A Proteomic Survey of *Chlamydomonas reinhardtii* Mitochondria Sheds New Light on the Metabolic Plasticity of the Organelle and on the Nature of the α-Proteobacterial Mitochondrial Ancestor

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Mitochondria play a key role in the life and death of eukaryotic cells, yet the full spectrum of mitochondrial functions is far from being fully understood, especially in photosynthetic organisms. To advance our understanding of mitochondrial functions in a photosynthetic cell, an extensive proteomic survey of Percoll-purified mitochondria from the metabolically versatile, hydrogen-producing green alga *Chlamydomonas reinhardtii* was performed. Different fractions of purified mitochondria from *Chlamydomonas* cells grown under aerobic conditions were analyzed by nano-liquid chromatography–electrospray ionization–mass spectrometry after protein separation on sodium dodecyl sulfate polyacrylamide gel electrophoresis or on blue-native polyacrylamide gel electrophoresis. Of the 496 nonredundant proteins identified, 149 are known or predicted to reside in other cellular compartments and were thus excluded from the molecular and evolutionary analyses of the *Chlamydomonas* proteome. The mitochondrial proteome of the photosynthetic alga reveals important lineage-specific differences with other mitochondrial proteomes, reflecting the high metabolic diversity of the organelle. Some mitochondrial metabolic pathways in *Chlamydomonas* appear to combine typical mitochondrial enzymes and bacterial-type ones, whereas others are unknown among mitochondriate eukaryotes. The comparison of the *Chlamydomonas* proteins to their identifiable homologs predicted from 354 sequenced genomes indicated that *Arabidopsis* is the most closely related nonalgal eukaryote. Furthermore, this phylogenomic analysis shows that free-living α-proteobacteria from the metabolically versatile orders Rhizobiales and Rhodobacterales better reflect the gene content of the ancestor of the chlorophyte mitochondria than parasitic α-proteobacteria with reduced and specialized genomes.

Introduction

Mitochondria are vital organelles that fulfil essential functions as diverse as energy production, metabolism of amino acids, lipids, iron, calcium homeostasis, and cell signaling (Neupert 1997; Scheffler 2001; Newmeyer and Ferguson-Miller 2003; Lill and Mühlenhoff 2006, Hajnoczky et al. 2006; Xiong et al. 2006). Genetic, proteomic, transcriptomic, and bioinformatic approaches focusing on these organelles have greatly expanded our knowledge of the mitochondrial functions and biogenesis among various eukaryotic lineages.

Comprehensive proteomic analyses have been reported for mitochondria isolated from mammals (Mootha et al. 2003; Taylor et al. 2003), yeast (Sickmann et al. 2003; Zahedi et al. 2006), higher plants (Brugière et al. 2004; Heazlewood et al. 2004; Millar et al. 2004, 2005; Huang et al. 2009), and from the ciliate *Tetrahymena thermophila* (Smith et al. 2007). Proteomic analyses of green algae, the ancestors of higher plants, have so far been lacking.

Chlamydomonas reinhardtii is the best investigated unicellular photosynthetic alga. *Chlamydomonas* is an excellent model organism for the study of photosynthesis and chloroplast biogenesis (Rochaix 1995, 2002; Wollman 2001; Choquet and Wollman 2002; Grossman et al. 2004), the assimilation and homeostasis of nutrients (Merchant et al. 2006), and the assembly and function of flagella (Snell et al. 2004). Clustering and assembly of *Chlamydomonas* expressed sequence tags (EST) (Jain et al. 2007) and the release of a 121-megabase draft genome sequence of *Chlamydomonas* (Merchant et al. 2007) have revealed the extraordinary complexity of the alga. New interest in the alga was sparked by the discovery of anaerobic metabolic pathways once thought to be specific to bacteria and anaerobic eukaryotes (Kreuzberg et al. 1987; Atteia et al. 2006), some of which being relevant in the context of biological hydrogen production (Melis and Happe 2004; Mus et al. 2007; Hemschemeier et al. 2008).

In contrast to the intensely studied chloroplast, mitochondria in *Chlamydomonas* have been so far poorly investigated. Little information derives from its mitochondrial DNA, which encodes seven respiratory proteins and a reverse transcriptase-like protein (Boer and Gray 1991). Due to the difficulty to obtain sufficiently highly enriched material for biochemical and biophysical studies, only the most abundant proteins have been characterized to date, such as the subunits of the oxidative phosphorylation complexes (OXPHOS) (Atteia 1994; Funes et al. 2002; van Lis et al. 2003; Cardol et al. 2004). Little work has been dedicated to the mitochondrial metabolic pathways and their adaptive responses to environmental changes. As yet, a total of 60 mitochondrial proteins have been identified experimentally in *Chlamydomonas*.

To better understand the metabolic diversity and the origin of the organelle, we developed a large-scale proteome analysis of the mitochondria from *Chlamydomonas*. Different fractions of Percoll-purified mitochondria were analyzed by mass spectrometry (MS) after separation

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of the proteins on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or on blue-native polyacrylamide gel electrophoresis (BN)-PAGE. Our findings provide insights into the mitochondrial functions of this metabolically versatile chlorophyte and uncover biochemical aspects novel to mitochondria in general. Moreover, the data yield a significant list of genuine mitochondrial proteins, a learning set required for the retraining of the publicly available targeting prediction programs that are not currently reliable for *Chlamydomonas* proteins. Finally, a comparative phylogenomic analysis was performed to identify the α-proteobacterial species that best relate to the algal mitochondrion.

Materials and Methods

Chlamydomonas Strain and Culture Conditions

Cell wall–deficient *C. reinhardtii* strain 83.82 (SAG Göttingen) was grown with orbital agitation at 25 °C, under continuous light in Tris–acetate–phosphate (TAP) medium (Harris 1989), supplemented with 1% (w/v) sorbitol. For mitochondrial isolation, 1.6 l of late-exponential phase grown cells were mixed with 4.4 l of fresh TAP medium supplemented with 34 mM of acetate with the initial pH adjusted to 7.0 (with KOH). The cells were grown further for 40 h in the presence of light.

Isolation and Subfractionation of *Chlamydomonas* Mitochondria

Mitochondria were isolated as described (van Lis et al. 2005). Cells were harvested by centrifugation at 2,000*g* for 10 min, resuspended in nebulizing buffer (50 mM 2-(*N*-morpholino)ethanesulfonic acid, 50 mM Tris, 0.25 M sorbitol, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 3 mM KH_2PO_4, 2 mM ethylenediaminetetraacetic acid [EDTA], pH 7.2) and disrupted using the BioNeb cell disruption system (Glas-Col) with N_2 (20 psi). The cell lysate was briefly centrifuged in a GSA rotor (Du Pont-Sorvall) by accelerating the rotor to 5,000 rpm and then immediately stopping the run. The supernatant was first centrifuged at 1,500*g* for 10 min in an SS34 rotor (Du Pont-Sorvall) to pellet cell debris and chloroplasts, and then the supernatant was centrifuged at 10,000*g* for 10 min. The pellet from this centrifugation contained mitochondria that were then purified on self-forming Percoll gradients, as described by Eriksson et al. (1995). Percoll-purified mitochondria were washed once in 0.25 M Sorbitol, 1 mM potassium phosphate, pH 7.2, 2 mM EDTA. Two distinct preparations were produced, one for organelle fractionation and the other for BN-PAGE analysis.

Mitochondria were resuspended in 10 mM 3-(*N*-morpholino)propanesulfonic acid–NaOH, pH 7.8, 4 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, and 0.5 mM ϵ-amino caproic acid, sonicated three times for 10 s and ultracentrifuged for 1 h at 70,000*g* (Beckman SW41-Ti rotor). The membrane fraction was resuspended in the same buffer in the presence of 1 M NaOH and kept on ice for 30 min. The sample was vortex mixed and then centrifuged for 15 min at 100,000*g*. The pellet was washed in the same buffer and again centrifuged for 10 min. Proteins in the soluble mitochondrial fraction and the NaOH-treated membranes were separated on SDS-PAGE for subsequent MS analysis.

Protein Electrophoresis

Proteins of the mitochondria and in the subfractions were boiled for 5 min in the presence of 2% SDS and then centrifuged for 10 min at 12,000*g*. Proteins (20 μg) in each sample were separated by glycine–SDS-PAGE (12% [w/v] acrylamide) (Laemmli 1970) in a Mini-PROTEAN 3 cell (Bio-Rad) (gel dimensions: 8 × 7.3 × 0.1cm; 10-well comb). After electrophoresis, gels were stained with Coomassie Blue R250.

For BN-PAGE, samples and polyacrylamide gel were prepared as described (van Lis et al. 2003) with the following modification: Mitochondria were solubilized in the presence of 2% (w/v) *n*-dodecyl maltoside to a final protein concentration of 5 mg/ml. The solubilized samples were ultracentrifuged at 40,000*g* for 20 min at 4 °C. Solubilized proteins (100 μg per well) were loaded on a gel with a 4–12% acrylamide gradient separating gel and a 3% acrylamide stacking gel in a Mini-PROTEAN 3 cell (Bio-Rad) (10 wells). One BN-PAGE lane was cut out and used for MS analysis.

Protein Analysis

Protein concentrations were estimated using the DC Protein Assay Kit 2 (Bio-Rad). Apparent molecular masses were estimated using the Precision Plus Protein Standards Dual Color (Bio-Rad). Proteins in the different samples were separated by SDS-PAGE and subsequently transferred onto Hybond C nitrocellulose membranes (Amersham Pharmacia Biotech Inc.) for immunodetection or stained with Coomassie Brilliant Blue. Blots were incubated for 1 h with primary antibodies as follows: 1:20,000 for anti-*C. reinhardtii* βF1-ATPase (Atteia et al. 2006) and 1:50,000 for anti-*C. reinhardtii* light harvesting complex (LHC) proteins (Dr O. Vallon, IBPC, France). Immunodetection was carried out using the enhanced chemiluminescence system (Durrant 1990).

MS and Protein Identification

Gel pieces were excised from the Coomassie Brilliant Blue-stained gel and subjected to tryptic digestion as described (Ferro et al. 2003). Tryptic peptides were resuspended in 0.5% aqueous trifluoroacetic acid. The samples were injected into a CapLC (Waters) nano-LC system and first preconcentrated on a 300-μm × 5-mm PepMap C_{18} precolumn. The peptides were then eluted onto a C_{18} column (75 μm × 150 mm). The chromatographic separation used a gradient from solution A (2% acetonitrile, 98% water, and 0.1% formic acid) to solution B (80% acetonitrile, 20% water, and 0.08% formic acid) over 60 min at a flow rate of 200 nl/min. The nano-LC system was directly coupled to a Q-ToF Ultima mass spectrometer (Waters). MS and MS/MS

data were acquired and processed automatically using MassLynx 4.0 software. Database searching was performed with Mascot 2.0 (www.matrixscience.com), using the NCBInr, the Joint Genome Institute (JGI) *C. reinhardtii* version 2.0 gene models (finalmodels V2) (http://genome.jgi-psf.org/Chlre2/Chlre2.download.html), the EST and assembled contigs (ACE) databases. The ACE database consists of translations of contigs built from at least four ESTs from the 20021010 assembly (full-length and 5′-only ACEs) and was kindly provided by Charles Hauser.

The score threshold for automatic validation of peptide matches was fixed at 40 for the following reasons: 1) for all data sets, this value is just above the Mascot score above which there is identity or extensive homology for peptide assignment with a probability of at least 95% as quoted by the Mascot output and 2) at this threshold setting, the false positive rate, computed on a spectra basis as described (Peng et al. 2003) was estimated to be 0.09%, 0.72%, and 1.12% for the whole mitochondria, soluble fraction, and NaOH-treated membrane samples, respectively. Consequently, we estimated that proteins identified by at least two peptides with a score higher than 40 were significantly present in our samples. For proteins identified by only one peptide having a score higher than 40, the peptide sequence was checked manually. Furthermore, we manually enriched the peptide identification data set with peptides with scores higher than 20 and lower than 40. These peptides were systematically checked and/or interpreted manually to confirm or cancel the Mascot suggestion.

The sequence of each identified entry was subjected to Blast-based similarity searches at NCBI to confirm annotation or to propose a function. For each protein identified, Blast searches were performed to identify the V3 gene model (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html).

Protein Characterization and Targeting Predictions

All the identified proteins were run through a series of prediction programs to provide theoretical molecular masses, p*I*, GRAVY score, and putative transmembrane segments. Masses, p*I*, and GRAVY score were determined with http://expasy.org/tools/protparam.html. Transmembrane segments were predicted by HMMTOP at http://www.enzim.hu/hmmtop/. Predictions of subcellular localization were done using: PREDOTAR 1.03 (http://urgi.versailles.inra.fr/predotar/french.html) (Small et al. 2004), MultiLoc plant (http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc/; Hoeglund et al. 2006), TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2007) with "no cutoff" set, and the "Plant" option selected, PSORT plant (http://psort.ims.u-tokyo.ac.jp/form.html). MitoPROT2 (http://ihg.gsf.de/ihg/mitoprot.html; Claros and Vincens 1996) with a discriminant function for mitochondrial proteins cutoff between 0.7 and 1.0. Motif searches were carried out with PFAM at http://pfam.jouy.inra.fr/.

Homolog Searches

Blast searches to identify homologs of the *Chlamydomonas* proteins were done using the following databases: http://mips.gsf.de/genre/proj/yeast (*Saccharomyces cerevisiae*) http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid = 9606 (Human), http://www.arabidopsis.org/Blast/index.jsp (*Arabidopsis*), and at http://seq.ciliate.org/cgi-bin/blast-tgd.pl (*T. thermophila*). Pairwise global alignments were calculated using ClustalW at http://www.ebi.ac.uk/Tools/clustalw/index.html (Thompson et al. 1994) and proteins with at least 20% amino acid identity were kept as homologs. In a few cases, such as for the proteins of the import systems, homologs that showed between 10% and 15% sequence identity were kept.

Alignment and Trees

Homologs to *Chlamydomonas* mitochondrial proteins were identified by reciprocal best Blast (Altschul et al. 1997) hits (rBBHs) on individual databases for 354 fully sequenced organisms including 286 eubacteria, 24 archaebacteria, and 44 eukaryotes (organism names given in supplementary data set S2 (Supplementary Material online). Reciprocal best hits with an *E*-value $\leq 10^{-20}$ were aligned pairwise using ClustalW (Thompson et al. 1994), pairs with 20% amino acid identity were retained. For the identity matrix in figure 5*A*, pairwise amino acid identities were written into a 347 × 354 matrix. MATLAB was used to create a color-coded image of the pairwise amino acid identities. Rows of *Chlamydomonas* proteins with homologs only in eukaryotes were arranged using a dictionary sort; remaining rows were sorted by descending sum of amino acid identity among α-proteobacterial rBBHs. For phylogenetic analyses, multiple alignments of *Chlamydomonas* proteins having homologs in at least three reference genomes were generated using MUSCLE (Edgar 2004) with a maximum of 16 iterations; gapped alignment positions were removed prior to tree reconstruction. Trees were inferred with PHYML (Guindon and Gascuel, 2003) using the Jones Taylor Thornton matrix (Jones et al. 1992) with rate variation among the protein positions according to a Gamma distribution with eight categories. The shape of the distribution (alpha parameter) was estimated from the data and invariable sites were taken into account. Trees were plotted with the DRAWTREE tool of the GCG package.

Results and Discussion

Isolation and Purity of *Chlamydomonas* Mitochondria

Mitochondria of *Chlamydomonas* were isolated from cells grown under mixotrophic conditions and purified on a self-generating Percoll density gradient. In photosynthetic organisms, the major source of contamination in mitochondrial preparations typically stems from the chloroplast. To assay this contamination, we carried out immunoblotting with antibodies raised against the LHC proteins. As shown in supplementary figure S1 (Supplementary Material online), the levels of LHCII proteins (the most abundant thylakoid proteins) were very low in our preparations, suggesting only a minor contamination by thylakoids membranes. This result was corroborated by the limited number of peptides from chloroplast proteins obtained through this proteomic study (supplementary table S1,

Supplementary Material online). Surprisingly, the main contaminants of these *Chlamydomonas* mitochondrial preparations were of cytosolic origin, likely resulting from a tight association with the mitochondrial outer surface.

Analysis of the *Chlamydomonas* Mitochondrial Proteome

To enhance the chances of identifying proteins with different physicochemical properties, we analyzed various fractions derived from two independent preparations of *Chlamydomonas* mitochondria, as outlined in supplementary figure S2 (Supplementary Material online). Mitochondria were disrupted by sonication and fractionated into their soluble and membrane components by ultracentrifugation. Membranes were further treated with 1 M NaOH to wash off the poorly hydrophobic proteins. Proteins in the soluble fraction and the NaOH-treated membranes were separated on SDS-PAGE. Mitochondria from a distinct preparation were solubilized by dodecylmaltoside, and solubilized proteins were then separated on one-dimensional (1D)-BN-PAGE. The two SDS-PAGE lanes and the BN-gel lane were cut into a number of bands according to apparent molecular masses. Gel bands were subjected to trypsinolysis, and proteins were further identified by nano-LC–ESI–MS/MS analyses followed by Mascot (Perkins et al. 1999) search against the NCBInr, the version 2.0 of the *Chlamydomonas* gene models (V2) at the JGI, and the assembled contigs database. BN-PAGE was used here to identify proteins that could have been missed in the analysis of mitochondrial subfractions separated on SDS-PAGE because of their physicochemical properties, their strong association into complexes or their low abundance. This approach was founded because a number of genuine mitochondrial proteins were detected only on BN-PAGE (supplementary table S1, Supplementary Material online). The relevance of the MS identification of proteins resolved on 1D BN-PAGE was illustrated using *Chlamydomonas* mitochondrial ATP synthase (complex V). All 16 complex V subunits were identified in a single band on 1D BN-PAGE, whereas all subunits but one (subunit δ') were identified in the analysis of discrete bands from the 2D-BN/SDS-PAGE (supplementary fig. S3, Supplementary Material online).

A total of 496 nonredundant proteins were identified with 387 proteins on native gel and 296 proteins on denaturing gels (supplementary fig. S2, Supplementary Material online). The analyses shared 187 proteins. Supplementary table S1 (Supplementary Material online) lists the proteins identified together with the mitochondrial sample in which they were found.

The proteomic analysis presented here covers a wide range of p*I* (4.25–11.7) and molecular mass (6.5–500 kDa) (supplementary table S1, Supplementary Material online). Proteins in the 10–50 kDa range represent approximately 60% of all proteins. The smallest known mitochondrial protein identified was the 6.8-kDa subunit Vc of respiratory complex IV (COX5c). Several proteins were also identified with a predicted molecular mass above 100 kDa, such as the photorespiratory glycolate dehydrogenase (GyDH) of 120 kDa and the P-protein of the glycine cleavage system of 111 kDa (GSCP). The identification of subunit I of complex IV with 12 predicted transmembrane domains (TMDs) and of the NAD(P) transhydrogenase (NADTH) with 15 predicted TMDs (supplementary table S1, Supplementary Material online) showed that highly hydrophobic proteins, often underrepresented in proteomic studies, were accessible with our approach. Four of the seven respiratory proteins encoded by *Chlamydomonas* mitochondrial DNA were identified and proteins of all mitochondrial subcompartments were found (supplementary table S1, Supplementary Material online).

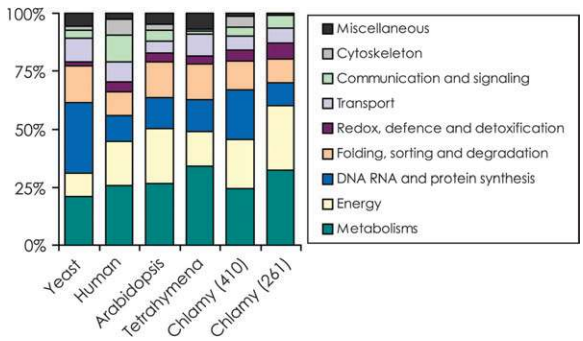

FIG. 1.—Functional classification of the proteins identified by a proteomic approach in *Chlamydomonas* mitochondria, and in mitochondria isolated from yeast (613 proteins; Sickmann et al. 2003), human (500 proteins; Taylor et al. 2003), *Arabidopsis* (344 proteins; Heazlewood et al. 2004), and *Tetrahymena* (317 proteins; Smith et al. 2007). For *Chlamydomonas*, two stacked columns are shown: *Chlamy* (410) corresponds to all proteins identified and *Chlamy* (261), to all proteins but the proteins deemed to be contaminants. Proteins with currently no assigned function were excluded from the analysis; these proteins represent 18.3% of the yeast proteome, 18.6% of the human proteome, 17.3% of the *Arabidopsis* proteome, 44% of the *Tetrahymena* proteome, and 17.3% of the *Chlamydomonas* proteome.

Overall Comparison with Large-Scale Mitochondrial Proteomes of Other Lineages

All 496 *Chlamydomonas* proteins identified were classified into 10 functional categories according to GenBank annotations and Blast searches (supplementary table S1, Supplementary Material online). Figure1 shows the respective distribution of the identified proteins among all categories but the category of unknown function (*Chlamy 410*). In order to compare the present findings with previous data, the proteins identified by MS in mitochondria isolated from yeast (Sickmann et al. 2003), human (Taylor et al. 2003), *Arabidopsis* (Heazlewood et al. 2004), and *Tetrahymena* (Smith et al. 2007) were classified according to the same functional categories. Although different experimental strategies were followed to uncover the mitochondrial proteomes of these five organisms, 55–65% of the proteins in each proteome were found to belong to the same three functional categories: "metabolisms," "energy and DNA," and "RNA and protein synthesis" (fig. 1).

The proteins with no assigned function ("unknown function" category) represent about 17% of the proteins identified in *Chlamydomonas* samples, which is similar to findings in other mitochondrial proteomes except in *Tetrahymena* where these proteins represent approximately 44% of all proteins identified.

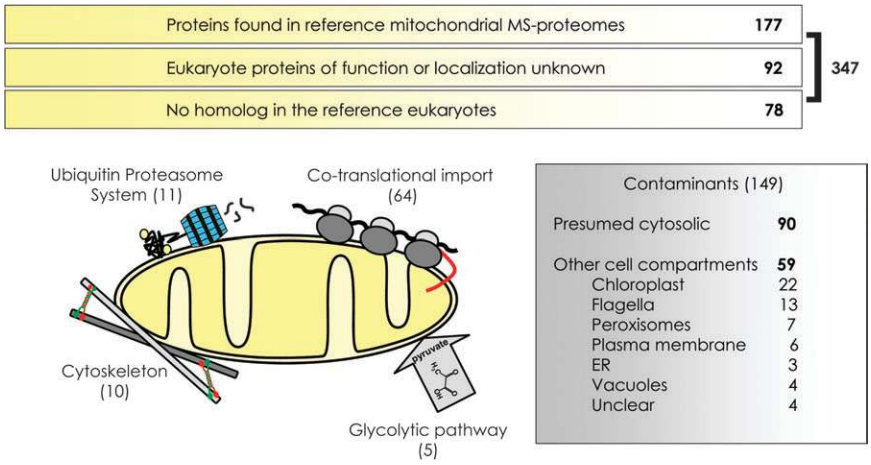

FIG. 2.—Overview of the proteins identified in *Chlamydomonas* mitochondria. Proteins regarded as mitochondrial were classified into three groups based upon the presence of homologs in the reference eukaryotes yeast, human, *Arabidopsis*, and *Tetrahymena*, and their detection in large-scale proteomics surveys. The source of the proteins currently considered as contaminants of the mitochondrial preparations is detailed in the gray box. As depicted, the proteins that stem from the cytosol are proposed to be in close contact with the mitochondrial outer membrane; the number of proteins belonging to each cytosolic machinery or macromolecular complex is indicated.

Overview of the *Chlamydomonas* Proteome

A categorized description of the *Chlamydomonas* mitochondrial proteome is given in figure 2. This description is proposed based on 1) our current knowledge of the green alga, 2) extensive Blast searches, and 3) comparisons with large-scale proteomic surveys carried out on mitochondria from four model organisms (see above).

Of the 496 *Chlamydomonas* proteins identified, 149 are known or predicted to reside in cellular compartments other than mitochondria (supplementary table S1, Supplementary Material online), as illustrated in figure 2. About two-thirds of these contaminants stem from the cytosol and belong to machineries or macromolecular complexes reported to be functionally associated with the mitochondria such as the 80S ribosome in yeast and human (Kellems et al. 1974, 1975; Crowley and Payne 1998; Marc et al. 2002; MacKenzie and Payne 2004) or the cytoskeleton in various organisms (Anesti and Scorrano 2006; Boldogh and Pon 2006). The other contaminants are from diverse cellular compartments among others the chloroplast and the vacuoles (fig. 2). We consider these 149 proteins as true contaminants but the potential contribution of dual targeting (Mackenzie 2005; Dinur-Mills et al. 2008) must be kept in mind. In the following, these 149 proteins potentially not of mitochondrial origin were excluded from the molecular and evolutionary analyses of algal proteome.

The remaining 347 proteins were classified into three groups based on the existence of clear counterparts in the four reference eukaryotes (yeast, human, *Arabidopsis*, and *Tetrahymena*) and their identification in the respective MS-mitochondrial proteomes. Among the 269 *Chlamydomonas* proteins that have a direct counterpart (amino acid identity >20%) in the reference eukaryotes, 177 have been found in the reference MS-proteomes (fig. 2 and supplementary table S2, Supplementary Material online). The overlap of the *Chlamydomonas* proteins with the MS-identified mitochondrial proteins in the reference eukaryotes is illustrated with a Venn diagram (fig. 3). The diagram shows that the *Chlamydomonas* proteome shares about 100 proteins with each experimental proteome. The 42 proteins common to all five surveys are mainly subunits of the OXPHOS chain and abundant metabolic enzymes (supplementary table S2, Supplementary Material online). Seventy-eight proteins identified in the *Chlamydomonas* samples have no known homolog in the four reference eukaryotes (fig. 2) and in some cases have no homolog outside the chlorophyte lineage. Among them, two-thirds have no assigned function

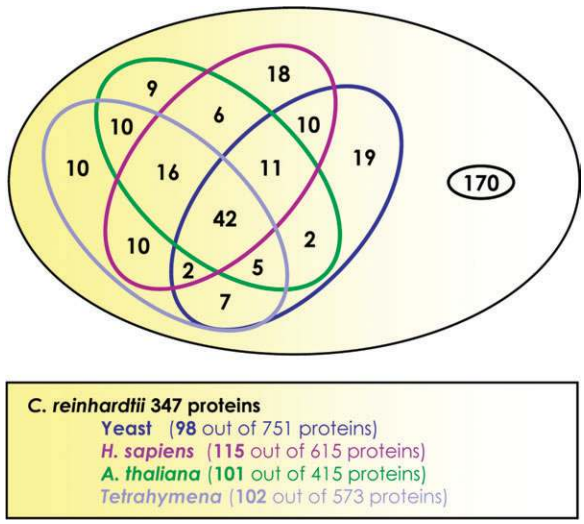

FIG. 3.—Venn diagram illustrating the overlap between the *Chlamydomonas* proteins and their homologs found in the large-scale mitochondrial proteomic analyses of yeast, *Tetrahymena*, human, and *Arabidopsis*. For *Chlamydomonas*, the proteins deemed to be contaminants were excluded from the analysis. The number of *Chlamydomonas* proteins for which no homolog was found in the other proteomics surveys is circled. For each reference eukaryote, the number of proteins shared with the *Chlamydomonas* proteome is indicated in bold followed by the total number of proteins identified by MS in isolated mitochondria. See supplementary table S2 (Supplementary Material online) for protein distribution.

(supplementary table S2, Supplementary Material online). Although these 347 proteins have not been previously detected as major components of other subcellular compartments in *Chlamydomonas* or other organisms, some of them (particularly those which have no known homolog in the reference eukaryotes) might not be genuine mitochondrial proteins but components of a compartment that is enriched in our mitochondrial preparations.

This description of the *Chlamydomonas* mitochondrial proteome appeared as the best suited considering our current knowledge on the photosynthetic alga. Furthermore, this description was valuable to uncover the metabolic abilities and the unique attributes of *Chlamydomonas* mitochondria.

New Insights into the Composition of the OXPHOS Complexes

In this *Chlamydomonas* proteome, 73 proteins were assigned to the category "energy production." Sixty-eight proteins are subunits of the OXPHOS complexes, of which 19 had not been previously identified by experimental work.

Chlamydomonas complex I is currently believed to comprise 41 subunits (Cardol et al. 2004, 2005). Among the 39 subunits identified here, eight were identified for the first time: ND2, NUO11, ND5, NUOB22, NUOS5, NUOA8, NUOP4, and NUOB14 (supplementary table S1, Supplementary Material online). We found five proteins (NUOP1, NUOP4, NUOP5, NUO17, and NUOB4) that were proposed earlier to be complex I subunits although they have no homolog in the four model organisms. The assignment of all five proteins but NUOP4 as complex I subunits was based on their MS identification in a BN-PAGE band containing enzymatically active complex I (Cardol et al. 2004). In this work, where most of a BN-gel lane was subjected to MS analysis, these five *Chlamydomonas*-specific proteins were found only where complex I migrates (970 kDa) (data not shown), providing further support to the earlier assignment. Genes for homologs of these five atypical complex I subunits are found in the *Volvox* genome but not in the fully sequenced genomes of three other unicellular photosynthetic organisms, the chlorophytes *Ostreococcus tauri* or *Ostreococcus lucimarinus* and the diatom *Thalassiosira pseudonana*.

Although complex II has not been characterized biochemically in *Chlamydomonas*, the genes for the core subunits SDH1–SDH4 could be identified in its genome (Cardol et al. 2005). Specific peptides for subunits SDH1, SDH2 and SDH3 were detected here (supplementary table S1, Supplementary Material online). MS peptides specific of SDH1, SDH2, and SDH3 were found in multiple BN-PAGE slices (data not shown), suggesting the aggregation of the complex or its destabilization under the experimental conditions.

Chlamydomonas complex III is predicted to comprise 10 subunits (Cardol et al. 2005). In addition to the three complex III subunits identified previously by their *N*-terminal sequences (CYC1, QCR1, and QCR5) (Atteia 1994; van Lis et al. 2003), three other subunits were found: cytochrome *b* (COB), the core II protein (QCR2), and a 14 kDa subunit (QCR7). BN-PAGE data indicated that complex III is found chiefly at 500 kDa as reported earlier (van Lis et al. 2003), and that the core protein QCR1 is prone to dissociation as QCR1 MS peptides were also found in the lowest part of the gel, where proteins of 50–60 kDa migrate (data not shown).

Chlamydomonas complex IV is predicted to contain nine subunits (Cardol et al. 2005). This work identified three subunits hitherto experimentally unidentified: COXI, COX5c, and COX90. COX90 has no known homolog in the four model eukaryotes or any other organism but *Volvox* to date. Its assignment as a complex IV subunit was suggested based on the spectroscopic analysis of a respiratory mutant with a disrupted cox90 gene that showed a decreased absorption peak at 609 nm, arising from the hemes a and a3 (Lown et al. 2001). Here, BN-gel showed that the distribution of the 11 kDa-COX90 follows that of the other complex IV subunits (not shown).

Chlamydomonas complex V is found on BN-PAGE as a highly stable dimer of 1,600 kDa (van Lis et al. 2003). The algal enzyme lacks the subunits for a typical stator stalk as well as the subunits required for the dimerization of the complex in fungi and mammals but exhibits several proteins that have no homolog in complex V of the reference eukaryotes (Vázquez-Acevedo et al. 2006; van Lis et al. 2007), and which are known as ASAs for ATP synthase associated proteins. Here, we identified the seven known typical complex V subunits ($\alpha, \beta, \gamma, \delta', \epsilon$, oligomycin sensitivity conferring protein, and ATP6) along with the seven already described ASAs (ASA1–ASA7) (van Lis et al. 2003; Vázquez-Acevedo et al. 2006). In addition, we identified two low molecular mass proteins proposed to be novel ASAs (ASA8 and ASA9) on the basis of their presence in enriched complex V (supplementary fig. S3, Supplementary Material online) (van Lis et al. 2007).

Metabolic Pathways

Of the 84 *Chlamydomonas* proteins assigned to the functional category "metabolisms," 50 have also been found in at least one of the four reference MS-mitochondrial proteomes (supplementary table S2, Supplementary Material online).

Amino Acid and Nitrogen Metabolism

Amino acid metabolism has been poorly investigated in the green alga and the subcellular localization of the various enzymes involved is virtually unknown. *Chlamydomonas* has genes for the two known isoforms of methionine synthase, the vitamin B_{12}–dependent enzyme (METH; EC 2.1.1.13) and the vitamin B_{12}–independent enzyme (METE; EC 2.1.1.14), in clear contrast to animals, plants, and yeast that contain only one isoform. In the mitochondrial samples analyzed here, which were obtained from cells grown in the absence of vitamin B_{12}, METE was the only isoform identified. A mitochondrial localization for METE in the alga contrasts with the situation in yeast where METE localizes to the cytosol, and in *Arabidopsis* where the enzyme is found in both the chloroplast and the cytosol (Ravanel et al. 2004). In mammals, the methionine synthase is a vitamin B_{12}–dependent enzyme that

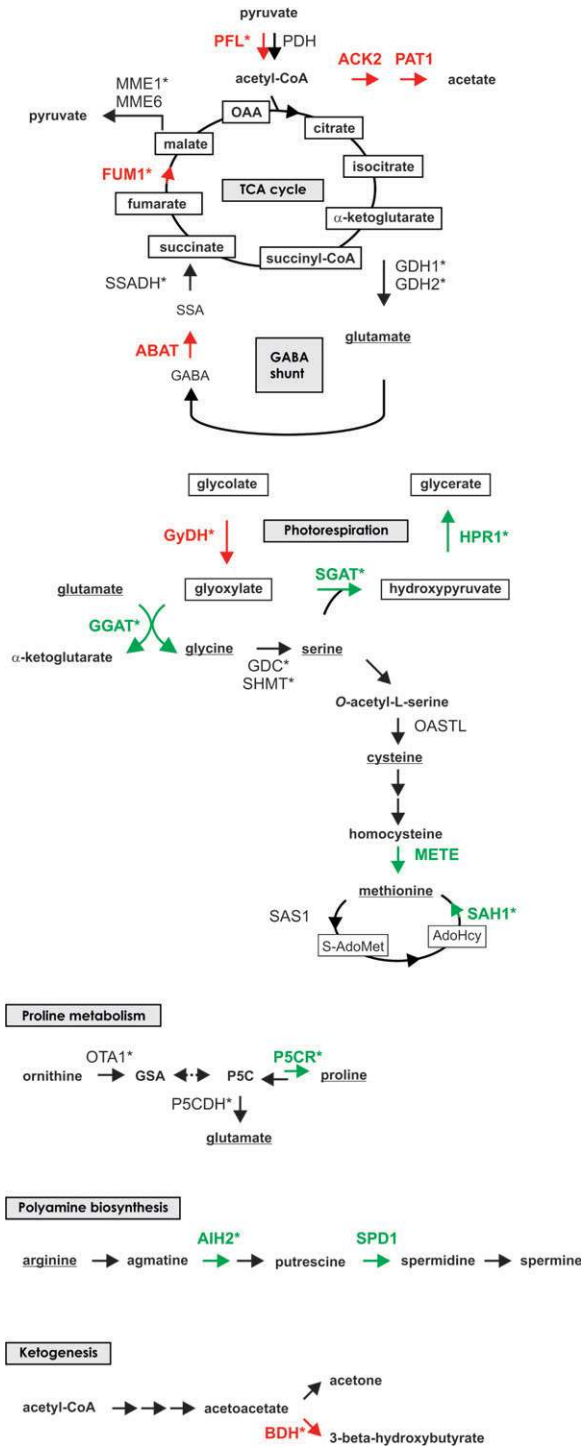

FIG. 4.—A selection of metabolic pathways in *Chlamydomonas* mitochondria as revealed by the proteomic survey. Color codes indicate the enzymes that are clearly related to bacterial enzymes (in red) and the enzymes that are not commonly described as mitochondrial proteins (in green). The phylogeny of the bacterial-type enzymes is given in supplementary data set S1 (Supplementary Material online). Of the TCA cycle enzymes only the fumarate hydratase (FUM1) is indicated because it shows no sequence identity to mitochondrial fumarate hydratases. *Protein identified by MS in the two independent analyses (BN-PAGE and SDS-PAGE). ABAT, 4-aminobutyric aminotransferase; ACK2, acetate kinase; AIH2, agmatine iminohydrolase; BDH, D-beta-hydroxybutyrate dehydrogenase; FUM1, class-I-fumarate hydratase; GDC/SHMT, glycine decarboxylase and serine hydroxymethyltransferase; GDH, glutamate dehydrogenase; GGAT, glutamate:glyoxylate aminotransferase; GyDH, glycolate dehydrogenase; HPR1, hydroxypyruvate reductase; METE, cobalamin-dependent methionine synthase; MME, malic enzyme; OASTL, cystathionine-beta-synthase; OTA1, ornithine aminotransferase; P5CDH, 1-Pyrroline-5-carboxylate dehydrogenase; P5CR, 1-pyrroline-5-carboxylate reductase; PAT1, phosphotransacetylase; PDH, pyruvate dehydrogenase complex; PFL, pyruvate formate-lyase; SAH1, *S*-adenosyl-L-homocysteine hydrolase; SAS1, *S*-adenosylmethionine synthetase; SGAT, serine:glyoxylate aminotransferase; SPD1, spermidine synthase; SSADH, succinate semialdehyde dehydrogenase.

localizes to the mitochondrion. A mitochondrial localization of METE in *Chlamydomonas* would give the organelle autonomy for methionine synthesis as well as for the regeneration of the AdoMet methyl group because a *S*-adenosylmethionine synthase (SAS1; EC 2.5.1.6) and a *S*-adenosyl-L-homocysteine hydrolase (SAH1; EC 3.3.1.1) were also found in the same samples (fig. 4). Neither a methionine synthase nor an *S*-adenosyl-L-homocysteine hydrolase has been detected in the reference mitochondrial proteomes.

In yeast, humans, and plants, de novo proline synthesis takes place in the cytosol, whereas proline degradation occurs in the mitochondrion (Brandriss and Magasanik 1981; Elthon and Stewart 1981). 1-Pyrroline-5-carboxylate dehydrogenase (P5CDH; EC 1.5.1.12), the second enzyme for proline degradation was identified in the *Chlamydomonas* samples (fig. 4) as well as in three of the four reference mitochondrial proteomes (supplementary table S2, Supplementary Material online). In addition, we identified in both mitochondrial preparations a 1-Pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2), which catalyzes the final step in proline synthesis and an ornithine aminotransferase (OAT1; EC 2.6.1.13) that produces L-glutamate 5-semialdehyde, the substrate of P5CR. Thus, it seems that proline synthesis may occur in *Chlamydomonas* mitochondria by the sequential action of OTA1 and P5CR (fig. 4). The localization of P5CR and P5CDH in the same cellular compartment implies a tight regulation of their enzymatic activities to prevent futile cycling between the biosynthetic and degradative pathways.

Several enzymes of the branched chain amino acid (BCAA) catabolism were identified in *Chlamydomonas* mitochondria: an isovaleryl-CoA dehydrogenase (IVD; EC 1.3.99.10) and the β subunit of methylcrotonyl-CoA carboxylase (MCC-B; EC 6.4.1.4), both involved in the initial degradation steps of the BCAA, as well as a hydroxymethylglutaryl-CoA lyase (HMG-CoAL; EC 4.1.3.4) and a methylmalonate semialdehyde dehydrogenase (EC 1.2.1.27), which catalyze the last degradation step of Leu and Val, respectively.

Polyamines are essential and ancient polycations required for cell division and proliferation, and for mitochondrial-mediated apoptosis (Thomas and Thomas 2001). Typical polyamines are the diamine putrescine, the triamine spermidine and the tetramine spermine. In eukaryotes, putrescine is synthesized de novo from ornithine by an ornithine decarboxylase (ODC; EC 4.1.1.17), usually in the cytosol. In plants and some fungi, putrescine can also be produced from arginine via an arginine decarboxylase

(ADC; EC 4.1.1.19), an agmatine iminohydrolase (AIH; EC 3.5.3.12) and an *N*-carbamoylputrescine amidohydrolase (NCPAH; EC 3.5.1.53). In plants, the localization of the ADC route is still under debate. The ADC has been found in various cellular compartments including chloroplast, cytosol, and mitochondria (Bagni and Tassoni 2001). AIH and NCPAH have been proposed to localize in the chloroplast (Borrell et al. 1995), but so far, no experimental evidence has been obtained. *Chlamydomonas* possesses both the ornithine and the arginine pathways to putrescine and, in light-adapted cells, the activity of ODC was found to be 1,000-fold higher than that of ADC (Voigt et al. 2000). In our samples, we identified the agmatine iminohydrolase AIH2, suggesting that the conversion of agmatine into *N*-carbamoylputrescine might take place in the algal mitochondria (fig. 4). In good agreement with such a localization, the identified *Chlamydomonas* AIH2 protein (V3 gene model) is predicted to be targeted to mitochondria with high score values by three different prediction tools (MitoProt, Predotar, and MultiLoc) (data not shown). Assuming that ADC is cytosolic in *Chlamydomonas* as proposed by Voigt et al. (2000), the presence of an AIH in the mitochondria would require the transport of agmatine to the organelle. Agmatine transport in liver mitochondria is well known in mammalian cells (Salvi et al. 2006). In the *Chlamydomonas* mitochondrial samples, a spermidine synthase (SPD1; EC 2.5.1.16), which converts putrescine into spermine, has also been identified (fig. 4). Polyamines are ubiquitous components in all cells. They are transported from their site of synthesis to the various cellular compartments. In mitochondria, polyamines exert protective functions and are also involved in apoptosis. Mitochondrial polyamine biosynthesis in *Chlamydomonas*, as suggested by the MS identification of an AIH and an SPD, might allow a tighter control of the mitochondrial functions.

In photosynthetic cells grown under low-CO_2 conditions, photorespiration results in the oxidation of glycolate and the release of carbon dioxide. A remarkable difference between the photorespiratory pathways in higher plants and some chlorophytes including *Chlamydomonas* resides in the glycolate-oxidizing enzyme (Frederick et al. 1973). In plants, this enzyme is an oxidase that localizes in the peroxisomes, whereas in *Chlamydomonas* and other chlorophytes, the glycolate-oxidizing enzyme is a dehydrogenase (GyDH; EC 1.1.99.14) (Nakamura et al. 2005). *Chlamydomonas* GyDH was identified in our survey supporting a mitochondrial localization of the enzyme. In addition to GyDH, three other proteins highly related to the plant photorespiratory enzymes were identified in the mitochondrial samples. These are a glutamate:glyoxylate aminotransferase (GGAT; EC 2.6.1.2), a serine:glyoxylate aminotransferase (SGAT; EC 2.1.6.45), and a hydroxypyruvate reductase (HPR1; EC 1.1.1.81) (supplementary table S1, Supplementary Material online). Interestingly, *Chlamydomonas* HPR1, SGAT, and GGAT exhibit an extended *N*-terminus and lack typical peroxisomal targeting signals as compared with their plant homologs (supplementary fig. S4, Supplementary Material online), which all localize to the peroxisome (Liepman and Olsen 2001, 2003). The experimental identification of photorespiratory enzymes in mitochondrial samples support earlier hypotheses proposing that photorespiration is a mitochondrial process in chlorophytes (Bruin et al. 1970; Beezley et al. 1976). Furthermore, the identification of these enzymes provides the necessary molecular tools to investigate further the metabolic route in chlorophyte algae and reconstruct the evolutionary history of photorespiration.

Hybrid-cluster proteins (HCP) are redox proteins found in all three domains of life (Andersson et al. 2006). So far, *Chlamydomonas* and the diatom *T. pseudonana* are the only eukaryotes known to possess HCPs. *Chlamydomonas* genome encodes four HCPs (HCP1–HCP4) that share 83–94% amino acid identity. In our survey, HCP2 has been unambiguously identified. The function of the HCPs in eukaryotes is currently unknown, whereas in bacteria, potential roles in the nitrogen cycle (Wolfe et al. 2002) and in oxidative stress defense (Briolat and Reysset 2002; Almeida et al. 2006) have been proposed. In *Chlamydomonas*, it could be hypothesized that HCPs are important for the algal anaerobic metabolism because the expression of one of them (HCP4) increased significantly (a factor of 1,500) upon anoxia (Mus et al. 2007).

Core Carbon Metabolism

All the eight enzymes of the tricarboxylic acid (TCA) cycle were identified (supplementary table S1, Supplementary Material online). These enzymes are all typically mitochondrial, except the fumarate hydratase (EC 4.2.1.2). Two biochemically distinct classes of fumarate hydratase are currently known (Woods et al. 1988; Schnarrenberger and Martin 2002): class-I-fumarate hydratase occurs in bacteria, whereas class-II-fumarate hydratase occurs in eubacteria, archaebacteria, and in mitochondria. All four reference eukaryotes have only a class-II-fumarate hydratase, which was found in their respective MS-mitochondrial proteomes. *Chlamydomonas* possesses genes for the two classes of enzyme (supplementary fig. S5, Supplementary Material online). However, only the class-I-fumarate hydratase (FUM1) was detected in the analyzed mitochondrial samples. Notably, *Euglena gracilis* also has a mitochondrial class-I-fumarate hydratase (Shibata et al. 1985).

The γ-aminobutyric acid (GABA) shunt bypasses two successive steps of the TCA cycle catalyzed by the α-ketoglutarate dehydrogenase and the succinyl-CoA synthetase. In our proteomic survey, we identified a succinate semialdehyde dehydrogenase (SSADH; EC 1.2.1.16) and a 4-aminobutyrate aminotransferase (ABAT; EC 2.6.1.19) (fig. 4), the two mitochondrial components of the GABA shunt. The ABAT identified here is more closely related to the bacterial aminobutyrate aminotransferases than to the eukaryote ones (supplementary fig. S6, Supplementary Material online). SSADH (50 kDa) and ABAT (45 kDa) were found in the same BN-gel band at an estimated molecular mass of 240 kDa (data not shown), suggesting that these enzymes might interact as observed earlier in human (Hearl and Churchich 1984).

Previous and present studies showed the occurrence in *Chlamydomonas* mitochondria of three metabolic enzymes

known only in bacteria or anaerobic eukaryotes: a pyruvate formate-lyase (PFL; EC 2.3.1.54), an acetate kinase (ACK2; EC 2.7.2.1) and a phosphotransacetylase (PAT1; EC 2.3.1.8) (Atteia et al. 2006). We reported earlier the existence in *Chlamydomonas* of an aldehyde/alcohol dehydrogenase (ADHE) (Atteia et al. 2006), an enzyme that converts acetyl-CoA into ethanol in bacteria and anaerobic eukaryotes. In *Polytomella*, a colorless relative of *Chlamydomonas*, the ADHE was found in mitochondria (Atteia et al. 2003). Here, we failed to detect the ADHE in the *Chlamydomonas* mitochondrial samples, which might be explained by the absence of the protein under the physiological conditions or by a different cellular localization in the green alga with respect to the colorless alga.

Vitamins and Cofactors

Chlamydomonas does not require any vitamins for growth (Croft et al. 2006), indicating that the alga possesses all the enzymes for de novo vitamin biosynthesis with the exception of vitamin B_{12}, which no eukaryotes are known to synthesize but which might be required in some conditions for the activity of the methionine synthase METH, as discussed above. Four enzymes belonging to four distinct vitamin biosynthetic pathways were detected in the *Chlamydomonas* mitochondrial samples. Of the vitamin B_1 (thiamine) biosynthetic pathway, the thiazole synthase (THI4a) was identified. In *Arabidopsis*, the thiazole synthase (Thi1; At5g54770) is targeted to both chloroplasts and mitochondria via a posttranscriptional mechanism (Chabregas et al. 2001) but so far the enzyme has only been detected in chloroplast stroma (Peltier et al. 2006). *Chlamydomonas* THI4a (36 kDa) was found on BN-gel at an estimated mass of 200 kDa (not shown), as was *Arabidopsis* Thi1 (Peltier et al. 2006). Of the vitamin B_5 biosynthetic pathway, the ketopantoate hydroxymethyltransferase (KPHMT; EC 2.1.2.11) was detected in *Chlamydomonas* mitochondria. KPHMT has also been identified by MS in mitochondria of yeast (Sickmann et al. 2003) but not in those of *Arabidopsis* although two isoforms are known to reside in the mitochondria (Coxon et al. 2005). In plants, archaea, and protists, vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine) is synthesized by a heterodimeric enzyme composed of a synthase (PDX1) and of a glutaminase (PDX2) (Tambasco-Studart et al. 2005). In the algal samples, PDX1 was unambiguously identified suggesting that the vitamin B_6 biosynthesis might take place in the mitochondria in contrast with the situation in *Arabidopsis* where GFP-fused PDX1 and PDX2 were found in the cytoplasm by confocal microscopy (Tambasco-Studart et al. 2005). In the soluble fraction of *Chlamydomonas* mitochondria, we identified a homolog of *Arabidopsis* diaminopelargonate synthase (BIO1), which catalyzes the second of four steps in the synthesis of vitamin B_8 (biotin).

Mitochondria contain the machinery for the biogenesis of iron–sulfur clusters (Fe/S) and their assembly into the apoproteins (Lill and Mühlenhoff 2006). A *Chlamydomonas* genome survey identified 18 proteins potentially involved in the mitochondrial iron–sulfur protein machinery (Godman and Balk 2008). Here, only two proteins of this machinery were identified: the iron–sulfur cluster assembly protein (ISU1), and the adrenodoxin reductase (ARH1). In yeast, ARH catalyzes the reduction of adrenodoxin (ferredoxin) in the presence of NADPH (Lill and Mühlenhoff 2006). In plants, ARH was also shown to be essential in the mitochondrial biotin synthase reaction (Picciocchi et al. 2003). With respect to the other mitochondrial MS-proteomes, ISU1 was detected in *Tetrahymena* and ARH in yeast.

Fatty Acids and Ketone Bodies

Enzymes involved in fatty acid metabolism were poorly detected in *Chlamydomonas* mitochondria, which is in contrast to the mitochondria of human and yeast. Three of the four enzymes involved in fatty acid β-oxidation were found in the algal samples: an acyl-CoA dehydrogenase (ACAD; EC 1.3.99.3), an enoyl-CoA hydratase (E-CoAH1; EC 4.2.1.17), and a 3-ketoacyl-CoA-thiolase (ATO1; EC 2.3.1.). The *N*-terminus of *Chlamydomonas* 3-ketoacyl-CoA-thiolase seems to include an incomplete PST2 peroxisomal targeting sequence (Swinkels et al. 1991) (supplementary fig. S7, Supplementary Material online), supporting its mitochondrial localization.

Of the mitochondrial fatty acid synthesis pathway, the acyl carrier protein (ACP1) was the only protein identified in *Chlamydomonas* mitochondria. This protein was found in the soluble fraction, and on BN-gel, it was found exclusively at an apparent molecular mass of 970 kDa (not shown), likely associated with complex I. Mitochondrial ACP1 was found in all four MS-derived mitochondrial proteomes.

Of ketone metabolism, we found a beta-hydroxybutyrate dehydrogenase (BDH; EC 1.1.1.30), which interconverts acetoacetate into beta-hydroxybutyrate. *Chlamydomonas* BDH relates more closely to the bacterial enzymes (40–54% aa identity) than to the eukaryotic (mitochondrial) ones (<20% aa identity). Its closest relative so far is the BDH of the α-proteobacterium *Rhodopseudomonas palustris* (GI:39937266) with 55% sequence identity.

Nucleotide Metabolism

Synthesis and degradation of nucleotides are complex metabolic pathways that involve multiple steps and compartments. Here, only six enzymes involved in the algal nucleotide metabolism were found (supplementary table S1, Supplementary Material online). Among them are an inosine 5-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) and a GMP synthase (GMPS; EC 6.3.5.2), which together catalyze the consecutive enzymatic steps in the synthesis of GMP from inosine monophosphate.

Mitochondrial Protein Import and Processing in *Chlamydomonas*

Targeting Machinery

Our *Chlamydomonas* genome survey identified 33 homologs of proteins involved in mitochondrial protein import and processing in yeast and *Arabidopsis*

Table 1
Evaluation of Mitochondrial Targeting in *Chlamydomonas* by Five Bioinformatic Subcellular Predictors

	Subset A				Subset B			
Program	321 Proteins (V2)	Sensitivity	334 Proteins (V3)	Sensitivity	159 Proteins (V2)	Sensitivity	170 Proteins (V3)	Sensitivity
Mitochondrial predictions of mitochondrial proteins								
Predotar	104	32%	109	33%	61	38%	65	38%
TargetP	132	41%	144	43%	77	48%	87	51%
MitoProt II	110	34%	108	32%	65	41%	70	41%
MultiLoc	63	20%	78	23%	35	22%	43	25%
PSORT	70	22%	72	22%	37	23%	40	24%
Chloroplast predictions of mitochondrial proteins								
Predotar	26	8%	27	8%	12	8%	12	7%
TargetP	32	10%	29	9%	15	9%	13	8%
MitoProt II	Na	Na	Na	Na	Na	Na	Na	Na
MultiLoc	60	19%	59	18%	30	19%	32	19%
PSORT	38	12%	40	12%	16	10%	17	10%

Performance comparison of the five subcellular predictors with the *Chlamydomonas* experimentally derived mitochondrial set. Subset A, all proteins identified in purified mitochondria that exhibit an *N*-terminal Met (in the V2 or V3 gene models) excluding contaminants (supplementary table S1, Supplementary Material online) and subset B, all proteins of subset A for which a homolog was found in the other reference MS-mitochondrial proteomes. Two complementary tests were performed: 1) Efficiency of the five tools to predict correct mitochondrial localization for the *Chlamydomonas* mitochondrial proteins. Sensitivity is the percentage of the proteins in the subset correctly predicted by each program to be targeted to mitochondria; and 2) Efficiency of the four adapted tools to predict erroneous chloroplast localization for the *Chlamydomonas* mitochondrial proteins: Sensitivity is the percentage of the proteins in the subset predicted by each program to be targeted to plastids. Na: not adapted given that MitoProt II does not predict chloroplast targeting sequences.

(supplementary table S3, Supplementary Material online). The proteomics survey identified 12 components of these machineries: Tom40, Tom22′ (Tom9), Tom20, Tim8, Tim9, Tim21, Tim44, Mdj2, mtHsp70, Mge1, Tim22d, and Mdm38 (supplementary tables S1 and S3, Supplementary Material online). None of these proteins had been identified experimentally in the alga so far.

Two proteins with homologies to the subunits α and β of the matrix mitochondrial processing peptidase (MPP), which cleaves off the targeting sequences upon entry into the matrix, were detected in *Chlamydomonas* mitochondria. These proteins are the complex III core proteins QCR1 and QCR2 (see above), which show homology to β-MPP and α-MPP, respectively. No other MPP-related proteins were found in the MS-proteome. Thus, it is likely that the processing of the mitochondrial precursor proteins in green alga is performed by the complex III core proteins, as in land plants (Brumme et al. 1998; Glaser and Dessi 1999), and in contrast to yeast and mammals, where the MPP complex is soluble in the matrix.

Targeting Prediction

We used our experimental set of mitochondrial proteins to test the reliability of five targeting prediction programs (Predotar, TargetP, MitoProt II, MultiLoc, and PSORT). The performance of each program to predict the targeting of a protein to the mitochondria or to the chloroplast was examined. Two different protein subsets were considered: all proteins identified but known contaminants (Subset A) and all proteins of subset A for which a homolog was found in at least one of the other reference MS-mitochondrial proteomes (Subset B) (table 1).

Among the five programs, TargetP and then Predotar performed best in predicting mitochondrial localization (table 1). MitoProt II is also efficient but is not useful for photosynthetic eukaryotes because chloroplast targeting predictions are not included. With TargetP, an accurate prediction of mitochondrial targeting was obtained for 41–51% of the proteins depending on the protein subset and on the *Chlamydomonas* gene models (V2, V3) (table 1). Of the reduced protein set (Subset B, V3 models), TargetP predicted a mitochondrial localization for 87 of 170 proteins, whereas 13 proteins were predicted to localize to the chloroplast. Similarly, Predotar predicted a mitochondrial localization for 65 of 170 proteins and a chloroplast localization for 12 of them. The sensitivity for mitochondrial targeting is lower than that obtained with a subset of MS-identified *Arabidopsis* mitochondrial proteins, which was of 66% and 62% with Predotar and TargetP, respectively (Heazlewood et al. 2004). The relatively lower sensitivity obtained with *Chlamydomonas* proteins may be due to 1) misannotated *N*-termini, and/or 2) software that is not adapted for *Chlamydomonas*. In fact, the software were developed using plant targeting sequences.

TargetP and Predotar are rather reliable programs to predict mitochondrial localization in *Chlamydomonas* but are unfortunately erroneous in the case of chloroplast proteins, as reported by various research groups. These observations reinforce: 1) the need for subcellular proteomic approaches to increase experimental data and 2) the development of prediction tools adapted to the model organism *Chlamydomonas*. In that respect, our work provides a significant list of genuine mitochondrial proteins, a learning set for the retraining of the programs to generate tools suitable for the green alga.

Evolutionary Aspects of the *C. reinhardtii* Mitochondrial Proteome

Phylogenetic analyses of mitochondrial genes and proteins indicate that the mitochondrial endosymbiont was an α-proteobacterium (Gray et al. 1999; Embley and Martin 2006), but the issue of the closest modern relative

among the α-proteobacteria is so far unresolved. Identifying a close relative of the original endosymbiont is difficult as major modifications of its proteome have occurred to synchronize the activities of the proto-mitochondrion and its host. Furthermore, the integration of the endosymbiont in its host seems to have progressed to various extents in the different lineages as revealed by a comparison of the functions retained by yeast and mammalian mitochondria (Gabaldón and Huynen 2007). A study focusing on the yeast mitochondrial proteome suggested that α-proteobacterial parasites related to *Rickettsia* might be the closest relatives (Kurland and Andersson 2000). Later studies focusing on mitochondrially encoded proteins in large mitochondrial genomes suggested an equally close relationship between mitochondria and the purple nonsulfur bacterium *Rhodospirillum rubrum* (Esser et al. 2004). The vast majority of functions performed by mitochondria are specified by nuclear genes, hundreds of which have been identified as gene acquisitions from the α-proteobacterial ancestor of the organelle (Gabaldón and Huynen 2004) and α-proteobacterial lineage sampling in earlier studies was sparse. The availability of 40 genome sequences from diverse α-proteobacterial representatives in the context of proteome data for an algal mitochondrion that is far more metabolically diverse than that of yeast prompted reinspection of the issue.

We compared the *Chlamydomonas* proteins identified by MS as queries with a search set comprising the proteins encoded by a total of 354 sequenced genomes including 286 eubacteria, 24 archaebacteria, and 44 eukaryotes using reciprocal Blast (Altschul et al. 1997). The *Chlamydomonas* proteins analyzed correspond to all proteins identified but those described as contaminants that stem from the cytosol and other cell compartments (see fig. 2 and supplementary table S1, Supplementary Material online). For each query protein, the best Blast hit with an *E*-value $\leq 10^{-20}$ in each genome was recorded, aligned to the query using ClustalW, and the proportion of identical amino acids in each pairwise alignment was written into a table (supplementary data set S2, Supplementary Material online), sorted for clarity, and color coded. At this threshold, 62 proteins found hits only among eukaryotes, and 150 found hits both in prokaryotes and eukaryotes. The resulting matrix shown in figure 5*A* provides a summary overview of the results. Among the eukaryote-only proteins are the subunits of the protein import system (TIM/TOM) and the carriers of the mitochondrial carrier family, but also several proteins with unknown function (MAP04, 07, 14, 17, 18, 23, 24, and 25). *Arabidopsis* showed the most hits with *Chlamydomonas* proteins (54 of 62 eukaryote-only proteins).

From the matrix, it is clear that the question of which bacterium is the closest relative to mitochondria has two aspects: gene distribution (presence vs. absence) and sequence similarity. The gene distribution aspect relates to the circumstance that many of the genomes sampled do not contain homologs of the *Chlamydomonas* protein owing to lateral gene transfer (LGT), which is a natural and widespread phenomenon in prokaryote genome evolution (Doolittle 1999; Dagan and Martin 2007). From the standpoint of sequence similarity, it is clear that among the lineages sampled in figure 5, those having the greatest overall similarity to the *Chlamydomonas* query are 1) eukaryotes (unsurprisingly) and 2) α-proteobacteria, as endosymbiotic theory would predict.

In order to quantify the comparisons in the standard currency of phylogenetics, we aligned the homologs identified in figure 5*A* and constructed maximum likelihood trees for the 183 proteins having at least four taxa in the alignment. In 39 trees, only eukaryotes were represented, with *Arabidopsis* appearing as the sister of *Chlamydomonas* in 33 of them. In 41 trees, α-proteobacterial homologs were the sister to the *Chlamydomonas* sequence or the eukaryotes within which the *Chamydomonas* sequence branched. Among these proteins were the hybrid-cluster protein HCP2 and the class-I-fumarate hydratase FUM1 (supplementary data set S1, Supplementary Material online). In 22 additional trees, the same result was obtained but homologs from other prokaryotes interleaved with the α-proteobacterial sequences. In the remaining 81 trees, homologs from prokaryotes other than α-proteobacteria were the sister to the *Chlamydomonas* sequence or to the eukaryotes within which the *Chamydomonas* sequence branched. This latter result is often interpreted as LGT to eukaryotes, but LGT among prokaryotes can produce the same phylogenetic result for gene acquisitions from the genomes of the ancestral mitochondrion (Esser et al. 2007), as can phylogenetic artifacts. The photorespiratory GyDH belongs to such a group and branches with the *Desulfovribrio* species. The pyruvate formate-lyase, the acetate kinase, and the phosphotransacetylase, which provide the alga with a metabolic route to produce ATP under anaerobic conditions (Atteia et al. 2006), also belong to this group.

Which α-proteobacteria branch most frequently with *Chlamydomonas*? In figure 5*B*, the frequency distribution is shown for the number of times that homologs from the indicated α-proteobacterial genus occurred in the α-proteobacterial sister group. If several sequences from the same genus were present, the genus was only counted once, to avoid oversampling. *Bradyrhizobium* and *Rhodobacter* were present most frequently (19 times each, respectively) in the α-proteobacterial sister group, whereas *Sicilibacter* was present most frequently (9 times) in the sister group where other prokaryotes interleaved with the α-proteobacterial sequences. The corresponding numbers for the Rickettsias were 12 and 3, respectively. Clearly, members of the Rhizobiales and Rhodobacterales have more proteins that link them specifically to mitochondria than members of the Rickettsiales with their highly reduced genomes (Sallstrom and Andersson 2005) do, but sampling among α-proteobacterial genomes is still very sparse, with representatives from only 22 of currently >220 recognized α-proteobacterial genera sampled.

Concluding Remarks

This study reports the first broad proteomic survey of the mitochondrion from a photosynthetic alga; it clarifies the composition of some typically mitochondrial complexes such as OXPHOS complexes as well as it identifies

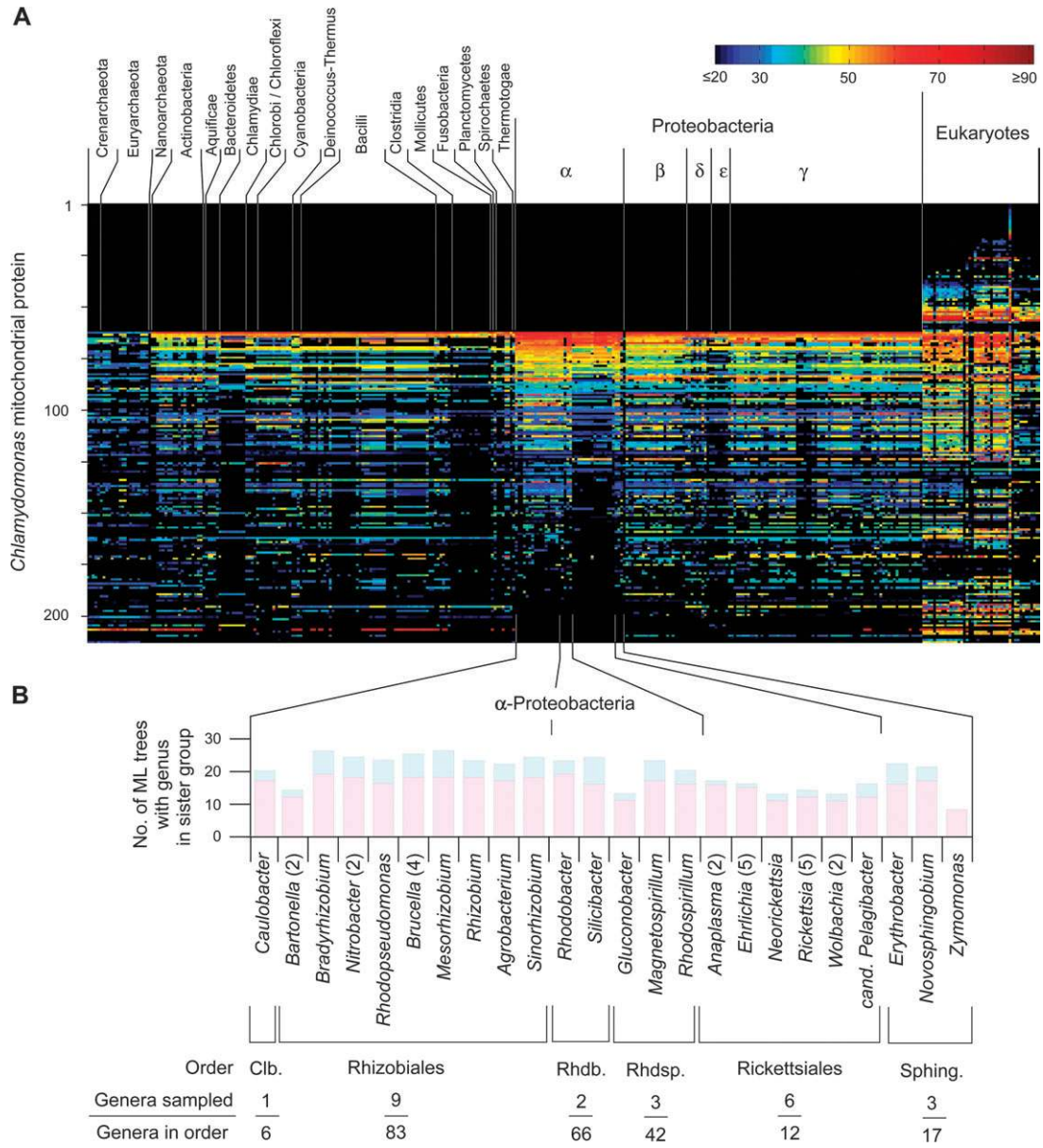

FIG. 5.—Sequence similarity and homolog distribution of *Chlamydomonas* mitochondrial proteins in sequenced genomes. (*A*) Amino acid sequence identity and protein presence or absence among 354 sequenced genomes for 212 *Chlamydomonas* mitochondrial proteins having homologs at the *E*-value threshold of $\leq 10^{-20}$. The amino acid identity in the pairwise comparison of the best Blast hit to each genome in the search set is color coded (scale bar at upper right). The eukaryote with the highest number of best hits is *Arabidopsis*. Twenty-seven *Chlamydomonas* mitochondrial proteins that found hits only in *Arabidopsis* below the *E*-value threshold of 10^{-20} are not shown. The full table with all species names, all protein symbols and all pairwise amino acid identity values is given as supplementary data set S2 (Supplementary Material online). (*B*) Frequency distribution of α-proteobacterial genera in ML trees constructed for homologs of *Chlamydomonas* mitochondrial proteins. The pink columns indicate the number of times that the homolog from the given genus occurred in the α-proteobacterial sister group to the *Chlamydomonas* mitochondrial protein, the blue columns indicate the number of times that the genus occurred in the α-proteobacterial sister group, but in which other prokaryotes interleaved with the α-proteobacterial sequences. The individual trees are given in the supplementary data set 1 (Supplementary Material online), the species identifiers correspond to those given in supplementary data set S2 (Supplementary Material online).

components of machineries unstudied in the alga so far, such as the TIM and TOM complexes. The comprehensive protein list reported here adds considerably to our understanding of the metabolism in the green alga while it further illustrates the metabolic diversity of the organelle with, for example, the identification of photorespiratory enzymes and bacterial-like enzymes. Finally, our data provide novel insights into the evolutionary history of the algal mitochondrion.

Chlamydomonas mitochondria exist in various shapes and sizes and have a complex morphodynamical behavior (Grobe and Arnold, 1975; Blank et al. 1980). Electron micrographs of the *Chlamydomonas* cells show that mitochondria closely interact with the chloroplast, and in some instances, mitochondrial arms penetrate the chloroplast (Ehara et al. 1995; Harris 2008, and references therein). Mitochondria purified with the routine protocol represent only a fraction of all mitochondria (Eriksson et al.

1995), and it is believed that most mitochondria remain associated with the chloroplasts. Immunoblot analyses of chloroplasts and mitochondria isolated from the same batch of cells have shown earlier the presence of genuine mitochondrial proteins in the chloroplast preparations (Atteia et al. 2006), confirming a tight association between the two organelles. Future proteomics studies should focus on the mitochondria that interact tightly with the chloroplasts to determine whether they exhibit metabolic specificities distinct from those of the mitochondria described in this work.

Supplementary Material

Supplementary tables S1–S3 and supplementary figures S1–S7, and data sets S1 and S2 are available at *Molecular Biology and Evolution* online (http://mbe.oxfordjournals.org/).

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