Proteomic investigations of complex I composition: how to define a subunit?

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INTRODUCTION

The NADH-Ubiquinone oxidoreductase (EC 1.6.5.3) is part of the respiratory chain present in all aerobic organisms. It is also known as NDH-1 in bacteria and complex I in eukaryotes because it is the first complex involved in the mitochondrial electron transfer chain. Its roles during respiration are to oxidize NADH, transfer the resulting electrons through a series of iron-sulfur (Fe/S) clusters to the quinone pool and export protons in the compartment located on the p-side of the membrane (reviewed in Brandt, 2006).

Complex I is known as the major entry point of electrons in the respiratory chain but surprisingly its mode of action is less understood than those of other respiratory complexes. It is composed of 14 subunits in bacteria and more than 40 subunits in higher eukaryotes. In E. coli, the 14 subunits are named NuoA–N and are encoded by the nuo operon. They are sufficient for the oxidation of NADH, the transfer of the electrons onto quinone and the pumping of protons into the periplasm. The structure of the bacterial enzyme has been solved only recently (Efremov and Sazanov, 2011b) and a functional mechanism, suggesting that complex I acts like a steam engine, has been proposed (Efremov and Sazanov, 2011a).

In eukaryotes, proteins homologous to these 14 “core” subunits are conserved and additional “accessory” subunits are found associated to the core subunits. Conservation of the core subunits suggests that the essential redox components and the basic mechanisms of electron and proton translocation described in bacteria are conserved across species. Structural work in Yarrowia lipolytica confirmed similarities between the bacterial complex and the core of eukaryotic complex I (Hunte et al., 2010) but provided no information on potential roles of the accessory subunits. These subunits can be divided into two groups. The first set contains subunits generally found in all eukaryotic complex I. In the second group are accessory subunits specific to plants, fungi, or animals. A comparative analysis of complex I composition in different organisms was undertaken to trace the evolution of the complex (Gabaldón et al., 2005). According to this analysis, bovine complex I contains 11 mammal-specific subunits while the Arabidopsis enzyme contains eight plant-specific subunits and Neurospora contains three fungi-specific subunits. The numbers of specific subunits have evolved with the release of new proteomic analyses [for example from 8 (Heazlewood et al., 2003) to 17 (Klodmann et al., 2010) in Arabidopsis]. The species-specific diversity in the composition of complex I suggests it has acquired additional functions in different organisms as mitochondria may have evolved differently in these organisms. However, a recent bioinformatic analysis questioned this variability in complex I composition and suggested a conserved subunit composition across species (Cardol, 2011). In the present review, an updated overview over proteomic approaches used to resolve the composition of Arabidopsis complex I is provided.

HISTORY OF THE PROTEOMIC ANALYSES OF PLANT COMPLEX I

Initial studies of higher plant complex I purified the enzyme from potato mitochondria using hydroxyapatite and gel filtration chromatography. Subsequent SDS-PAGE detected 32 protein bands, from which 10 N-terminal sequences were obtained (Herz et al., 1994). A similar number of bands was obtained when immunopurified wheat complex I was subjected to SDS-PAGE (Combettes and Griezenberger, 1999). The composition of complex I was later analyzed using Blue-Native gel electrophoresis.
A search for orthologs of mammalian complex I subunits in Arabidopsis genome allowed the identification of 14 additional subunits in different eukaryotes was investigated (Cardol, 2011). Recently, using a PSI-Blast approach, the composition of complex I contains eight mammal-specific subunits. Among the latter, six subunits were obtained for Arabidopsis homolog of the bovine 42 kDa subunit, encoded by the gene At1g72040. This protein, which is annotated as a member of the nucleoside triphosphate hydrolases superfamily, is predicted to be mitochondrial but has only been identified in the nuclear proteome in plants (Bae et al., 2003). The protein orthologous to the bovine B9 subunit, encoded by At2g46540, was identified in the mitochondrial proteome (Brugière et al., 2004; Heazlewood et al., 2004). The closest homolog of the B14.5a subunit, encoded by At5g8050, has been experimentally found in plastids (Ferro et al., 2010) but not in mitochondria. The protein homologous to the B22 subunit can drive a GFP fusion protein into mitochondria in vivo (Han et al., 2010) and has been found associated with a subcomplex of complex I (Sunderhaus et al., 2006). A mutant in the gene encoding this protein showed a respiration defect but the presence of complex I in this mutant was not investigated (Han et al., 2010). The closest homolog of the MLQR subunit, encoded by At3g29970, has not been identified in any proteomic study. Finally, the Acyl Carrier Protein (ACP, subunit SDAP in bovine) was found to be a soluble protein of the mitochondrial matrix in Arabidopsis (Meyer et al., 2007). In contrast, 43 proteins were identified in at least three proteomic approaches directed at the composition of Arabidopsis complex I. With the addition of the small hydrophobic mitochondrial-encoded ND4L, 44 Arabidopsis proteins are considered as bona fide subunits. A model of the organization of these subunits in the complex is proposed in Figure 1. The function of the accessory subunits has not been investigated in plants. Indeed, complex I mutants were used to analyze the consequences of the absence of complex I on respiration and plant metabolism (Dutilleul et al., 2003; Meyer et al., 2009) and to determine the assembly of complex I (Meyer et al., 2011).

CARBONIC ANHYDRASES

The main difference between plant and animal complex I is the presence of gamma-carbonic anhydrases (CAs) in the plant enzyme. The CAs were proposed to be involved in CO2 metabolism in plant mitochondria (Braun and Zabaleta, 2007). To date, no direct proof of this function has been obtained (Klodmann and Braun, 2011). Among the Arabidopsis CAs, two carbonic anhydrase-like (CAL) proteins have been identified and were considered to represent two distinct complex I subunits. Other higher plants, such as rice, maize, or poplar, possess only one gene encoding CAL. This suggests that the presence of two CALs in Arabidopsis is the result of a recent gene duplication event, and that the two CALs are isoforms of the same subunit. A similar analysis of the genes encoding CAs revealed that plants possess two (maize, sorghum) or three (Arabidopsis, rice, poplar) genes encoding CAs. Additional biochemical data and particularly the characterization of mutants are required to determine the exact composition of Arabidopsis complex I regarding CAs and CALs. To date, only CA2 and CAL2 have been studied. The ca2 mutant showed highly reduced levels of complex I (Perales et al., 2005) and a trimmed CA domain (Sunderhaus et al., 2006), suggesting that CA2 is not easily replaced by other CAs. The cal2 mutant contained wild-type levels of complex I (Meyer et al., 2011), in agreement with both CALs being isoforms. The CA domain of complex I may thus include CA2, CAL, and either CA1 or CA3.
Table 1 | Comparative analysis of the composition of complex I in bovine and *Arabidopsis*.

| *Arabidopsis* identifier | Bovine | Comments | Heazlewood et al. (2003) | Sundin et al. (2006) | Meyer et al. (2008) | Klodmann et al. (2010) | Klodmann and Braun (2011) | Klodmann et al. (2011) | Concensus (44 subunits) |
|--------------------------|--------|----------|--------------------------|----------------------|---------------------|------------------------|--------------------------|------------------------|------------------------|
| AtMg00990                | ND3    | E. coli NUOA | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At5g11770                | 20 kDa (PSST) subunit | E. coli NUOB | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00070                | ND9/30 kDa subunit | E. coli NUOC | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00510                | ND7/49 kDa subunit | E. coli NUOD | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At4g02580                | 24 kDa subunit | E. coli NUOE | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At5g08530                | 51 kDa subunit | E. coli NUOF | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At5g37510                | 75 kDa subunit | E. coli NUOG | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00516, AtMg01120,    | ND1    | E. coli NUOH | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg01275, AtMg079010,   | 23 kDa (TYKY) subunit | E. coli NUOI | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At1g16700                | 23 kDa (TYKY) subunit | E. coli NUOI | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00270                | ND6    | E. coli NUOJ | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00650, AtMg00060,    | ND4    | E. coli NUOK | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00513, AtMg00665     | ND5    | E. coli NUOL | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00580                | ND4    | E. coli NUOM | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg00828                | ND2    | E. coli NUON | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| AtMg01320                | 13 kDa subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g7690,                | 15 kDa (PFFD) subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At3g82790                | 15 kDa (PFFD) subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At5g67590                | 18 kDa subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g0360,                | 39 kDa subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At1g76200, At5g47570     | AGGG subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At1g14450, B12 subunit   | X       | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g02510                | B13 subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At3g12260                | B14 subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At4g20150                | B14.5b subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g42210                | B14.7 subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g31490                | B15 subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g33220, B16.6 subunit | X       | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At1g04630                | B172 subunit (DAP13) | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At3g03100                | B172 subunit (DAP13) | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g02050, B18 subunit   | X       | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At3g47890                | B8 subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At2g42310, ESS subunit   | X       | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At3g57785                | KFYI subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At4g05885                | MNLL subunit | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At3g8610, PDSW subunit   | X       | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |
| At3g18410, At1g49140     | X       | X            | X                        | X                    | X                   | X                      | X                        | X                      | X                      |

(Continued)
From six proteomic analyses of Arabidopsis complex I, 49 different proteins were identified (the identification is indicated with ‘X’). From this list, 44 proteins are part of a consensus composition as they were identified in at least three studies, with the exception of ND4L (*) that was never identified by MS, most probably due to its small size and extreme hydrophobicity. These proteins were identified in subcomplexes but not in mature complex I.

**Table 1 | Continued**

| Arabidopsis identifier | Bovine | Comments |
|------------------------|--------|----------|
| At3g06310, At5g18800   | PGIV subunit | X X X X X |
| At1g67785              | SGDH subunit | X X X X X |

**ARABIDOPSIS PROTEINS NOT FOUND IN BOVINE COMPLEX I**

| Arabidopsis identifier | Bovine | Comments |
|------------------------|--------|----------|
| At1g19580              | CA1: carbonic anhydrase 1 | X X X X X |
| At1g47260              | CA2: carbonic anhydrase 2 | X X X X X |
| At5g46510              | CA3: carbonic anhydrase 3 | X X X X X |
| At5g48680, At5g48510   | CAL: carbonic anhydrase-like | X X X X X |
| At5g63510              |                          |          |
| At1g67350              | P1                  | X X X X X |
| At2g27730              | P2                  | X X X X X |
| At5g14105              | P3                  | X X X X  |
| At1g6880               |                     | X        |
| At1g72170              |                     |          |
| At2g10110              | TIM22              | X        |
| At1g18320              |                     |          |
| At3g47930              | GLDH                | X$       |

**BOVINE SUBUNITS NOT FOUND IN MATURE ARABIDOPSIS COMPLEX I**

| Arabidopsis identifier | Bovine | Comments |
|------------------------|--------|----------|
| At1g72040              | 42 kDa subunit |          |
| At2g46540              | B9 subunit |          |
| At5g08050              | B14.5a subunit |          |
| At4g34700              | B22 subunit | X$       |
| At3g29970              | MLQR subunit |          |
| At1g65290, At2g44620   | SDAP subunit |          |
| At5g47630              |                     |          |

**NON-CONSERVED BOVINE COMPLEX I**

| Arabidopsis identifier | Bovine | Comments |
|------------------------|--------|----------|
| 10 kDa subunit         |        |          |
| B17 subunit            |        |          |

**OTHER PUTATIVE PLANT-SPECIFIC SUBUNITS**

Four other putative proteins have been described as complex I subunits in proteomic studies. The L-galactono-1,4-lactone dehydrogenase (GLDH, At3g47930) was found in a smaller version of complex I but not in the holocomplex (Heazlewood et al., 2003). GLDH was later identified as a complex I assembly factor as a mutant lacking this protein did not contain a fully assembled complex I (Pineau et al., 2008). Because GLDH is absent from the mature complex (Meyer et al., 2008; Klodmann et al., 2010), it cannot be considered as a complex I subunit. A protein encoded by the gene At1g68680 has only been detected in one proteomic study of complex I (Meyer et al., 2008). It was recently identified in the proteome of the mitochondrial outer membrane (Duncan et al., 2011) and may therefore not be a complex I subunit. TIM22 (encoded by At1g18320 and At3g10110) has been identified in the vertical containing complex I subunits on a BN-SDS-PAGE (Klodmann et al., 2011). As this protein acts as an import channel during the import of cytoplasmically translated carriers into the inner mitochondrial membrane (Murche et al., 2007), a role of TIM22 in the assembly of complex I is plausible. Another protein, encoded by At1g72170, has also been identified by Klodmann et al. (2011) as a putative new complex I subunit. This protein had previously been found in the Arabidopsis mitochondrial proteome (Heazlewood et al., 2004) and in the mitochondrial membrane proteome (Brügger et al., 2004) but no data are available regarding its function. More biochemical evidence is required to validate these proteins as complex I subunits.
FIGURE 1 | Model of Arabidopsis complex I. This model includes the 44 consensus subunits defined in Table 1. It was built using the shape of the complex obtained by single particle analysis (Dudkina et al., 2005), the presence of CAs in the extra matrix-facing domain (Sunderhaus et al., 2006), the structure of the bacterial enzyme for the localization of the 14 core subunits and the Fe/S clusters (Efremov and Sazanov, 2011b), the co-localization of subunits within the same subcomplex (Klodmann et al., 2010; Meyer et al., 2011) as well as bioinformatic prediction of transmembrane domains and topology using the TMHMM prediction service (Krogh et al., 2001). The nomenclature used is the one determined for bovine and plant-specific subunits are names as in Table 1. The core subunits are shown in light gray. Accessory subunits present in bovine and Arabidopsis complex I are shown in white and accessory subunits present in Arabidopsis but absent from bovine complex I are shown in dark gray.

HOW TO DEFINE A SUBUNIT?
DISTINGUISHING SUBUNITS FROM ASSEMBLY FACTORS
Defining the subunit composition of a large complex is a challenge, not only because of technological limitations. It is generally assumed that a subunit is essential for either the presence or the function (or both) of the complex. However, not all proteins that correspond to this definition are subunits. For example, all the assembly factors identified in mammals (for review see McKenzie and Ryan, 2010) or in plants (Bych et al., 2008; Pineau et al., 2008) are essential for the presence of the mature complex but are not present in the mature complex. On the other hand, assembly factors can be accessory subunits. For example, the MWFE subunit is required for the insertion of some mitochondrial-encoded subunits within the membrane (Yadava et al., 2004; Meyer et al., 2011). Importantly, while a combination of proteomic and genetic tools is suitable to define if a protein is present in and essential for the biogenesis of complex I, additional biochemical or proteomic studies are required to identify “true” subunits and distinguish them from assembly factors or interactants. For example, the characterization of a mutant will elucidate if a protein is important for the assembly/stability of the complex, defining interaction partners will determine if the protein is involved in other metabolic pathways and in vitro activity assay will highlight new functions of the complex.

IMPORTANCE OF THE TECHNIQUES USED FOR COMPLEX I PREPARATION
The principal characteristic of a subunit is its presence in the fully assembled complex. Proteomic analyses of the composition of a multiprotein complex critically depend on the availability of a highly purified complex. Remarkably, proteomic approaches using different techniques to isolate complex I identified sets of accessory subunits that only partially overlap. Several factors can influence the outcome of the purification strategy. While detergents, salts and buffers used for the solubilization of mitochondrial membranes and extraction of the complex are critical, the fractionation technique used to isolate the complex (immunoprecipitation, chromatography, or BN-PAGE) is also important. Notably, in all studies that identified ACP as part of complex I, the sample for the proteomic analysis was prepared by non-gel based techniques.
Abdrakhmanova, A., Zickermann, V., PAGE. Similarly, subunits found in bovine complex I (purified by Bridges et al., 2010) and in plants (Meyer et al., 2007). ACP therefore never found in Arabidopsis complex I preparations (always analyzed on BN-PAGE) could be soluble mitochondrial proteins that are loosely associated with the complex and detached during the electrophoresis.

ALTERNATIVE PROTEOMIC APPROACHES

Several MS techniques have been recently developed to discriminate between true subunits and proteins transiently interacting with a complex. Such approaches are based on differential labeling of the two samples to compare (i.e., comparing complex with interactors to complex alone) and the ratio labeled/non-labeled protein defines stable and dynamic components of complexes (Kito et al., 2008; Pflieger et al., 2008). Alternatively, the determination of the stoichiometry of the different subunits of a complex could help defining true subunits from interactors. In a total extract, an interactor will not be associated with all the complexes and thus have a lower stoichiometry than true subunits. Several approaches have been developed to determine the stoichiometry of proteins (Gerber et al., 2003; Hochleitner et al., 2004; Beynon et al., 2005; Nanavati et al., 2008). So far, none of these proteomic approaches have been tested to investigate the composition of respiratory complexes. The transient association between complex I and other mitochondrial proteins, such as ACP involved in lipid biosynthesis, implies possible interactions of the respiratory chain with other metabolic pathways.

CONCLUSIONS

More than 15 years after the first efforts to determine the subunit composition of complex I in plants, 44 confirmed complex I subunits are known in the model organism Arabidopsis thaliana. Knowledge of the composition of complex I is a prerequisite for defining strategies to characterize the functions that complex I fulfills in plant mitochondria.

Analyses of the composition of a multiprotein complex require proteomic approaches. The comparison of proteomic investigations of complex I composition in different organisms indicates that sample preparation is critical. In addition, thorough proteomic investigations are indispensable for determining the subunit composition of the complex and can provide indications toward which metabolic pathways are associated with the respiratory chain. Importantly, coupling proteomics with biochemical and genetic analyses will be essential for i) functional studies of proteins found in preparations of the complex, and for ii) defining if these proteins are true subunits, assembly factors or proteins that interact with the complex.

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