J Bioenerg Biomembr (2006) 38:271–282
DOI 10.1007/s10863-006-9046-x

ORIGINAL PAPER

The mitochondrial ATP synthase of chlorophycean algae contains eight subunits of unknown origin involved in the formation of an atypical stator-stalk and in the dimerization of the complex

Miriam Vázquez-Acevedo · Pierre Cardol · Araceli Cano-Estrada · Marie Lapaille · Claire Remacle · Diego González-Halphen

Received: 20 May 2006 / Accepted: 29 June 2006 / Published online: 8 December 2006

Abstract Mitochondrial F_1F_O-ATP synthase of *Chlamydomonas reinhardtii* and *Polytomella sp*. is a dimer of 1,600,000 Da. In *Chlamydomonas* the enzyme lacks the classical subunits that constitute the peripheral stator-stalk as well as those involved in the dimerization of the fungal and mammal complex. Instead, it contains eight novel polypeptides named ASA1 to 8. We show that homologs of these subunits are also present in the chlorophycean algae *Polytomella* sp. and *Volvox carterii*. Blue Native Gel Electrophoresis analysis of mitochondria from different green algal species also indicates that stable dimeric mitochondrial ATP synthases may be characteristic of all Chlorophyceae. One additional subunit, ASA9, was identified in the purified mitochondrial ATP synthase of *Polytomella* sp. The dissociation profile of the *Polytomella* enzyme at high-temperatures and cross-linking experiments finally suggest that some of the ASA polypeptides constitute a stator-stalk with a unique architecture, while others may be involved in the formation of a highly-stable dimeric complex. The algal enzyme seems to have modified the structural features of its surrounding scaffold, while conserving almost intact the structure of its catalytic subunits.

M. Vázquez.-Acevedo · A. Cano-Estrada · D. González-Halphen (✉)
Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-600, Delegación Coyoacán, 04510 México D.F., Mexico
e-mail: dhalphen@ifc.unam.mx

P. Cardol · M. Lapaille · C. Remacle
Genetics of Microorganisms, Institute of Plant Biology B22, University of Liège, B-4000 Liège, Belgium

Keywords Oxidative phosphorylation · F_1F_O-ATP synthase · Dimeric mitochondrial complex V · Chlorophycean algae · Stator-stalk · *Chlamydomonas reinhardtii* · *Volvox carterii* · *Polytomella* sp. · ASA subunits

Introduction

Mitochondrial F_1F_O-ATP synthase (complex V) plays a major role in oxidative phosphorylation (OXPHOS). Two multisubunit domains constitute the enzyme, a soluble fraction called the F_1 factor and a membrane bound sector F_O (Alfonzo et al., 1981). F_1F_O-ATP synthase is also a molecular motor in which a central rotor-stalk [$\gamma/\delta/\varepsilon/c_{10}$] (subunits of beef mitochondria) rotates around an axis perpendicular to the plane of the membrane, while other polypeptides constitute the fixed elements of the motor, subunit *a*, the catalytic core [α_3/β_3], the peripheral stator-stalk [OSCP/*b*/*d*/F_6], and the so-called minor subunits [A6L/*e*/*f*/*g*] (Golden and Pedersen, 1998; Ko et al., 2000; Walker and Dickson, 2006). Proton flux across subunit *a* causes a rotary movement of the membrane-embedded ring c_{10} and its bound, protruding central-stalk [$\gamma/\delta/\varepsilon$]. In a full cycle, three sequential 120° movements of subunit γ induce consecutive conformational changes in the three catalytic β subunits leading to substrate binding (ADP + Pi), ATP synthesis, and ATP release (Itoh et al., 2004). Additional subunits play a regulatory role, such as the inhibitory protein IF_1 (Gledhill and Walker, 2005); or are involved in the formation and stabilization of a dimeric complex, such as subunits *e* and *g* (Brunner et al., 2002; Fronzes et al., 2006).

While a dimeric form is observed *in vivo* (Schägger, 2002), complex V from different sources, including mammals, fungi and higher plants, usually migrates in Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

(Schägger, 1994) as a monomer of 550–600 kDa. In addition, it is often observed that in these conditions, the enzyme partially dissociates with the concomitant appearance of the free F_1 subcomplex (Jänsch et al., 1996; Arnold et al., 1998; Horvath et al., 2000; Eubel et al., 2004). In sharp contrast, complex V of the chlorophycean algae *Chlamydomonas reinhardtii* and *Polytomella* sp. migrates in BN-PAGE as a stable dimer of 1,600 kDa (van Lis et al., 2003; van Lis et al., 2005; Dudkina et al., 2005). Furthermore, monomeric F_1Fo or free F_1 moieties were never detected in BN-PAGE. Recently, an electron microscopy (EM) study (Dudkina et al., 2005) revealed that the structure of the ATP synthase of *Polytomella* sp. is different from the dimeric enzyme isolated from bovine mitochondria (Minauro-Sanmiguel et al., 2005).

Combining biochemical and computational analyses, the mitochondrial ATP synthase from *C. reinhardtii* was found to contain 14 subunits of 7 to 60 kDa (van Lis et al., 2003; Franzén and Falk, 1992; Nurani and Franzén, 1996; Funes et al., 2002a; Cardol et al., 2005). Seven polypeptides were identified as subunits α, β, γ, δ, *a* (ATP6), *c* (ATP9), and OSCP, while no homologs of the ε, *b*, *d*, *e*, *f*, *g*, IF_1, A6L, and F6 subunits were found. The other seven polypeptides, named ASA1 (for **A**TP **S**ynthase **A**ssociated protein), ASA2, ASA3, ASA4, ASA5, ASA6, and ASA7 (previoulsy NUOP6), in accordance with the *C. reinhardtii* genome project nomenclature, had no counterparts in the databases. This suggested that unique proteins were associated with chlorophycean mitochondrial ATP synthases (Cardol et al., 2005). The ASA1 polypeptide (61 kDa), initially named MASAP (for **M**itochondrial **A**TP **S**ynthase **A**ssociated **P**rotein), MAAP67, or P60, was found in the isolated mitochondrial ATP synthases of both *C. reinhardtii* and *Polytomella* sp. (Accession numbers CAD29654 and CAD90158). It was put forth that ASA1 stabilizes the chlorophycean ATP synthase dimer in BN-PAGE (van Lis et al., 2003; van Lis et al., 2005).

In this work, we demonstrate that the subunit composition of the mitochondrial ATP synthase of *Polytomella* sp. is similar to the one of the *C. reinhardtii* enzyme. Also, we describe the presence of the classical subunit ϵ and of the novel polypeptides ASA8 and ASA9. We also report that homologous genes are present in the chlorophycean alga *Volvox carterii*. In addition, we sought the presence of dimeric mitochondrial ATP synthases in some algal species, which could be indicative of enzymes with an atypical subunit composition. Heat-treatment dissociation and cross-linking experiments on the purified ATP synthase from *Polytomella* sp. indicated that some of the ASA1 to 9 subunits are constituents of the peripheral stator-stalk of the enzyme, while others may be involved in the dimerization of the complex. A model for the topological arrangement of the ASA subunits is presented. Altogether, the data suggest that chlorophycean algae contain novel polypeptide constituents in their mitochondrial ATP synthase which give rise to a highly-stable, dimeric enzyme with unique architectural features.

Materials and methods

Algal strains and growth conditions

Polytomella sp. (198.80, E.G. Pringsheim) was grown aerobically in wide-bottom flasks for 48 h at 25 °C in 2 L of MAP medium (pH 7.0) as previously described (van Lis et al., 2005). The other chlorophyte algae used in this work were all axenic cultures known to grow relatively rapidly: *Scenedesmus obliquus* (SAG 276–3b), *Chlorogonium elongatum* (SAG 12–2b), *Chlorococcum ellipsoidum* (SAG 63.80), *Chlamydomonas reinhardtii* (137C), *Chlamydomonas smithii* (cc-1373, Duke University), *Chlamydomonas moewusii* (SAG 21.90), *Tetraselmis chui* (SAG 8–6), *Pseudendoclonium basiliense* (SAG 466.1), and *Chlorella vulgaris* (UTEX 259). Strains were grown in liquid or on solid agar medium under continuous illumination (45 μE m^{-2} s^{-1}) at 25°C. Tris-acetate-phosphate medium (TAP) (Gorman and Levine, 1965) was used for all the green algae, except for *T. chui* which was grown on solid Marine medium (Difco Marine Broth 2216, BD).

Polytomella ATP synthase purification

Polytomella sp. cells were centrifuged at 2000 × *g* for 5 min, resuspended in 200 ml of a cold buffer containing 0.3 M sucrose, 4 mM potassium EDTA, and 20 mM Tris (pH 7.2) (SPT buffer) and centrifuged again. All steps were carried out at 4 °C. The pellet was resuspended in 10 to 20 ml of SPT buffer and the cells were broken mechanically with a Potter homogenizer with five to six gentle manual strokes of the teflon-pestle. The broken cells were centrifuged at 1000 × *g* for 10 min and the supernatant (S1) saved. The pellet was resuspended in SPT buffer and treated again with five to six strokes of the Potter homogenizer, in order to break the cells that were not lysed in the first treatment. The sample was centrifuged at 1000 × *g* for 10 min; the resulting supernatant (S2) was mixed with S1 and centrifuged at 17, 000 × *g* for 10 min. The resulting mitochondrial pellet was resuspended in a small volume of SPT buffer. The protein concentration of the mitochondrial fraction was estimated, and then diluted to a final concentration of 10 mg of protein/ml in 50 mM Tris-HCl (pH 8.0), 1 mM $MgSO_4$, 1mM phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml tosyl-lysyl-chloromethylketone (TLCK) and 100 mM NaCl. N-dodecyl-β-maltoside (added from a 10 mg/ml stock solution) was added drop by drop until it reached a final w/w ratio of 2 mg of detergent per mg of protein. The mixture was incubated with gentle stirring for 30 min, and centrifuged at 90, 000 × *g* for

20 min. The supernatant was recovered and dialyzed for two hours against 2 L of 50 mM Tris-HCl (pH 8.0) containing 1 mM $MgSO_4$. The dialyzed sample was loaded onto a DEAE-Biogel A column (70 ml) equilibrated with 50 mM Tris-HCl (pH 8.0), 1 mM $MgSO_4$, 0.1 mg/ml of n-dodecyl-β-maltoside, 1mM PMSF, and 50 μg/ml TLCK (TMT buffer). The column was washed with 3–4 column volumes of the same buffer and then eluted with a linear NaCl gradient (0 to 125 mM in TMT buffer). The fractions enriched in *Polytomella* sp. mitochondrial ATP synthase were recovered and concentrated by ultrafiltration in an Amicon chamber with a YM100 membrane. After adding glycerol to a final concentration of 10%, the sample was loaded onto seven-ml tubes containing 15 to 40% glycerol gradients in 20 mM Tris-HCl (pH 8.0), 1 mM sodium EDTA, 10 mM succinate, 35 mM NaCl, 2 mM ATP and 0.1 mg/ml of n-dodecyl- β-maltoside. The gradients were centrifuged at 40000 $\times$ g for 17 h in a swingout rotor. The purified ATP synthase complex was recovered in a band that migrated around 20% glycerol.

Protein analysis

To prepare the total membrane fraction from algae, about 5×10^6 cells were frozen in liquid nitrogen and mechanically disrupted in a mortar. The broken cells were suspended in a MOPS buffer (280 mM mannitol, 10 mM MOPS-KOH pH 7.4, 0.1% BSA, 1 mM PMSF) and the solution was homogenized by sonication (two times for 10 sec; Vibra Cell sonicator, Danbury, CT). The suspension was centrifuged at low speed (10 min at 480 $\times$ g followed by 5 min at 3000 $\times$ g) and the resulting supernatant was then centrifuged at high speed (27000 $\times$ g for 15 min). The final pellet containing membranes was suspended in the MOPS buffer. Proteins were solubilized in the presence of n-dodecyl-β-maltoside and subjected to BN-PAGE as described by Schägger (1994). The stacking gel contained 4% (w/v) acrylamide, and the separating gel was a gradient from 5 to 12% (w/v) acrylamide. Complex I resolved by BNE-PAGE was identified by its NADH dehydrogenase activity detected by incubating the gel in 100 mM MOPS-KOH (pH 8.0) containing 100 mM NADH and 1 mg per ml Nitro blue Tetrazolium (NBT). Complex V was identified by its ATP synthase activity detected by incubating the gel in 50 mM HEPES (pH 8.0) containing 10 mM ATP and 30 mM $CaCl_2$. When indicated, BN-PAGE was followed by 2D-Tricine-SDS-PAGE (Schägger, 1994). Protein concentrations were estimated according to Bradford (1976), or to Markwell et al. (1978). The isolation of polypeptides for N-terminal sequencing was carried out as previously described (Atteia et al., 1997). N-terminal sequencing was carried out by Dr. J. d'Alayer on an Applied Biosystems Sequencer at the Laboratoire de Microséquençage des Protéines, Institut Pasteur, Paris (France). Cross-linking experiments were carried out with the water-soluble, homobifunctional, thiol-cleavable reagent 3,3′-dithio bis-(sulfo succinimidyl propionate) (DTSSP). The enzyme (4 mg protein /ml), in a buffer containing 20 mM Hepes (pH 7.4), 1 mM sodium EDTA, 10 mM succinate, 35 mM NaCl, 2 mM ATP and 0.1 mg/ml of n-dodecyl-β-maltoside, was incubated in the presence of 0.75 mM DTSSP, for one hour, at 6 °C. The reaction was stopped by the addition of 25 mM Tris (pH 8.0) (final concentration). The sample (100 μg of protein) was subjected to Tricine-SDS-PAGE [7%(w/v) acrylamide] in non-reducing conditions. The lanes of interest were cut and incubated for one hour in the presence of 50 mM 1,4-dithiothreitol, 0.1% SDS, 0.1 M Tris, 0.1 M Tricine (pH 8.25) and loaded onto 2D-Tricine-SDS-PAGE [12% (w/v) acrylamide].

Results

Update on the subunit composition of mitochondrial ATP synthase from the chlorophycean alga *C. reinhardtii*

A new version of the genome sequence of *C. reinhardtii* has recently become available (*C. reinhardtii* v3.0 at the Department of Energy Joint Genome Institute, http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). So, we carried out an extensive search of *Arabidopsis thaliana* ATP synthase subunit homologs in the *Chlamydomonas* genome using BLAST (Altschul et al., 1990). Among the 16 polypeptides that form the mitochondrial F_1F_O-ATP synthase of *Arabidopsis*, including the plant-specific 24 kDa F_Ad subunit (Heazlewood et al., 2004), only eight homologous subunits and an isoform could be identified in the green alga [α, β, γ, δ, ϵ, OSCP, *a* (ATP6) and *c* (ATP9)]. The other eight constituents of the higher plant mitochondrial ATP synthase had no counterparts in *Chlamydomonas* [A6L (ATP8), IF_1, F_Ad, *b*, *d*, *e*, *f*, and *g*]. Instead the algal enzyme contains the seven novel polypeptides ASA1 to 7 whose corresponding genes were previously identified in the *C. reinhardtii* genome sequence (Cardol et al., 2005). An additional subunit, ASA8, was found based on its similarity to its *Polytomella* homolog (see below). Table 1 summarizes and updates the constituents of the mitochondrial ATP synthase of *C. reinhardtii*.

One can hypothesize that subunits ASA1 to 8 were recruited to the mitochondrial ATP synthase from pre-existing subunits belonging to another ATP synthase/ATPase of *C. reinhardtii* (either the chloroplast or the vacuolar enzymes). Nevertheless, none of the novel F_1F_O-ATP synthase subunits share any significant sequence similarity with subunits of CF_1F_O-ATP synthase or of V_1V_O-ATPase. Moreover, with the exception of ASA1 of *Polytomella* sp.,

Table 1 Polypeptides associated with *C. reinhardtii* F_1Fo-ATP synthase

Subunit name	Molecular mass (kDa) pp/ms	N-terminal sequence of the mature subunit (and reference)	*Chlamydomonas* genome identifier
ASA1	63.1/60.6	YVTALKVEF (*a*)	estExt_GenewiseW_1.C_160132
β (ATP2)	61.8/59.1	AEPAAAAT (*b*)	estExt_GenewiseW_1.C_130017
α (ATP1)	61.5/56.8	ASDAKALD (*c*)	estExt_GenewiseW_1.C_50221
ASA2	48.4/45.5	ATATFVPGVSGDASG (*d*)	estExt_fgenesh2_pg.C_300145
ASA3	39.7/36.3	GAPAGSHDHP (*d*)	Chlre2_kg.scaffold_50000016
ASA4	34.0/31.2	ATGAAPSKK (*d*)	estExt_fgenesh2_kg.C_20106
γ (ATP3)	35.1/30.8	ASNQAVKQRI (*d*)	estExt_fgenesh2_pg.C_570033
a (ATP6)	35.5/24.6	STAAQVQSGATVNSL (*d*)	estExt_gwp_1W.C_10357
OSCP (ATP5)	25.9/22.6	AKTAPKA (*d*)	estExt_fgenesh2_pg.C_280199
ASA7	22.2/19.5	LSTLVEKFTFGSAAD (*d*)	estExt_fgenesh2_pg.C_300161
δ (ATP16)	21.2/18.6	*EEVAVPAGPKE* (*e*)	estExt_fgenesh2_kg.C_410003
ASA5	14.3	MKLLPESLQQEAA (*d*)	estExt_fgenesh2_kg.C_320014
ASA6	16.1/13.3	EESSVANLVKS (*d*)	estExt_fgenesh2_pg.C_70146
ASA8	10.0	*MTLGEAYLKDIL* (*e*)	estExt_fgenesh2_pg.C_250059
ϵ (ATP15)	8.3	*MCPPSGPFYRVA* (*e*)	estExt_gwp_1W.C_80137
c (ATP9)	9.9/7.3	*SVLAASKMVGA* (*e*)	e_gwW.68.39.1

Note. Molecular masses were calculated from the deduced sequences of the preproteins (pp) and from the mature sequences (ms). ASA1 to ASA8 subunits are the novel components of *C. reinhardtii* ATP synthase. Sequences in italics were not determined experimentally, but predicted from sequence similarities with the corresponding *Polytomella* sp. polypeptides. Subunit ASA7 corresponds to the previously identified subunit NUOP6, and *c* (ATP9) corresponds to the previously described ATP9B isoform (Cardol et al., 2005). ATP9A (*C. reinhardtii* genome identifier e_gwH.68.58.1) is not present in *V. carterii*, and is probably also not expressed in *C. reinhardtii*. The presence of an homolog of *Polytomella* ASA9 remains to be found in *C. reinhardtii*.
References: (a) Atteia (1994). (b) Franzén and Falk (1992). (c) Nurani and Franzén (1996). (d) Funes et al., (2002a). (e) This work.

Chlamydomonas subunits ASA1 to 8 have no homologs in the databases and therefore, their evolutionary origin remains obscure.

Subunit composition of the mitochondrial ATP synthase from the colorless chlorophycean alga *Polytomella* sp

To explore the subunit composition of mitochondrial ATP synthase in another chlorophycean alga, we obtained a highly homogeneous preparation of *Polytomella* sp. mitochondrial ATP synthase through a two-step purification procedure (see Materials and Methods). When the purified enzyme was run in BN-PAGE side by side with solubilized mitochondria from *Polytomella*, it exhibited the same mobility as complex V (Fig. 1A). Therefore, the *Polytomella* mitochondrial ATP synthase obtained from the glycerol gradients also behaves as a dimeric complex. This purified enzyme exhibited a polypeptide pattern after SDS-PAGE similar to the one previously characterized (Atteia et al., 1997; Funes et al., 2002; van Lis et al., 2005). In addition, the number and size of the *Polytomella* ATP synthase polypeptide constituents are similar to that of the *C. reinhardtii* enzyme (van Lis et al., 2005).

All the polypeptides of the purified *Polytomella* mitochondrial ATP synthase resolved by SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane and subjected to N-terminal sequence analysis. Except for ASA9 which exhibited a blocked N-terminus, N-terminal sequences–from 21 to 47 residues–were obtained for all polypeptides (Fig. 1B). Since each one matched a *C. reinhardtii* counterpart, the analysis allowed the unambiguous identification of 16 of the 17 polypeptides that constitute the mitochondrial ATP synthase of the colorless alga. The N-terminal sequence of an 8 kDa subunit, upon BLAST analysis, revealed a *C. reinhardtii* homolog, (gene identifier estExt_fgenesh2_pg.C_250059). This novel polypeptide was named ASA8.

Some minor differences were found between the *Chlamydomonas* and *Polytomella* subunits. For example, *Polytomella* subunit ASA3 was found to be partially proteolyzed, since its N-terminal sequence determined by Edman degradation is similar to a *C. reinhardtii* mature ASA3 subunit that would have lost its MTS plus 44 additional residues from its N-terminus.

The polypeptide patterns of the *Polytomella* sp. enzymes obtained from glycerol gradients and by BN-PAGE (by

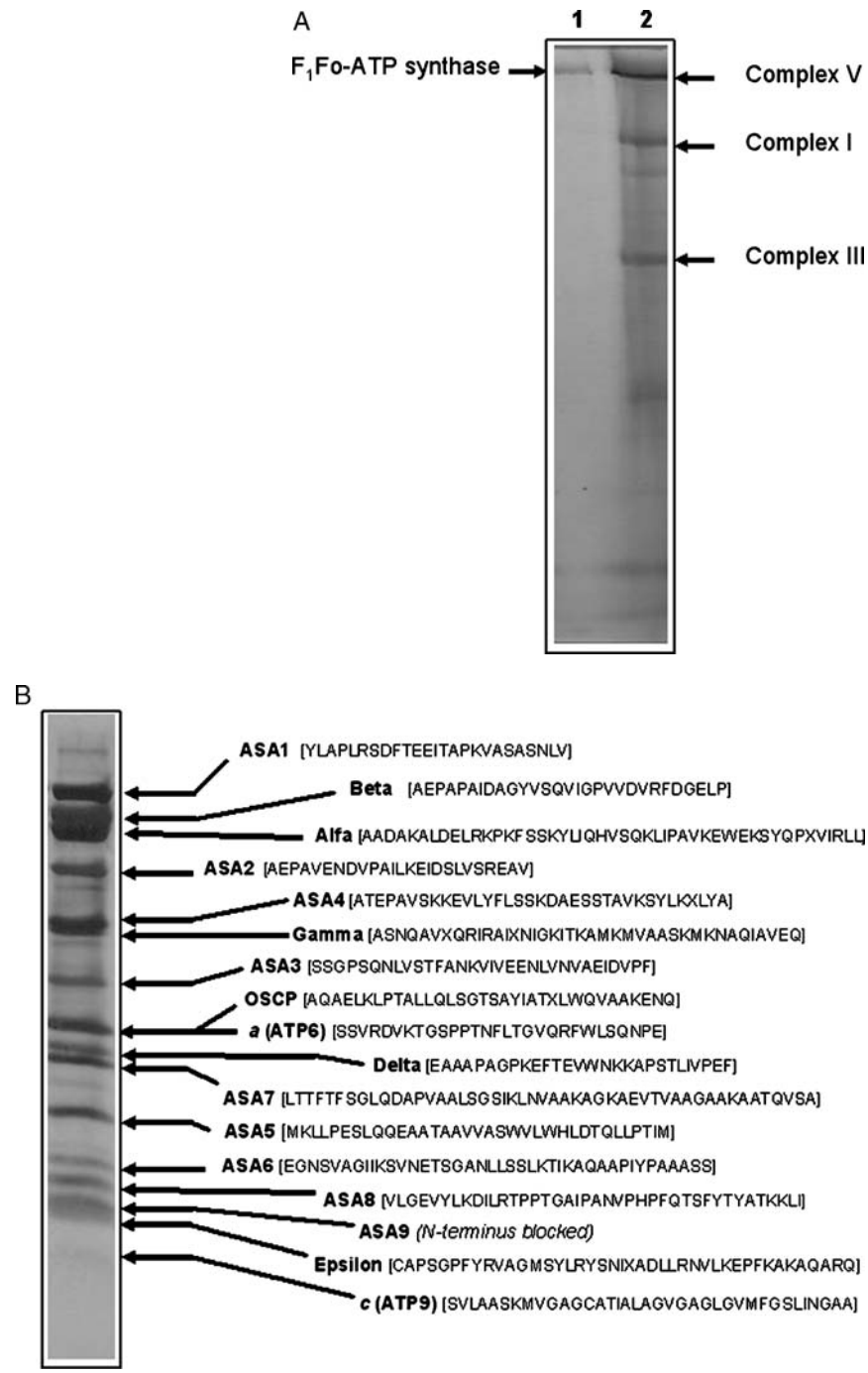

Fig. 1 *Coomassie Blue stained electrophoretic patterns of purified mitochondrial ATP synthase from Polytomella sp.* (A) Blue native-PAGE of purified ATP synthase obtained from glycerol gradients (30 μg of protein) (lane 1) and total mitochondrial proteins from *C. reinhardtii* (0.5 mg of protein) (lane 2). (B) The subunits of the *Polytomella* ATP synthase complex after electrophoresis through a Tricine-SDS polyacrylamide gel and transfer onto a PVDF membrane. The N-terminal sequences of the polypeptides were determined and the identified subunits are indicated. The sequences of the β and α subunits were taken from Atteia et al. (1994). The additional faint bands found in the preparation were considered to be contaminants and were not further explored

cutting the corresponding complex V band from the first dimensional gel and loading it onto SDS-PAGE) were compared and were found identical (data not shown). This suggests that ASA1 to 9 polypeptides are genuine components of ATP synthase, since they remain associated with the enzyme complex isolated by two different purification procedures.

Considering the theoretical molecular masses of the *Chlamydomonas* subunits, the observed apparent molecular mass of the *Polytomella* dimeric complex as judged by BN-PAGE (1600 kDa), is consistent with a stoichiometry $[\alpha_3/\beta_3/\gamma/\delta/\epsilon/a/c_{10}$/OSCP/ASA1/ASA2/ASA3/ASA4/ASA5/ASA6/ASA7/ASA8/ASA9] for each monomer, with a theoretical molecular mass of 787.9 kDa (1575.8 kDa for the dimer). Altogether, the data suggest that the newly-identified polypeptides ASA2 to 8 are real subunits of the mitochondrial ATP synthase of the colorless alga *Polytomella* sp. The sequence of the *Polytomella* ASA9 subunit and the possible presence of a counterpart in *C. reinhardtii* remain to be explored.

Homologs of the genes encoding the *C. reinhardtii* ATP synthase subunits are also present in the genome of the chlorophycean alga *V. carterii*

The genome of the chlorophycean alga *V. carterii*, another close-relative of *C. reinhardtii*, has been subjected to a first round of sequencing using a shot-gun approach. Comparisons between *Chlamydomonas* and *Volvox* sequences are publicly available at the *C. reinhardtii* genome web page. We searched the *V. carterii* genome for homologs of the genes encoding the 16 subunits of the *C. reinhardtii* mitochondrial ATP synthase. *V. carterii* contains gene homologs encoding subunits α, β, γ, δ, ε, *a*, *c*, OSCP, and subunits ASA1 to 8 (data not shown). Of the two *c* (ATP9) isoforms, ATP9A and ATP9B described for *C. reinhardtii* (Cardol et al., 2005), only ATP9B has a counterpart in *V. carterii*. No homologs for the classical ATP synthase subunits, *b*, *d*, *e*, *f*, *g*, IF_1, A6L, and F6 could be found in the *Volvox* genome. Thus, the data indicate that the novel subunits ASA1 to 8 are characteristic of at least three chlorophycean algae: *C. reinhardtii*, *Polytomella* sp., and *V. carterii*. These findings led us to hypothesize that the presence of a highly-stable dimeric mitochondrial ATP synthase with an atypical subunit composition may be characteristic of the whole lineage of chlorophycean algae, and led us to explore additional algal species.

Highly stable dimeric mitochondrial ATP synthases are present in several algae from the chlorophycean lineage

The Chlorophyta lineage comprises four major classes of green algae with more than two thousand species: Chlorophyceae, Prasinophyceae, Trebouxiophyceae, and Ulvophyceae. We assumed that the presence of a stable dimeric complex V (with apparent molecular masses close to 1,600,000 Da) could represent mitochondrial ATP synthases with an atypical subunit composition. Since it is not feasible to purify the mitochondrial ATP synthase from all green algal species, we choose at least one representative from each class, and resolved their OXPHOS complexes by BN-PAGE from crude membrane fractions. Specific activity stainings were used for the detection of complexes I and V. Dimeric complex V, stained with Coomassie-Blue is visualized only for the Chlorophycean species. Figure 2 shows the electrophoretic patterns after BN-PAGE of solubilized complexes from six chlorophycean algae of various orders (*Scenedesmus obliquus*, *Chlorogonium elongatum*, *Chlorococcum ellipsoidum*, *Chlamydomonas moewusii*, *C. reinhardtii*, *and Chlamydomonas smithii*), the Trebouxyphycea *Chlorella vulgaris*, the Prasinophycea *Tetraselmis chui*, and the Ulvophycea *Pseudendoclonium basiliense*. The position of complex I (NADH- ubiquinone oxidoreductase, about 1000 kDa), detected as a control of the presence of

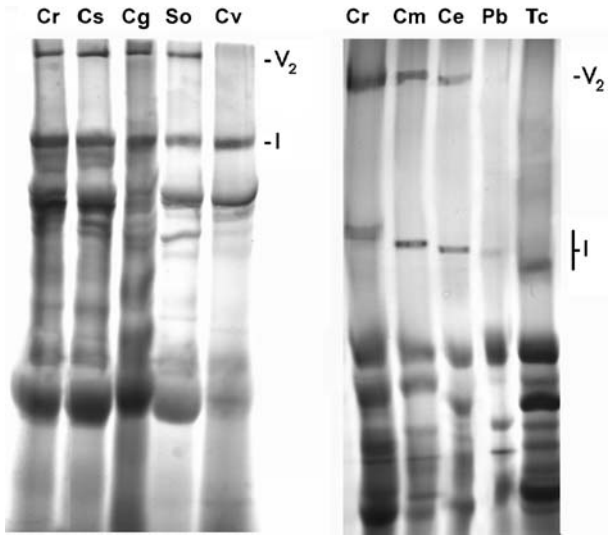

Fig. 2 *Coomassie-Blue stained image of the native membrane protein complexes from different Chlorophyta separated by BN-PAGE.* In each lane 120 μg of protein were loaded. The positions of mitochondrial dimeric complex V (V_2) and complex I (I) are indicated. Both complexes were identified by their specific activity stainings. Cr: *Chlamydomonas reinhardtii,* Cs: *Chlamydomonas smithii*; Cg: *Chlorogonium elongatum*; So: *Scenedesmus obliquus*; Cv: *Chlorella vulgaris*; Cm: *Chlamydomonas moewusii*; Ce: *Chlorococcum ellipsoidum*; Pb: *Pseudendoclonium basiliense*; Tc: *Tetraselmis chui*

respiratory-chain complexes in the protein extracts, is indicated on the figure. Only the chlorophycean algae showed the presence of dimeric complex V. No high molecular mass bands related to complex V and running above complex I could be identified in *C. vulgaris*, *T. chui*, or *P. basiliense*.

Table 2 summarizes the data concerning subunit composition of mitochondrial F_1F_O-ATP synthase among eukaryotes. Since the *b* (ATP4) and A6L (ATP8) subunits are absent from the three chlorophycean species *C. reinhardtii*, *Polytomella sp.* and *Volvox carterii* investigated in this work, we suggest that the presence of mitochondrial genes coding for these two subunits is a clue indicating the presence of a classical enzyme. Both the *atp4* and *atp8* genes are found in the mitochondrial genomes of non-chlorophycean green algal classes and of higher plants. Although specific subunits remain to be genetically or biochemically identified, the available data suggest that subunits ASA1 to 8 are components characteristic of the chlorophycean algae and responsible for the highly-stable dimeric form of the enzyme in this lineage.

Some of the novel subunits ASA1 to 9 are constituents of the peripheral stator-stalk of *Polytomella* sp. mitochondrial ATP synthase, while others may participate in its dimerization

To test the stability of the purified *Polytomella* mitochondrial ATP synthase, the enzyme was subjected to incubation at 60 °C for various periods of time. Samples were subjected

Table 2 The subunit composition of mitochondrial F1Fo-ATP synthase from various organisms is indicated. Subunits present in an organism are marked as (●) if mitochondria encoded or as (○) if nucleus encoded. Missing subunits are marked as (–). Blank spaces indicate that neither biochemical nor molecular genetic evidence for the presence or for the absence of a particular subunit is available. Subunits marked in bold over gray background are the ones conserved in all mitochondrial F_1F_O-ATP synthases. Except for *V. carterii* (*V.c.*) and *Polytomella* sp. (*P.s.*), representative species whose mitochondrial genomes have been fully sequenced were chosen to construct this table. Therefore, some algal species differ from those used in Fig. 2. Abbreviations (and accesion numbers of the mtDNA sequences): *B.t., Bos taurus* (NC_006853)*; S.c., Saccharomyces cerevisiae* (NC_001224)*; A.t., Arabidopsis thaliana* (NC_001284)*; N.o., Nephroselmis olivacea* (AF110138)*; P.w., Prototheca wickerhamii* (NC_001613)*; P.a., Pseudodendoclonium akinetum* (NC_005926)*; S.o., Scenedesmus obliquus* (NC_002254)*;* and *C.r., Chlamydomonas reinhardtii* (NC_001638). The mtDNA sequences of *Polytomella* sp. and *V. carterii* are assumed to be highly similar to the ones of *Polytomella parva* (AY062933) and *C. reinhardtii* respectively.

	Metazoa	Fungi	Viridiplantae							
			Streptophyta	Chlorophyta						
				Prasinophyceae	Trebouxiophyceae	Ulvophyceae	Chlorophyceae			
Subunit Name	*B.t.*	*S.c.*	*A.t.*	*N.o.*	*P.w.*	*P.a.*	*S.o.*	*C.r.*	*P.s.*	*V.c.*
α (ATP1)	○	○	●	●	●	●		○	○	○
β (ATP2)	○	○	○					○	○	○
γ (ATP3)	○	○	○					○	○	○
δ (ATP16)	○	○	○					○	○	○
ε (ATP15)	○	○	○					○	○	○
OSCP (ATP5)	○	○	○					○	○	○
A6L (ATP8)	●	●	●	●	●	●		-	-	-
F6	○	○	-					-	-	-
IF_1	○	○	○					-	-	-
F_Ad (24 kDa)	-	-	○					-	-	-
***a* (ATP6)**	●	●	●	●	●	●	●	○	○	○
b (ATP4)	○	○	●	●	●	●		-	-	-
***c* (ATP9)**	○	●	●	●	●	●	●	○	○	○
d	○	○	○					-	-	-
e	○	○	○					-	-	-
f	○	○	○					-	-	-
g	○	○	○					-	-	-
ASA1	-	-	-					○	○	○
ASA2	-	-	-					○	○	○
ASA3	-	-	-					○	○	○
ASA4	-	-	-					○	○	○
ASA5	-	-	-					○	○	○
ASA6	-	-	-					○	○	○
ASA7	-	-	-					○	○	○
ASA8	-	-	-					○	○	○
ASA9	-	-	-						○	

to BN-PAGE followed by 2D SDS-PAGE. Figure 3A shows a typical pattern of dissociation of the enzyme. The high molecular mass species corresponded to the intact, dimeric enzyme of 1,600 kDa. After 20 s of incubation, a second band with an estimated apparent molecular mass of 800 kDa appeared; followed by a pair of bands around 400 kDa and 200 kDa that appeared between 30 and 40 s, and several additional bands of 70 kDa or less that increased after longer times of incubation at 60 °C. Complex I present as a residual contaminant in this particular preparation disappeared after 30 s incubation at 60 °C. Analysis by 2D SDS-PAGE of the polypeptide pattern generated by each band allowed its identification (Fig. 3B). The 800 kDa band gave rise to a polypeptide pattern identical to the one of 1,600 kDa, suggesting that in short incubation times at high temperature, the dimeric ATP synthase dissociates into two monomers. Longer times of incubation gave rise to soluble F_1 (400 kDa band) formed by subunits $[\alpha_{3/}\beta_{3/}\gamma/\delta]$ (Fig. 3C). In some experiments, subunit ϵ remained attached to the F_1 subcomplex. In contrast, the 200 kDa band resolved into five polypeptides. Four of them could be readily identified by their mobility in SDS denaturing gels: ASA1, ASA3, ASA5, ASA8, and *c* (ATP9) (Fig. 3C). The fifth subunit could correspond to either OSCP or *a* (ATP6), since they both exhibit the same mobility in the

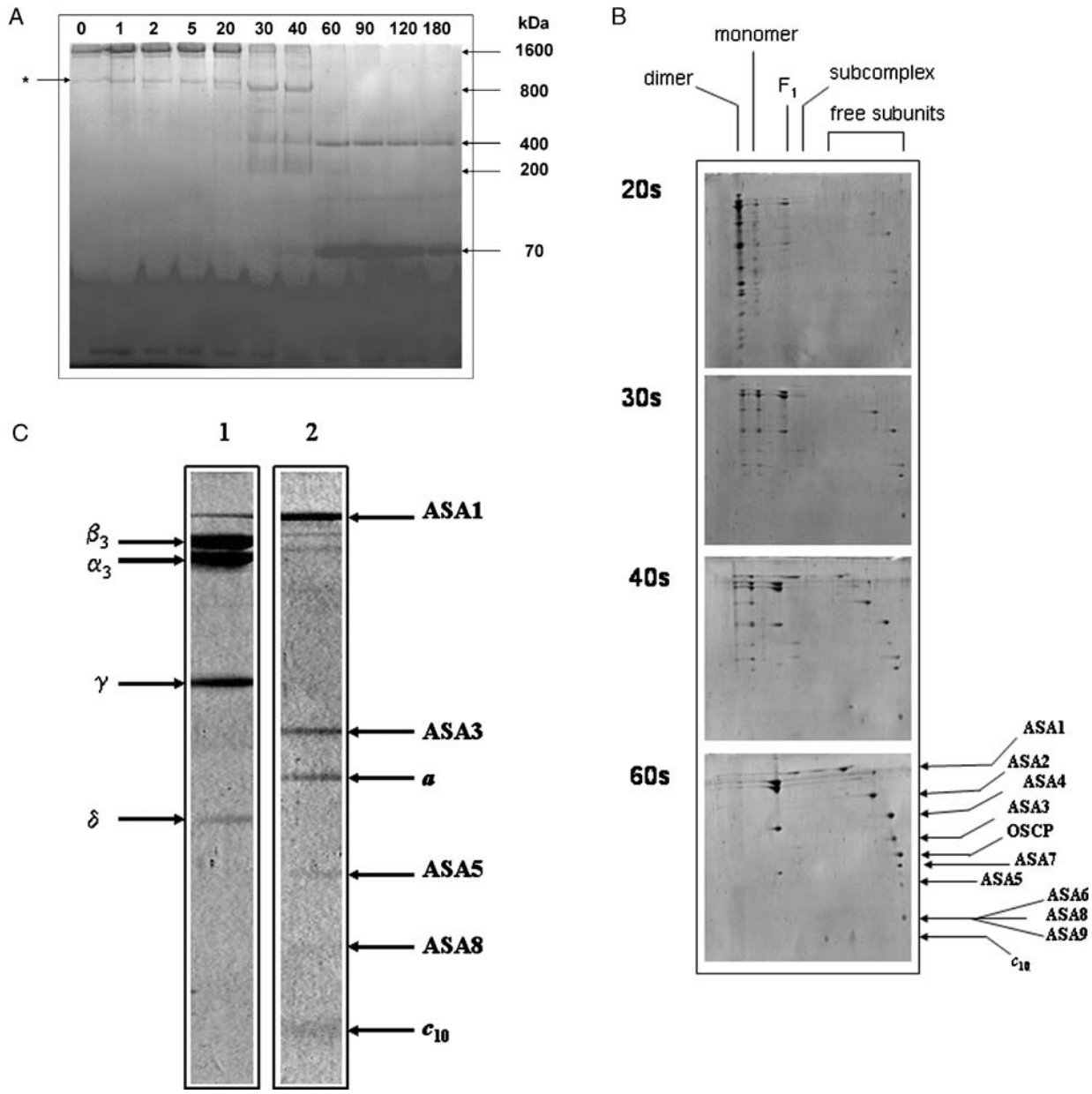

Fig. 3 *Electrophoretic patterns obtained for the time course of heat-induced dissociation of the isolated ATP synthase from Polytomella sp.* (A) Blue native-PAGE of purified ATP synthase incubated at 60 °C for the indicated periods of time (in seconds). The control without heating is labelled zero. The approximate apparent molecular masses of the identified complexes and subcomplexes are indicated. The asterisk indicates the presence of residual complex I in this particular ATP synthase preparation. (B) Two-dimensional resolution of the ATP synthase protein subcomplexes of samples obtained after different incubation times at 60° C. (C) Amplification of a 2D-Tricine-SDS gel showing the subunit composition of the two main subcomplexes generated during heat dissociation. Lane 1 shows the 400 kDa F_1 subcomplex, lane 2 the 200 kDa subcomplex

gel system used (see Fig. 1B). In order to ascertain the fifth subunit identity, a 2D gel was run and then transferred to a PVDF membrane. The band of interest was subjected to N-terminal sequence analysis. The sequence SSVRD allowed the unambiguous identification of the fifth component of the 200 kDa subcomplex as subunit *a* (ATP6). We thus conclude that subunits [ASA1/ASA3/ASA5/ASA8/*a*/c_{10}] form a transient 200 kDa subcomplex during the dissociation process of the enzyme at high temperatures. In contrast, subunits ASA2, ASA4, ASA6, ASA7 and ASA9 readily dissociated from the ATP synthase complex and migrated independently towards the front of the first dimension Blue Native gel.

To gain further insights into the vicinity and the possible interactions of subunits ASA1 to 9, purified *Polytomella* ATP synthase was incubated with the water-soluble,

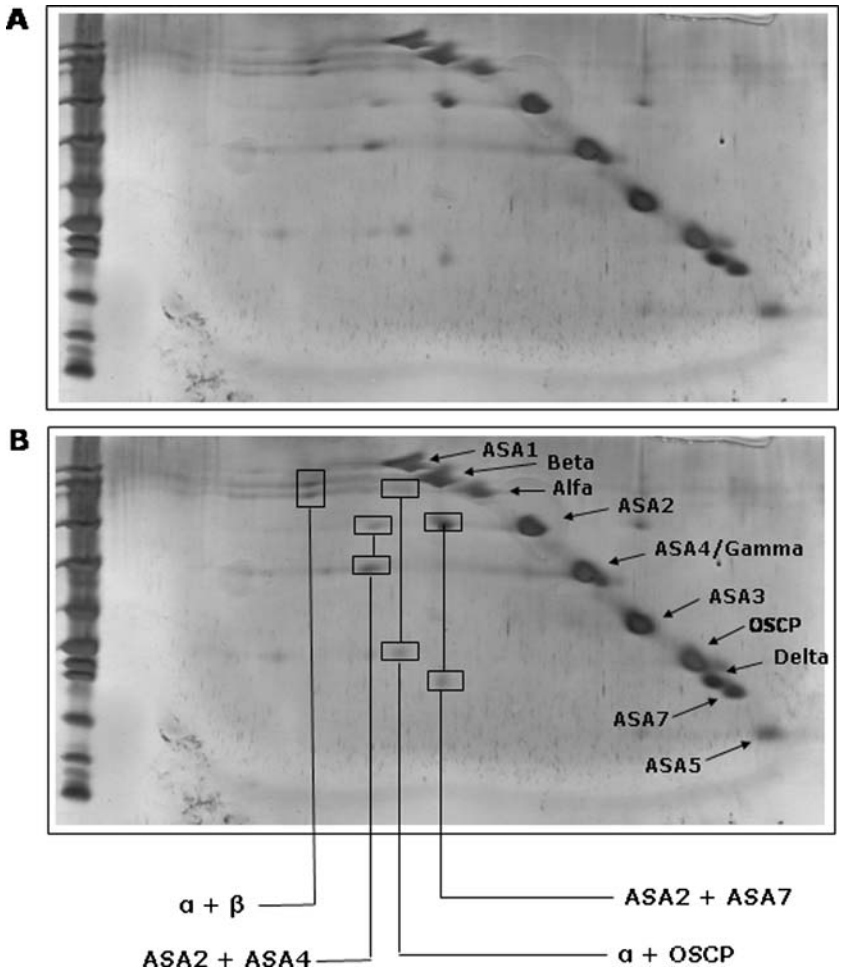

Fig. 4 *Second dimensional analysis of the Polytomella ATP synthase subunits cross-linked with DTSSP. Polytomella* ATP synthase treated with the cross-linking agent was resolved in a Tricine-SDS gel in non-reducing conditions (100 μg of protein). The 1D gel was then incubated in the presence of DTT to release the cross-linked products and subjected to 2D Tricine-SDS-PAGE and silver staining. Panel A shows the original 2D gel. Panel B shows the same gel, indicating the intact ATP synthase subunits that run on the diagonal. The identified cross-linked subunits, released upon DTT treatment and running out of the diagonal, are indicated by boxes and connecting lines

homobifunctional, thiol-cleavable reagent DTSSP (containing a spacer arm of 12 Å). The cross-link products were then released in the presence of DTT in order to analyze the resulting polypeptide pattern with 2D-Tricine-SDS gels. Other than the expected major cross-link product between subunits α and β, three additional interactions were revealed: ASA2 with ASA4, α with OSCP, and ASA2 with ASA7 (Fig. 4).

Discussion

In this work, we demonstrate that the eight novel subunits (ASA1 to 8), absent from the typical mitochondrial ATP synthase, are genuine components of the enzyme in at least three members of the chlorophycean lineage. In contrast, subunits A6L (ATP8), F6, IF_1, *b*, *d*, *e*, *f*, and *g* typical of ATP synthase, are absent from the three algal enzymes. Recently, the structures of two dimeric forms of the ATP synthase were obtained by EM studies. Beef heart dimeric ATP synthase purified from digitonin-solubilized mitochondria showed associations between its monomers at the level of both the F_1 and Fo sectors, with a closer packing in the Fo-Fo interface (Minauro-Sanmiguel et al., 2005). In contrast, the dimeric ATP synthase of the alga *Polytomella* sp. exhibited two large, protruding arms that extend from the membrane to the upper regions of the F_1 moieties (Dudkina et al., 2005). Based on the results described here, the large structural differences observed between the beef and algal dimeric enzymes should be ascribed to the different polypeptides, other than the catalytic core [α_3/β_3], that constitute the fixed subunits of the enzyme [*a*/*b*/OSCP/A6L/F_6/*d*/*e*/*f*/*g*] in bovine, versus [*a*/OSCP/ASA1/ASA2/ASA3/ASA4/ASA5/ASA6/ASA7/ASA8/ASA9] in the alga. The different subunit compositions imply that the molecular mass of the algal fixed subunits (560.5 kDa) is twice that of the beef dimeric enzyme (272.1 kDa). The temperature-dissociation and cross-linking experiments carried out with the isolated *Polytomella* ATP synthase, reinforce the idea that some of the novel subunits are the building blocks of the stator-stalk, while others are involved in the dimerization of the complex. Upon incubation at high temperature for short periods of time, the enzyme

dissociated into its monomers and subsequently into two large subcomplexes: the 400 kDa F_1 moiety [$\alpha_{3/}\beta_{3/}\gamma/\delta$], and the 200 kDa subcomplex [ASA1/ASA3/ASA5/ASA8/a/c_{10}]. In addition, subunits ϵ, ASA2, ASA4, OSCP, ASA6, ASA7, and ASA9 seem to dissociate independently. Nevertheless, cross-linking experiments suggest that subunits ASA2, ASA4, and ASA7 are in close proximity and may form their own subcomplex. Each of the two long protruding arms of the *Polytomella* ATP synthase was interpreted to be formed by the dimer-specific component MASAP (ASA1), and a classical peripheral stalk (Dudkina et al., 2005). However, we clearly demonstrate here that the classical peripheral stalk components are absent in chlorophycean algae. Therefore, the structure of the *Polytomella* ATP synthase dimer observed by EM must be reinterpreted. Most probably, the two F_O moieties are held together through their *a* subunits by subcomplex [ASA1/ASA3/ASA5/ASA8], while the protruding arms formed by two [ASA2/ASA4/ASA7/OSCP] subcomplexes are the stator-stalks that stabilize the F_1 sectors (see models in Fig. 5).

The drastic modification in composition and architecture of a highly-conserved, universally-distributed energy transducing enzyme, that is found exclusively in a particular lineage of eukaryotes, is remarkable. For yet unknown evolutionary reasons, chlorophycean algae have recruited the eight novel polypeptides ASA1 to 8 to its mitochondrial ATP synthase complex, while keeping the orthodox, universal components in its catalytic core [$\alpha_{3/}\beta_3$], in its rotary central-stalk [$c_{10}/\gamma/\delta/\epsilon$], and in the two subunits that are the binding sites for the peripheral stator-stalk (*a* and OSCP). Notably, subunit *a* and OSCP of the algae are the polypeptides that exhibit the least sequence similarity when compared with their higher plant counterparts (data not shown). Very likely, their sequences have been modified in order to efficiently interact with the new stator-stalk components (at least, with subunits ASA2, ASA4 and ASA7). Apparently, during the course of evolution, chlorophycean algae have designed a novel ATP synthase molecular motor, in which the peripheral stator-stalk is built with different components in a rather unique architecture, albeit the basic rotary and catalytic elements are conserved. This type of structural transformation in an oligomeric complex is, to our knowledge, without precedent in enzymology.

The dimeric nature of chlorophycean mitochondrial ATP synthase and mitochondrial cristae morphology

Mitochondrial dimeric F_1F_O-ATP synthase of *C. reinhardtii* behaves as a highly-stable dimer, even when it is solubilized with relatively high concentrations of n-dodecyl-β-maltoside (van Lis et al., 2003). It requires incubation at high-temperature in order to dissociate. The presence of some of the novel subunits (like ASA1 and ASA3) seems to be responsible for the extraordinary stability of the enzyme.

Eukaryotic organisms may be divided into three groups according to the morphology of their mitochondrial cristae: (*i*) discoidal as in trypanosomatids, (*ii*) flattened as in several fungi, and (*iii*) tubular as in photosynthetic organisms and in many protists (Gray et al., 1998). When dimerization of the yeast mitochondrial ATP synthase is abolished by deletion of the genes encoding the *e* and *g* subunits, the mutants exhibit mitochondria with altered cristae morphology (Arselin et al., 2004). Remarkably, the structures of the dimeric enzymes of both beef (Minauro-Sanmiguel et al., 2005) and *Polytomella* (Dudkina et al., 2005) show a sharp angle between the two F_O sectors of each monomer, which is thought to induce a curvature in the inner membrane and thus contribute to mitochondrial cristae morphology. A recent EM study of ultrathin sections of *Polytomella* mitochondria indicated that ATP synthase seems to arrange in a helical fashion in the tubular-shaped cristae membranes of this colorless alga (Dudkina et al., 2006). With notable exceptions, mitochondrial ATP synthases from chlorophycean algae are "all nucleus-encoded" OXPHOS complexes.

In the majority of the mitochondrial DNAs (mtDNA) of the chlorophycean algae characterized to date – *Chlamydomonas eugametos* (Denovan-Wright and Lee, 1994) [AF008237], *Chlamydomonas reinhardtii* (Boer and Gray, 1988; Michaelis et al., 1990) [U03843], *Chlorogonium elongatum* (Kroymann and Zetsche, 1998) [Y07814, Y13643, and Y13643], and *Polytomella parva* (Fan and Lee, 2002) [AY062933, AY062934] – all genes encoding ATP synthase subunits (*atp* genes) are absent. However, there are two notable exceptions: the mtDNA of *Scenedesmus obliquus* (Kück et al., 2000; Nedelcu et al., 2000) [AF204057] has retained the genes *atp6* and *atp9*, and the mtDNA of *Pedinomonas minor* (Turmel et al., 1999) [AF116775] has retained *atp6* and *atp8*. Nevertheless, these two algae exhibit unique mitochondrial genetic codes, and are therefore thought to be evolutionary intermediates that interrupted prematurely the migration of mitochondrial genes to the nucleus, due to codon code incompatibilites (Kück et al., 2000; Nedelcu et al., 2000; Funes et al., 2002b). In addition, the *atp8* gene found in the *P. minor* mtDNA encodes an extremely small A6L subunit (of only 49 residues) as compared to the 134 residues or more in the rest of Viridiplantae (Gray et al., 1998); it is therefore a pseudogene, or a gene that expresses a truncated polypeptide. Aside from *S. obliquus* and *P. minor,* it may be assumed that the vast majority of the chlorophycean ATP synthases have become "all nucleus-encoded" H^+ translocating complexes. No other eukaryotic organism exhibits this remarkable property, except the apicomplexan parasites, which also lack all *atp* genes in their mtDNAs (Feagin, 2000), but whose mitochondrial ATP synthases remain to be characterized.

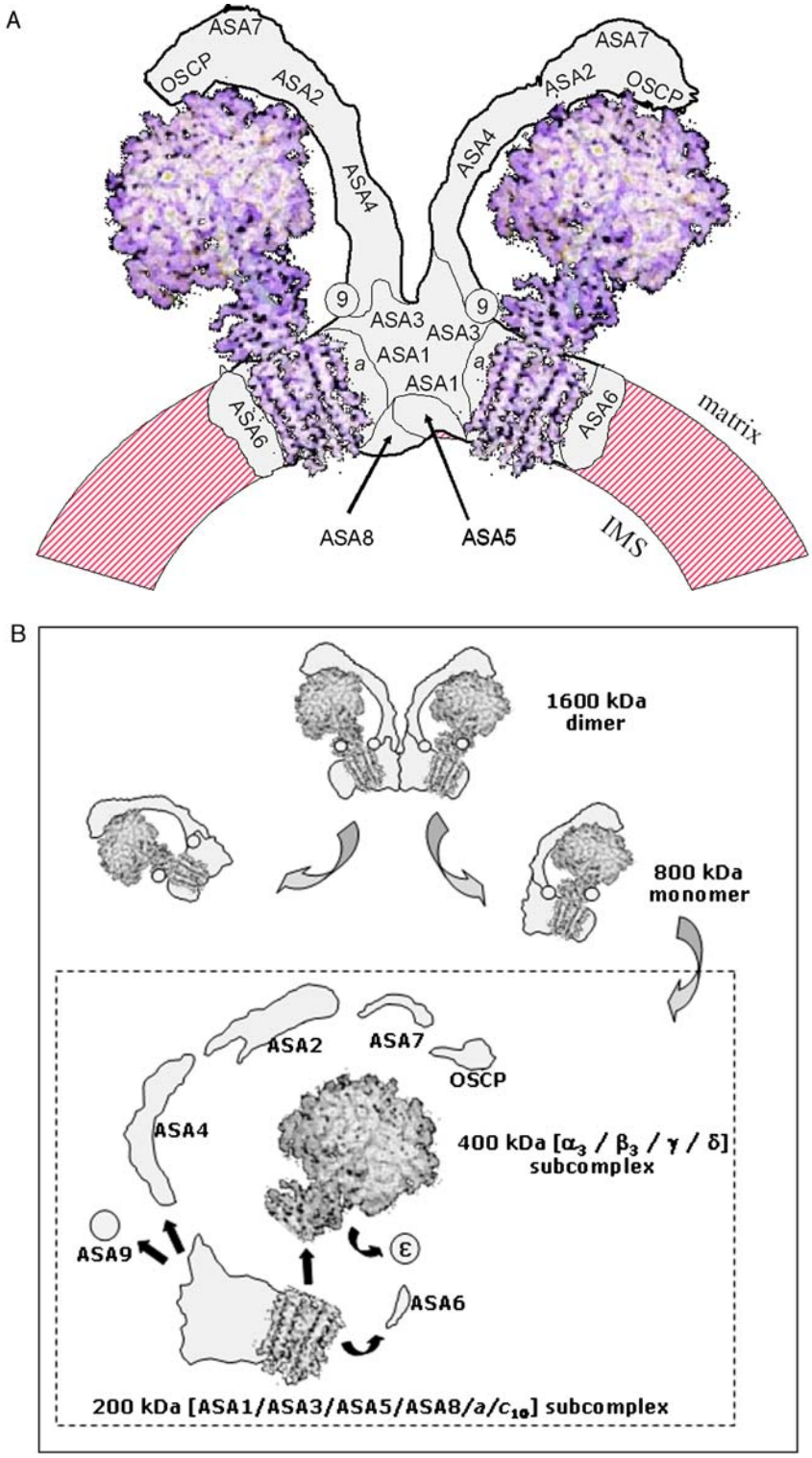

Fig. 5 *Models for the arrangement of subunits in the mitochondrial F1Fo-ATP synthase of Polytomella sp. and for the dissociation of the complex after heat treatment*. (A) Subunit arrangement of the dimeric algal mitochondrial ATP synthase. The model is based on the overall structure of the complex found in the EM study of Dudkina et al. (2005), and on the data obtained from the heat treatment dissociation assays and cross-linking experiments (this work). For illustration purposes, the F_1F_O moiety comprising subunits [$\alpha_3/\beta_3/\gamma/\delta/\epsilon/c_{10}$] corresponds to the yeast crystallographic model obtained by Stock et al. (1999). Subunit ASA6 is shown as an integral membrane protein, since hydrophobic analysis suggests the presence of one transmembrane helix. Number 9 indicates subunit ASA9. Subunits ASA8 and ASA5 are shown located towards the intermembrane space (IMS), since both polypeptides lack a MTS, and are therefore potential candidates to be inserted through the IMS. (B) Model for the sequential dissociation of mitochondrial ATP synthase from *Polytomella* sp. The subunit composition of the two subcomplexes was inferred from the 2D gel patterns shown in Fig. 3

Concluding remarks

The loss of the subunits involved in the dimerization of the mitochondrial ATP synthase, the loss of its classical stator-stalk constituents, the appearance of the nucleus-encoded polypeptides ASA1 to 8, and the loss of the mitochondrial genes encoding ATP synthase components, are all characteristic of several chlorophycean algae. Therefore, it may be hypothesized that at some point in evolution eight novel polypeptides were recruited to the mitochondrial ATP synthase of these algae. The integration of these new constituents to the enzyme may have brought evolutionary advantages that eventually led to the loss of the genes encoding the original components of the complex (subunits *b*, *d*, *e*, *f*, *g*, F6, F_Ad, IF_1 and A6L, or those that were actually present in the ancestor of the chlorophycean lineage). The evolutionary origin of the novel chlorophycean ATP synthase subunits ASA1 to 8 still remains obscure, as well as the way the corresponding genes were acquired: either by duplication of pre-existing genes or by horizontal gene transfer. Whatever their origin may be, these subunits are probably essential constituents of mitochondrial F_1Fo-ATP synthase in chlorophycean algae. To our knowledge, this is the only example of an oligomeric enzyme that has recruited new non-conserved subunits, thus largely modifying the structural features of its scaffold, while conserving almost intact the structure of its catalytic subunits. In addition, while the vast majority of mitochondrial ATP synthases are invariably encoded by both nuclear and organellar genomes, to date, the chlorophycean enzyme is a unique example of an "all nucleus-encoded" H^+ translocating OXPHOS complex.

Acknowledgements The authors wish to thank Dr. J. d'Alayer (Institut Pasteur) for his expert help in sequencing peptides. The authors are grateful Drs. G. Dreyfus, D. Georgellis and A. Gómez-Puyou (IFC, UNAM), to Dr. D. W. Krogmann (Purdue University), and to Dr. R. F. Matagne (University of Liège) for their critical comments on the manuscript. This research was supported by grants TW01176 from the Fogarty International Center at NIH (USA); HL59646 from NHLBI, NIH, (USA); 40696-Q from CONACyT (Mexico) IN218705-3 from DGAPA, UNAM (Mexico) and by grants from the Belgian FRFC (2.4587.04, 2.4582.05 and F.4735.06). P.C. is a postdoctoral researcher of the FNRS, Belgium. M.L. is a Fellow of FRIA, Belgium.

References

Alfonzo M, Kandrach MA, Racker E (1981) J Bioenerg Biomembr 13:375–391
Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) J Mol Biol 215:403–410
Arnold I, Pfeiffer K, Neupert W, Stuart RA, Schägger H (1998) EMBO J 17:7170–7178
Arselin G, Vaillier J, Salin B, Schaeffer J, Giraud MF, Dautant A, Brethes D, Velours J (2004) J Biol Chem 279:40392–40399
Atteia A (1994) C R Acad Sci III 317:11–19
Atteia A, Dreyfus G, González-Halphen D (1997) Biochim Biophys Acta 1320:275–284
Boer PH, Gray MW (1988) Curr Genet 14: 583–590
Bradford MM (1976) Anal Biochem 72:248–254
Brunner S, Everard-Gigot V, Stuart RA (2002) J Biol Chem 277:48484–48489
Cardol P, González-Halphen D, Reyes-Prieto A, Baurain D, Matagne RF, Remacle C (2005) Plant Physiol 137:447–459
Denovan-Wright EM, Lee RW (1994) J Mol Biol 241:298–311
Dudkina NV, Heinemeyer J, Keegstra W, Boekema EJ, Braun HP (2005) FEBS Lett 579:5769–5772
Dudkina NV, Sunderhaus S, Braun HP, Boekema EJ (2006) FEBS Lett 580:3427–3432
Eubel H, Heinemeyer J, Braun HP (2004) Plant Physiol 134:1450–1459
Fan J, Lee RW (2002) Mol Biol Evol 19:999–1007
Feagin JE (2000) Int J Parasitol 30:371–390
Franzén L-G, Falk G (1992) Plant Mol Biol 19:771–780
Fronzes R, Weimann T, Vaillier J, Velours J, Brethes D (2006) Biochemistry 45:6715–6723
Funes S, Davidson E, Claros MG, van Lis R, Pérez-Martínez X, Vázquez-Acevedo M, King MP, González-Halphen D (2002a) J Biol Chem 277:6051–6058
Funes S, Davidson E, Reyes-Prieto A, Magallon S, Herion P, King MP, González-Halphen D (2002b) Science 298:2155
Gledhill JR, Walker JE (2005) Biochem J 386:591–598
Gorman DS, Levine RP (1965) Proc Natl Acad Sci (USA) 54:1665–1669
Golden TR, Pedersen PL (1998) Biochemistry 37:13871–13881
Gray MW, Lang BF, Cedergren R, Golding GB, Lemieux C, Sankoff D, Turmel M, Brossard N, Delage E, Littlejohn TG, Plante I, Rioux P, Saint-Louis D, Zhu Y and Burger G (1998) Nucleic Acids Res 26:865–878
Groth G, Walker JE (1996) Biochem J 318:351–357
Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Millar AH (2004) Plant Cell 16:241–256
Horvath A, Kingan TG, Maslov DA (2000) J Biol Chem 275:17160–17165
Itoh H, Takahashi A, Adachi K, Noji H, Yasuda R, Yoshida M, Kinosita K (2004) Nature 427:465–468
Jänsch L, Kruft V, Schmitz UK, Braun HP (1996) The Plant J 9:357–368
Ko YH, Hullihen J, Hong S, Pedersen PL (2000) J Biol Chem 275:32931–32939
Kroymann J, Zetsche K (1998) J Mol Evol 47:431–440
Kück U, Jekosch K, Holzamer P (2000) Gene 253:13–18
Markwell MAK, Hass SM, Biber LL, Tolbert NE (1978) Anal Biochem 87:206–210
Michaelis G, Vahrenholz C, Pratje E (1990) Mol Gen Genet 223:211–216
Minauro-Sanmiguel F, Wilkens S, García, JJ (2005) Proc Natl Acad Sci (USA) 102:12356–12358
Nedelcu AM, Lee RW, Lemieux C, Gray MW, Burger G (2000) Genome Res 10:819–831
Nurani G, Franzén, L-G (1996) Plant Mol Biol 6:1105–1116
Schägger H (2002) Biochim Biophys Acta 1555:154–159
Schägger H (1994) In A Practical Guide to Membrane Protein Purification. In von Jagow G, Schägger H (eds) Academic Press, San Diego, pp. 59–166
Stock D, Leslie AG, Walker JE (1999) Science 286:1700–1705
Turmel M, Lemieux C, Burger G, Lang BF, Otis C, Plante I, Gray MW (1999) Plant Cell 11:1717–1730
van Lis R, Atteia A, Mendoza-Hernández G, González-Halphen D (2003) Plant Physiol 132:318–330
van Lis R, González-Halphen D, Atteia A (2005) Biochim Biophys Acta 1708:23–34
Walker JE, Dickson VK (2006) Biochim Biophys Acta 1757:286–296