentation as an effective tool to unravel the photophosphorylation apparatus in higher plant chloroplasts (e.g., Refs. 36, 37, and 42). Indeed, the results described in these reports showed a differential solubilization of the thylakoid membrane proteins if the digitonin treatment was performed under conditions similar to those of the present study. In particular, Anderson and Boardman (37) investigated in a comprehensive survey the efficiency of digitonin to solubilize spinach chloroplasts. These authors varied each digitonin concentration from 0.5 to 2% (w/v) as well as solubilization time from 30 min to 72 h at 0 °C in 25–50 mM phosphate buffer (pH 7.2), 5–10 mM KCl (37). Essentially, the solubilization of chlorophyll-containing thylakoid membrane proteins was not quantitative, and the extractability of PSI was far better than that of photosystem II, particularly after 30 min of solubilization, whereas the detergents Triton X-100 and Nonidet P-10 proved much more efficient but less gentle than digitonin (37). It has to be mentioned that some variations in the amounts and nature of extracted thylakoid membrane protein complexes by digitonin treatment are conceivable because the thylakoid membrane architecture and their protein distribution in higher plants can change due to environmental conditions (43), which was also discussed in a recent report (44). A more detailed study and discussion of the effects of different non-ionic detergents (e.g., digitonin, DM, DS) to solubilize spinach chloroplasts for the characterization of photophosphorylation supercomplexes will be published elsewhere.

As the most important consequence of the above-discussed results, it has to be emphasized that the investigation of plant mitochondrial protein complexes and, in particular, of plant OXPHOS supercomplexes is greatly facilitated because an extensive time- and material-consuming purification of mitochondria is not necessary for most photosynthetic organisms. In fact, such an extended purification procedure may lead to some damage of mitochondria, because swelling and rupture of mitochondria is more likely, which might affect the stability of membrane protein complexes in subsequent biochemical analyses. Damage of mitochondria might be an important factor contributing to the failure of Eubel et al. (25, 26) to detect significant amounts, if any, of complex IV-containing supercomplexes from three plant species, including Arabidopsis green leaves. Accordingly, these authors noted that optimal conditions (freshly prepared mitochondria, etc.) were necessary for detection of significant amounts of complex IV-containing supercomplexes in potato mitochondria (26).

Phylogenetic Conservation of the Respirasome Architecture of the Standard Respiratory Chain—To challenge the generally accepted “random collision model” (2), according to which the standard respiratory complexes as well as the alternative plant and fungal respiratory enzymes are distributed independently from each other in the inner mitochondrial membrane, it is of paramount importance to identify and characterize specific aggregates of the respiratory enzymes in a wide range of phylogenetically distant eukaryotes. We demonstrate that in spinach green leaves most of the standard respiratory complexes I, III, and at least a smaller proportion of complex IV are assembled into supercomplexes, which apparently correspond to the building blocks of the respirasome as a large supramolecular network found in mitochondria of mammals (3–6) and the filamentous fungus P. anserina (7), a close relative of the well known Neurospora crassa, both possessing complex I in contrast to S. cerevisiae. Comparable results were obtained in a recent study by analysis of non-green tissue potato mitochondria (26). Thus, it appears that fundamental features of the supramolecular organization of the standard respiratory complexes I, III, and IV as a respirasome are conserved between mammals, fungi, and higher plants.

Is the Plant Respiratory Chain Organized in a More Dynamic Manner than That in Mammals and Fungi?—The ratio of the small supercomplexes I$_1$III$_2$ and III$_2$IV$_1$ to total supercomplexes separated after BN-PAGE is significantly larger in spinach than observed in mammalian and fungal mitochondria under essentially the same conditions, leading in the latter cases to a significantly higher proportion of the large supercomplexes I$_1$III$_2$IV$_1$$_2$ (3–7). The highly abundant or exclusive presence, respectively, of I$_1$III$_2$ and III$_2$IV$_1$ was also found by the analysis of four other plants (25, 26). Combined with our observation that a particularly gentle CN-PAGE could not significantly increase the yield of preserved OXPHOS supercomplexes (Fig. 2E), in contrast to those of mammals and fungi (7),

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*4 N. H. Reischneider, S. Rexroth, H. J. Schwassmann, H. Seeleit, N. A. Dencher, and F. Krause, unpublished result.*
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those results suggest that the distribution of small and large respiratory supercomplexes obtained by native electrophoresis of digitonin extracts approximately reflects their state in the native membrane. Anyway, all the abovementioned results indicate that the plant respiratory supercomplexes are relatively labile in comparison with their mammalian and fungal counterparts. Taken together, it can be speculated that the respirosome architecture in higher plant mitochondria might be a more dynamic structure that allows reorganization of the respiratory complexes I, III, and IV as a functional advantage of the respirasome (3, 9–11). This seems plausible for two major reasons. First, the plant respiratory complexes have plant-specific non-respiratory functions. Most notably, complex III is a bifunctional protein complex in green plants, which is also the site of light-dependent respiratory chain in the filamentous fungus P. anserina (51), as in some other fungi (52), is composed of the smaller ones, which are distinct from the respirasome supercomplexes I, II, III, and IV, I 1III2, and/or per -

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