J Bioenerg Biomembr (2009) 41:1–13
DOI 10.1007/s10863-009-9203-0

The fully-active and structurally-stable form of the mitochondrial ATP synthase of *Polytomella* sp. is dimeric

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Received: 1 February 2009 / Accepted: 5 February 2009 / Published online: 26 February 2009

Abstract Mitochondrial F_1F_O-ATP synthase of chlorophycean algae is a stable dimeric complex of 1,600 kDa. It lacks the classic subunits that constitute the peripheral stator-stalk and the orthodox polypeptides involved in the dimerization of the complex. Instead, it contains nine polypeptides of unknown evolutionary origin named ASA1 to ASA9. The isolated enzyme exhibited a very low ATPase activity (0.03 Units/mg), that increased upon heat treatment, due to the release of the F_1 sector. Oligomycin was found to stabilize the dimeric structure of the enzyme, providing partial resistance to heat dissociation. Incubation in the presence of low concentrations of several non-ionic detergents increased the oligomycin-sensitive ATPase activity up to 7.0–9.0 Units/mg. Incubation with 3% (*w*/*v*) taurodeoxycholate monomerized the enzyme. The monomeric form of the enzyme exhibited diminished activity in the presence of detergents and diminished oligomycin sensitivity. Cross-linking experiments carried out with the dimeric and monomeric forms of the ATP synthase suggested the participation of the ASA6 subunit in the dimerization of the enzyme. The dimeric enzyme was more resistant to heat treatment, high hydrostatic pressures, and protease digestion than the monomeric enzyme, which was readily disrupted by these treatments. We conclude that the fully-active algal mitochondrial ATP synthase is a stable catalytically active dimer; the monomeric form is less active and less stable. Monomer-monomer interactions could be mediated by the membrane-bound subunits ASA6 and ASA9, and may be further stabilized by other polypeptides such as ASA1 and ASA5.

Keywords Mitochondrial F_1F_O-ATP synthase · Dimeric complex V · Monomeric complex V · Chlorophycean algae · *Chlamydomonas reinhardtii* · *Polytomella* sp. · ASA subunits

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Introduction

Mitochondrial F_1F_O-ATP synthase (complex V), a key participant in oxidative phosphorylation, is a heteromultimeric protein complex embedded in the inner membrane. The enzyme is a proton-driven molecular motor that utilizes the electrochemical gradient generated by the respiratory

chain to synthesize ATP. Two of the best characterized ATP synthases, the beef heart enzyme and rat liver are comprised of at least 15 subunit types, i.e., α, β, γ, δ, ε, *a*, *b*, *c*, *d*, *e*, *f*, *g*, F_6, A6L, and OSCP. They may also bind one or more regulatory peptides, e.g., IF1 and factor b, under certain physiological conditions (Golden and Pedersen 1998; Ko et al. 2000; Walker and Dickson 2006; Hong and Pedersen 2008).

The mitochondrial ATP synthase from chlorophycean algae, like the one from *C. reinhardtii* and *Polytomella* sp., is a dimer of 1,600 kDa (van Lis et al. 2003; van Lis et al. 2005; Dudkina et al. 2005; Vázquez-Acevedo et al. 2006; van Lis et al. 2007). Electron microscopy studies evidenced the unique dimeric structure of the algal enzyme (Dudkina et al. 2005; Dudkina et al. 2006). The structure exhibits two robust peripheral stalks, not evident in the dimers of bovine or yeast ATP synthases (Minauro-Sanmiguel et al. 2005; Dudkina et al. 2006). In addition, the algal enzyme differs strongly in its polypeptide composition from the mitochondrial ATP synthases of the vast majority of eukaryotes. It only contains the eight orthodox subunits α, β, γ, δ, ε, *a* (ATP6), *c* (ATP9), and OSCP, and in addition, it has nine atypical polypeptides of unknown evolutionary origin named ASA1 to ASA9 (Cardol et al. 2005; Vázquez-Acevedo et al. 2006). Subunits ASA1 to ASA9 are thought to substitute for subunits *b*, *d*, *e*, *f*, *g*, IF_1, A6L, and F_6, which in the conventional enzymes are involved in the formation of the peripheral stalk (*b*, *d*, *f*, A6L, and F_6), in the dimerization of the complex (*e* and *g*), and in the regulation of the enzyme activity (IF_1). Therefore, some of the ASA subunits are thought to be the building blocks of a distinct peripheral stalk, others may participate in the dimerization of the complex, and others may have a yet unknown regulatory function (Vázquez-Acevedo et al. 2006; van Lis et al. 2007).

In this work, we explored the ATP hydrolytic activity of the mitochondrial ATP synthase purified from *Polytomella* sp. The isolated enzyme exhibited a very low ATPase activity that increased upon heat treatment, due to its dissociation and the concomitant release of the F_1 sector. Oligomycin was found to stabilize the dimeric structure of the enzyme, providing partial resistance to heat dissociation. Incubation in the presence of several non-ionic detergents increased the ATPase activity up to 7.0–9.0 Units/mg. The detergent-activated enzyme is fully sensitive to oligomycin and is readily inactivated by heat treatment. It was previously shown that upon incubation at 60°C for short periods of time the mitochondrial ATP synthase purified from *Polytomella* sp. dissociated into monomers, and subsequently disassembled into F_1 moieties and free polypeptides (Vázquez-Acevedo et al. 2006).

The role of monomeric versus dimeric forms of ATP synthases remains obscure. Here, it was found that incubation with 3% taurodeoxycholate (TDOC) in ice for 30 min also monomerized the algal ATP synthase, a process that was ocassionally accompanied by the partial release of some of the small, membrane-bound subunits. Once the algal ATP synthase was obtained in a predominant monomeric form, the properties of the monomeric and dimeric enzymes were compared. The effects of heat treatment, high hydrostatic pressure, and protease digestion upon the monomeric and dimeric forms of the enzyme were assayed. While the dimeric enzyme was relatively resistant to different treatments, the monomeric enzyme was found to be extremely prone to dissociation. The data suggest that the fully functional form of the enzyme is dimeric, and that the monomeric form represents a labile form of the enzyme with diminished ATPase activity and diminished oligomycin sensitivity. The data also suggest that monomer-monomer interactions are mainly mediated by several small, membrane-bound subunits (ASA6 and ASA9) that may be further stabilized by larger polypeptides like ASA1 and ASA5.

Materials and methods

Algal strains and growth conditions Polytomella sp. (198.80, E.G. Pringsheim) was grown as previously described (van Lis et al. 2005).

Detergents Octaethylene-glycoldodecyl ether ($C_{12}E_8$) and n-dodecanoyl sucrose were obtained from Calbiochem. Lauryldimethylamine oxide (LDAO) was from Millmaster Onyx International (New Jersey, USA). Octanoyl-N-methylglucamide and Decanoyl-N-methylglucamide were obtained from Boehringer Mannheim. The rest of the utilized detergents were from Sigma.

Polytomella ATP synthase purification The procedure previously described was followed (Vázquez-Acevedo et al. 2006).

Protein analysis Proteins were solubilized in the presence of n-dodecyl-β-maltoside and subjected to BN-PAGE (Schägger 1994). BN-PAGE was usually carried out in 4%–12% acrylamide gradient gels. Denaturing SDS-Tricine-PAGE was carried out as in Schägger (1994). Protein concentrations were estimated according to Markwell et al. (1978). Cross-linking experiments were carried out with the water-insoluble, homo-bifunctional, thiol-cleavable reagent dithiobis(succinimidyl)propionate (DSP). The enzyme, in its dimeric or monomeric form (3 mg protein /ml), was incubated 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, in the

presence of 0.2 mM DSP, for 30 min, at 4 °C. The reaction was stopped by the addition of 25 mM Tris (pH 8.0) (final concentration). The sample (120 μg of protein) was subjected to SDS- Tricine-PAGE [7% (*w*/*v*) acrylamide] in non-reducing conditions. The lanes of interest were cut and incubated for 1 h 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-SDS-Tricine-PAGE [12% (*w*/*v*) acrylamide].

Chymotrypsin cleavage experiments were carried out at room temperature in a 1:100 ratio (mg protein of protease: mg protein ATP synthase), in a buffer containing 20 mM

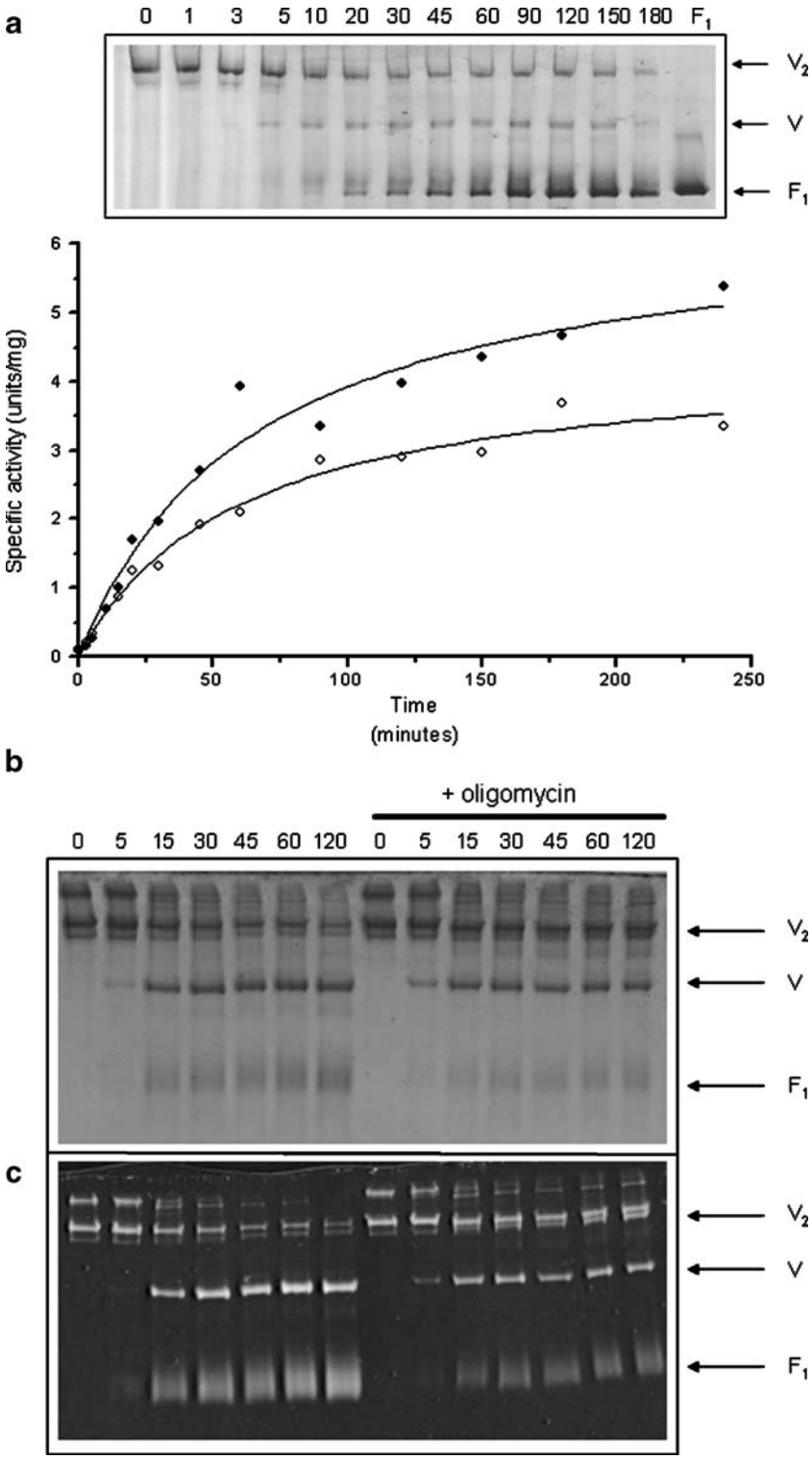


Fig. 1 *Heat activation and effect of oligomycin upon the heat-induced dissociation of the ATP synthase from Polytomella sp.* Panel A) ATPase activity of the purified algal ATP synthase as a function of time at 45 °C. Filled rhomboids, activity in the absence of oligomycin; open rhomboids, activity in the presence of oligomycin. The ATPase assay medium did not contain any added detergent. Inset: BN-PAGE showing samples retrieved from the time course of incubation at 45 °C. Numbers indicate time in minutes. Lane marked as F_1 was loaded with the algal enzyme incubated at 60 °C for 2 min. V_2 denotes the dimer, V the monomer, and F_1 the F_1 sector. Panel B) BN-PAGE showing samples retrieved from a time course of incubation (in minutes) of the algal ATP synthase at 37 °C in the absence and presence of oligomycin (preincubation for 30 min with 40 μg/ml). The positions of dimeric (V_2) and monomeric (V) complex V and of the F_1 subcomplex are indicated. 50 μg of protein were loaded in each lane. Panel C) In-gel ATPase activity staining of the gel shown in Panel B. Additional oligomycin was not added to the medium during the in-gel ATPase activity assay. 50 μg of protein were loaded in each lane. Dimer (V_2), monomer (V); F_1 sector (F_1)

Tris-HCl (pH 7.5), 2 mM ATP, 1 mM sodium EDTA and 0.1 mg/ml lauryl maltoside (LM). Protease cleavage was stopped by the addition of 1 mM phenylmethanesulphonyl-fluoride (PMSF) and 1 mM Nα-Tosyl-Lys-chloromethylketone HCl (TLCK) final concentrations.

ATPase activity measurements ATPase activity was measured spectrophotometrically following the oxidation of NADH at 340 nm, using the ATP regenerating system described by Pullman et al. (1960) with modifications. The assay medium contained 3 mM $MgSO_4$, 30 mM K_2SO_4, 25 mM Tris-sulfate (pH 7.4), 3 mM sodium ATP, 3 units of pyruvate kinase, 4.5 units of lactate dehydrogenase, 1.5 mM of phosphoenol pyruvate, and 0.3 mM NADH. Where indicated, detergents at different concentrations were added. ATPase activity is expressed in Units/mg (μmoles ATP hydrolyzed min^{-1} mg $protein^{-1}$). Usually, assays were carried out with 20 μg of protein. To assay oligomycin sensitivity, samples were preincubated 30 min in the presence of 10 μg/ml oligomycin. The inhibitor was also added to the assay medium at the same final concentration to attain maximal inhibition (98%). Preincubation alone in the presence of the inhibitor resulted only in a 50% loss of activity. DMSO was used as vehicle when adding the inhibitor.

In-gel ATPase activity ATPase activity was visualized after BN-PAGE (Zerbetto et al. 1997; Wittig and Schägger 2005). The gels were incubated for 3 h in a buffer containing 270 mM glycine and 35 mM Tris-HCl (pH 8.4). Then, the following reagents were added: 8 mM ATP, 14 mM $MgSO_4$, and 0.2% $Pb(NO_3)_2$ until the white precipitates of lead phosphate were visible. The reaction was stopped by adding 50% methanol.

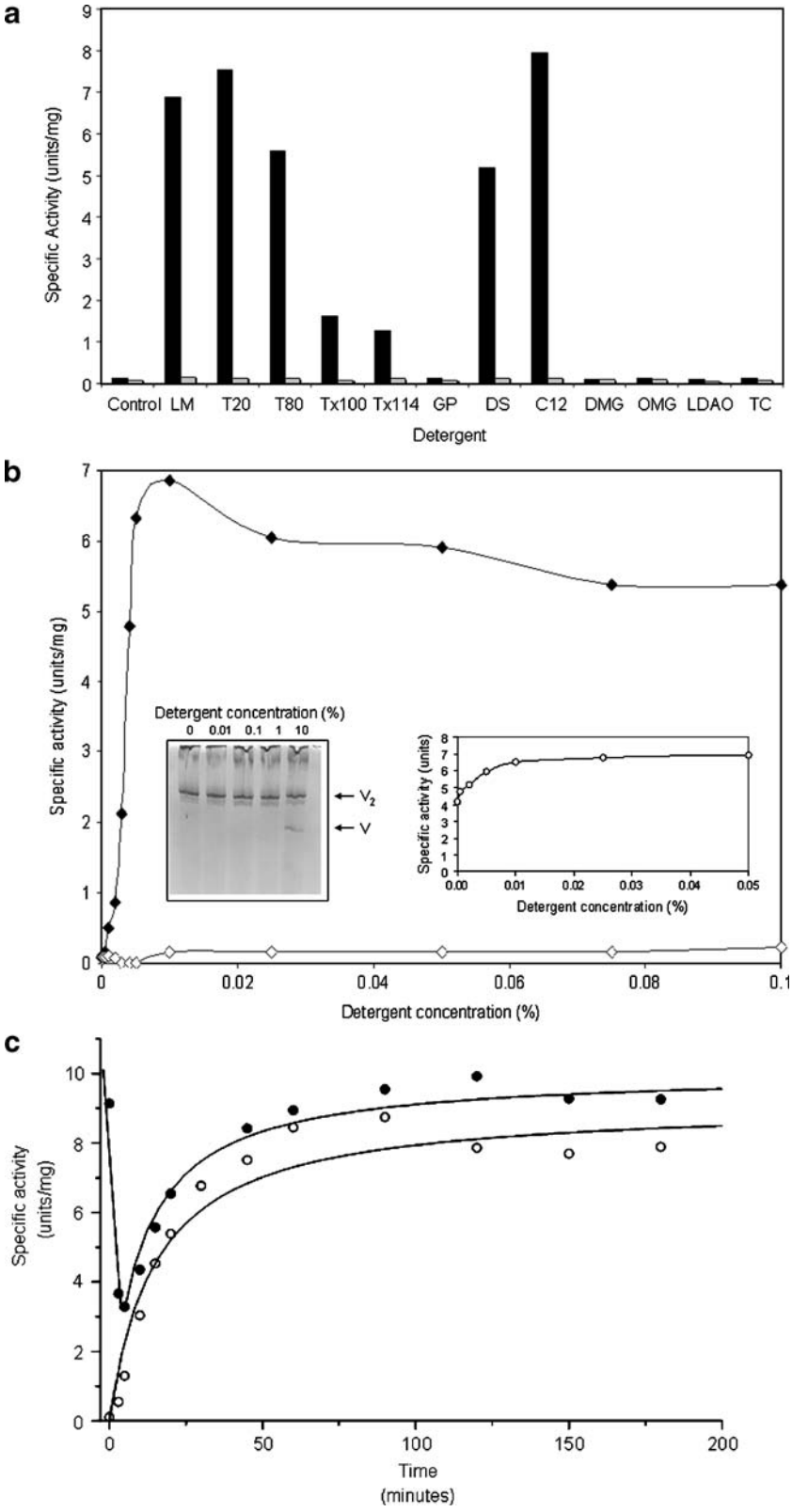
Compression of the algal mitochondrial ATP synthase Experiments were carried out in the high pressure pump previously described (Souza et al. 2004) at a constant temperature of 25 °C. Samples of the dimeric or monomeric algal ATP synthase (100 μg of protein) were compressed for 15 min at the indicated pressures. All samples (1 mg/ml of protein) were compressed in a buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM ATP, 1 mM sodium EDTA and 0.1 mg/ml LM.

Results

The mitochondrial ATP synthase from Polytomella sp., as isolated, exhibits a very low ATPase activity that increases upon heat treatment The algal enzyme, as obtained from the glycerol gradient centrifugation step (Vázquez-Acevedo et al. 2006), exhibits a very low ATPase activity, of 0.03 to 0.07 Units/mg, in the absence of detergent in the assay medium. The hydrolytic activity of the enzyme increased by heat treatment. Incubation at 45 °C activated the enzyme as a function of time accompanied by a concomitant loss of oligomycin sensitivity (Fig. 1A). In order to explore the changes in the oligomeric state of the complex, samples retrieved from a time-course of thermal activation were subjected to BN-PAGE. The electrophoretic pattern revealed that during the time of heating there was a progressive diminution of the dimeric enzyme that paralleled accumulation of the monomeric species and F_1 subcomplexes. Therefore, heat activation and loss of oligomycin sensitivity seem to be due to the progressive release of free, active F_1 sector. BN-PAGE electrophoretic patterns and in-gel ATPase activity staining also revealed that the enzyme monomerizes as a function of time when it is incubated at 36 °C; and oligomycin partially prevented the heat-induced dissociation of the dimeric enzyme (Fig. 1B). Both the dimers and monomers were catalytically active, as shown in-gel ATPase activity staining (Fig. 1C). To allow the expresion of the in-gel ATP hydrolytic activity, no oligomycin was added to the in-gel activity assay medium.

The algal mitochondrial ATPase is also activated by non-ionic detergents We explored if there are other conditions in which the enzyme may be activated without being

Fig. 2 *Effect of detergents on the ATPase activity of the ATP synthase from Polytomella sp.* Panel A) ATPase activity at 25 °C of the algal enzyme in the presence of various detergents (black bars). Detergents were added to the ATPase activity assay medium at a final concentration of 0.01% (*w*/*v*). Control: no detergent added; LM, lauryl maltoside; T20, Tween 20; T80, Tween 80; Tx100, Triton X100; Tx114, Triton X114; GP, n-octyl-β-D-glucopyranoside; DS, n-dodecanoyl sucrose; C_{12}, octaethylene glycol monododecyl ether; DMG, decanoyl-N-methylglucamide; OMG, octanoyl-N-methylglucamide, LDAO, lauryl-dimethylamine oxide, and TC, taurodeoxycholate. To assay oligomycin sensitivity (gray bars), samples were preincubated 30 min in the presence of 10 μg/ml oligomycin. The inhibitor was also added to the assay medium at a final concentration of 5 μg/ml. Panel B) Effect of increasing concentrations of LM in the assay medium on the ATPase activity of the enzyme. Filled rhomboids, activity in the absence of oligomycin; open rhomboids, activity in the presence of oligomycin. Left side inset : BN-PAGE electrophoretic pattern of the dimeric algal ATP synthase (V_2) incubated for 30 min in the presence of increasing concentrations of LM; 60 μg of protein were loaded in each lane. Only at concentrations as high as 10% LM, the appearance of a discrete band of the monomeric form (V) was observed. Right side inset : effect of increasing concentrations of LM in the assay medium on the ATP hydrolysis of the F_1 subcomplex. The F_1 sector was prepared by incubating the algal ATP synthase for two minutes at 60 °C. The assayed ATP hydrolytic activity was completely insensitive to oligomycin. Panel C) ATP hydrolytic activity of the purified algal ATP synthase as a function of time at 45 °C. The ATPase assay medium contained 0.01% LM. Filled rhomboids, activity in the absence of oligomycin; open rhomboids, activity in the presence of oligomycin


a
Specific Activity (units/mg)
Control LM T20 T80 Tx100 Tx114 GP DS C12 DMG OMG LDAO TC
Detergent
b
Specific activity (units/mg)
Detergent concentration (%)
0 0.01 0.1 1 10
V2
V
Specific activity (units)
Detergent concentration (%)
Detergent concentration (%)
c
Specific activity (units/mg)
Time (minutes)

dissociated. To this end, different detergents were added to the ATPase activity assay medium. Some non-ionic detergents induced a large increase in the ATPase activity of the complex (Fig. 2A). The detergent-activated enzymes were fully sensitive to oligomycin. The non-ionic detergents dodecyl-β-D-maltopyranoside (lauryl maltoside, LM), octaethylene glycol monododecyl ether ($C_{12}E_8$), Tween 20, Tween 80, and n-dodecanoyl sucrose strongly activated the enzyme. In contrast, Triton X100 and Triton X114 induced a modest activating effect, while n-octyl-β-D-glucopyranoside, octanoyl-N-methylglucamide decanoyl-N-methylglucamide, lauryldimethylamine oxide (LDAO) and sodium taurodeoxycholate had no noticeable effect on the activity (Fig. 2A). The activation by LM was further explored. Figure 2B shows titration curves with increasing concentrations of LM in the assay medium in the absence and presence of oligomycin. Maximal activations were observed at concentrations of 0.01% (*w*/*v*) of LM (7.0 Units/mg). The detergent-activated enzyme was fully sensitive to oligomycin (Fig. 2B). The activation of the enzyme induced by detergents was not due to dissociation of the algal ATP synthase, since increasing detergent concentrations (up to 1%) did not disrupt the dimeric enzyme, as judged by BNE-PAGE (Fig. 2B, inset). Only when the enzyme was incubated in ice for 30 min in the presence of very high concentrations of detergent (10% LM), the appearance of a discrete population of monomeric forms of the enzyme became evident (Fig. 2B, inset). LM also activated the ATP hydrolytic activity of the F_1 sector. The F_1 sector was prepared by heating the dimeric enzyme for 2 min at 60 °C. In these conditions, the algal F_1Fo-ATP synthase is disrupted, giving rise to an active F_1 subcomplex that exhibits a relatively high ATPase activity (around 4.0 Units/mg). Increasing concentrations of LM to the ATPase assay medium further increased this activity up to values around 7.0 Units/mg (Fig. 2B, inset).

When the algal enzyme was incubated at 45 °C for increasing periods of time and its activity measured in the presence of detergent in the assay medium (LM 0.01%), a different pattern of activation was observed (Fig. 2C). In a first phase, the original activity, which was fully sensitive to oligomycin, decreased sharply during the first minutes, simultaneous to a discrete loss in oligomycin sensitivity. In a second phase, the ATPase activity of the enzyme increased as a function of time; this was accompanied by a parallel loss of oligomycin sensitivity (Fig. 2C). We attribute this second phase to the progressive disassembly of the F_1Fo-ATP synthase complexes and the consequent release of free, active, oligomycin-insensitive F_1 sectors.

Taurodeoxycholate monomerizes the algal mitochondrial ATP synthase When the dimeric enzyme is incubated at relatively high temperatures (i.e., 60 °C) for brief periods of time, it dissociates into its monomers, and subsequently, into F_1 moieties and free independent subunits (Vázquez-Acevedo et al. 2006). We searched for conditions, other than heat treatment, that induce dissociation of the enzyme into stable monomers. The incubation of the enzyme with sodium taurodeoxycholate (TDOC), the hydrophylic, bile salt-related anionic detergent, induced a concentration-dependent dissociation of the enzyme into monomers (Fig. 3A); at relatively high concentrations of TDOC, F_1 was released, and the amount of free, dissociated subunits increased. Treatment with TDOC has the advantage over heat treatment in that it allows for a more precise control of the dissociation process, and in consequence, it is possible to work with a population of enzymes in which the monomeric form predominates, albeit in coexistance with undissociated dimers and free F_1 moieties (Fig. 3B). A concentration of 3% (*w*/*v*) taurodeoxycholate was chosen for all further experiments. The mixture of ATP synthases generated by treatment with 3% TDOC will be referred to as the monomeric form of the enzyme, although it actually represents a population with 50% monomers and 50% of a mixture of dimers, F_1 sectors and free subunits (Fig. 3B). TDOC-induced monomerization was irreversible, since treatment with the detergent, followed by extensive dialysis in the presence of 0.1% LM, did not restore the dimeric form of the complex (data not shown). The 3%-TDOC-treated enzyme exhibited an ATPase activity of 0.6 Units/mg in the absence of detergent in the assay medium, and increased in the presence of 0.01% LM to 2.0–4.0 Units/mg. In addition, the monomer was only partially sensitive to oligomycin (45% inhibition). The contrasting behavior between the dimeric and monomeric forms of the enzyme could also be observed by BNE-PAGE followed by in-gel ATPase activity staining (Fig. 3C). Both the dimer and the monomer exhibited in-gel ATP hydrolysis activity, however, the dimer was more sensitive to oligomycin than the monomer.

Neither heat treatment nor TDOC-induced monomerization elicited major changes in the subunit composition of the enzyme. The polypeptide patterns of the dimer and the monomer in SDS-Tricine-PAGE were essentially the same (data not shown). In some experiments, partial loss of the small, membrane bound subunits ASA6 and/or ASA9 was observed.

Crosslinking experiments suggest that subunit ASA6 may be involved in monomer-monomer interactions In order to identify possible close-neighbor interactions between the *Polytomella* ATP synthase subunits, the dimeric enzyme was incubated with dithiobis(succinimidyl)propionate (DSP) a homobifunctional, thiol-cleavable and membrane permeable crosslinker that has a spacer arm of 12 Å. The cross-link products were analyzed by two-dimensional gel electrophoresis. A first-dimension SDS-PAGE was run in the absence of

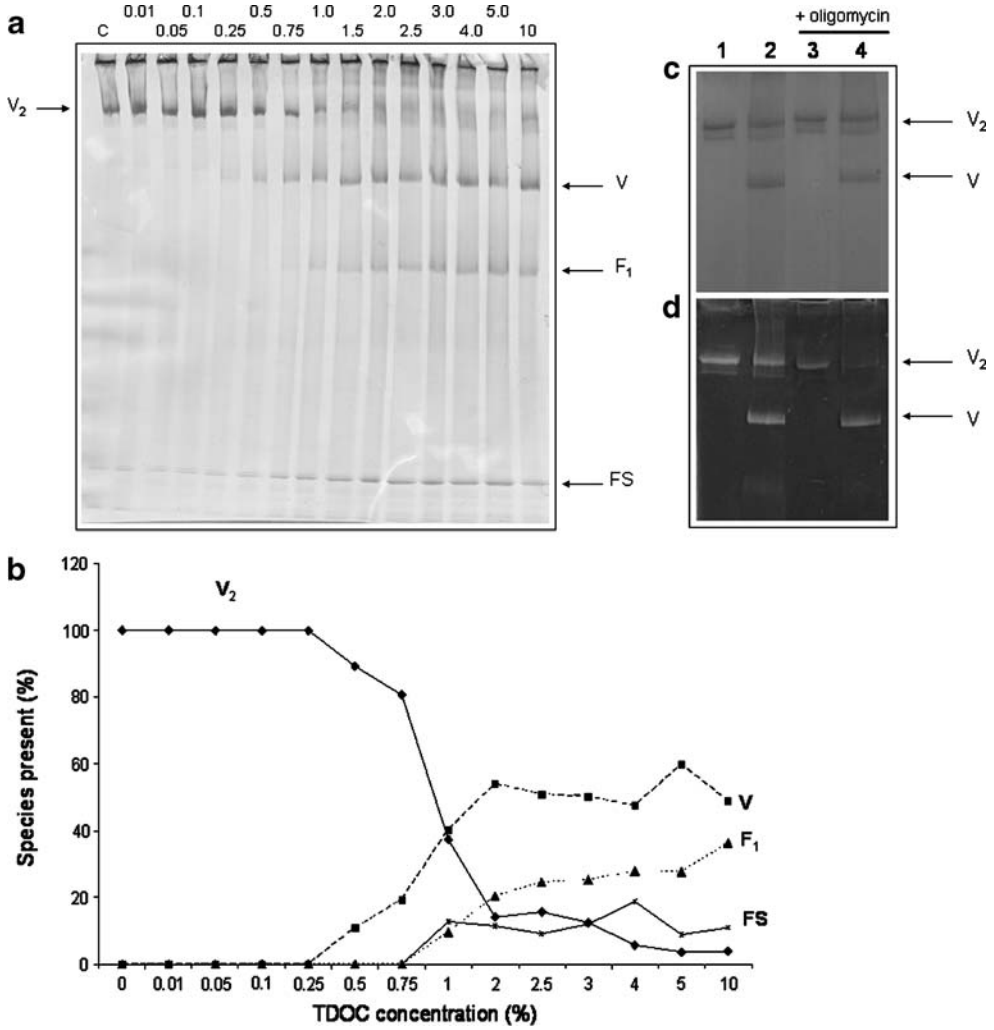


Fig. 3 *Effect of increasing TDOC concentration on the oligomeric state of the ATP synthase from Polytomella sp.* Panel A) BN-PAGE of purified ATP synthase samples incubated in the presence of TDOC at increasing concentrations (% *w*/*v*) in ice, for 30 min; 100 μg of protein were loaded in each lane. The control lane in the absence of the detergent is labelled C. Dimer (V_2), monomer (V); F_1 sector (F_1), Free subunits (FS). Panel B) Quantitation of the oligomeric forms of the algal ATP synthase as a function of TDOC concentration. Data were taken from the densitometric analysis of the gel shown in panel A. Curves denote the % of the different forms of the enzyme; 100% is the sum of all species detected. Dimers (V_2, filled rhomboids), monomers (V, filled squares), F_1 sectors (F_1, filled triangles) and free, dissociated subunits (FS, crosses). Panel C) BN-PAGE of dimeric and monomeric forms of the enzyme. Lanes 1 and 3 were loaded with the dimer (V_2), lanes 2 and 4 with the monomer (V) prepared by incubation with 3% TDOC in ice for 30 min. The samples of lanes 3 and 4 were preincubated with oligomycin (10 μg/ml). 50 μg of protein were loaded in each lane. Panel D) In-gel ATPase activity staining of the samples shown in panel C

a reducing agent. Subsequently, a lane of the gel was incubated with DTT, in order to cleave the cross-link products, which were then resolved in 2D-Tricine-SDS gels. The major cross-linked products in the algal ATP synthase are shown in Fig. 4A. Of particular significance was the presence of a polypeptide spot with an apparent molecular mass of 26 kDa that was identified as a ASA6-ASA6 cross-linked product. The presence of the ASA6-ASA6 cross-linked product was observed in several different experiments carried out with the dimer (Fig. 4B). In contrast, no ASA6-ASA6 cross-linked product could be observed when the reaction with the bifunctional reagent was carried out with the monomeric enzyme (Fig. 4C). The rest of the cross-linked products were formed in both the dimeric and the monomeric enzyme. Therefore, it is evident that the ASA6-ASA6 cross-linked product is formed between ASA6 subunits of two monomers that are in close vicinity in the intact, dimeric ATP synthase. This confirms that the interaction between ASA6 subunits, as originally suggested by van Lis et al. (2007), may contribute to the stability of the dimeric enzyme.

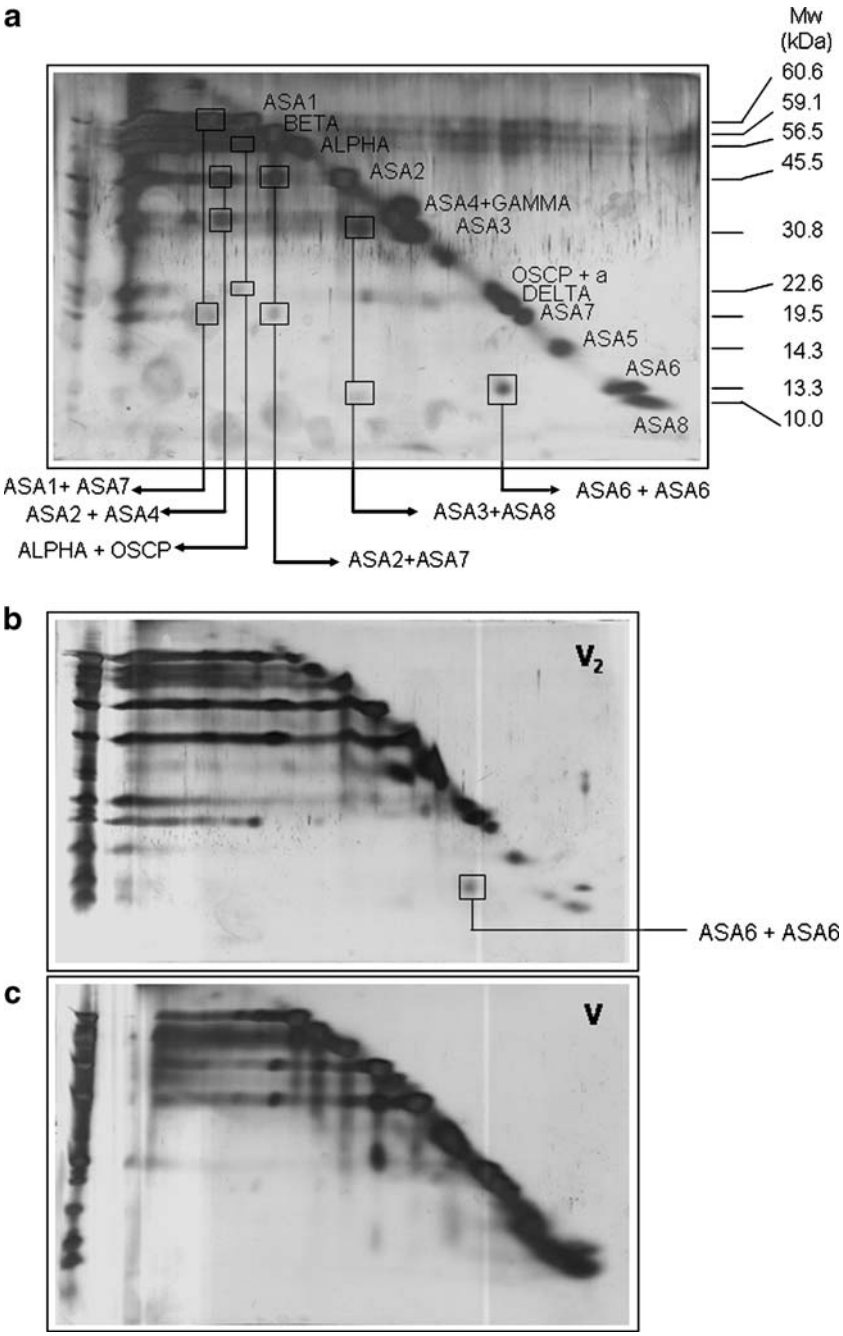


Fig. 4 *Second dimensional analysis of the Polytomella ATP synthase subunits cross-linked with DSP.* Panel A) *Polytomella* ATP synthase treated with the bifunctional reagent was resolved by SDS-Tricine-PAGE in non-reducing conditions (100 μg of protein). The 1D gel was then incubated in the presence of DTT to cleave the cross-linked products and subjected to 2D-SDS-Tricine-PAGE and silver staining. Panel A shows the original 2D gel. The identified cross-linked subunits that were released by DTT treatment and ran out of the diagonal, are indicated by boxes and connecting lines. Pure ATP synthase was used as molecular mass marker, and the apparent molecular masses (in kDa) are shown on the right hand side. Panels B and C) comparative 2D-SDS-Tricine-PAGE showing the ASA6-ASA6 cross-link product generated by DSP on the dimer (V_2) and the absence of such cross-link product when the experiment was carried out with the monomeric form of the enzyme (V)

Properties of the dimeric and monomeric mitochondrial ATP synthase of Polytomella sp. The dimeric and monomeric forms of the enzyme were subjected to different treatments: high temperature, high hydrostatic pressures, and proteolysis. While the dimeric enzyme was relatively stable to incubation for up to 1 h at 36 °C, the monomer rapidly dissociated and liberated F_1 moieties under the same conditions (Fig. 5). Thus, the thermostability of the dimer is higher than that of the monomer. A higher oligomeric form of the enzyme (V_x) was observed in certain preparations (Fig. 5). This high molecular species (possibly tetramers) migrate into the gel when the acrylamide gradients are slightly modified.

In a further characterization of the algal ATP synthase, the enzyme was subjected to high hydrostatic pressures, a method that is used to disrupt subunit interactions within oligomers (Silva et al. 1996). The algal ATP synthase was resistant to pressures up to 1,600 bar for 15 min. Treatment at higher hydrostatic pressures, 2,000 bar or more, disrupted the dimers into its polypeptide components

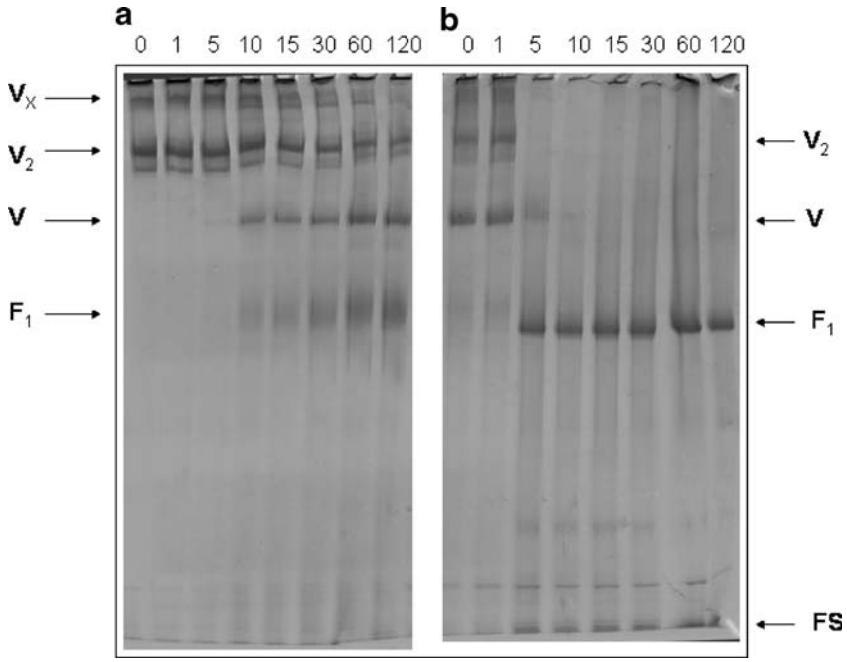


Fig. 5 *Effect of a time course of incubation at 36 °C for the dimeric and monomeric forms of the algal ATP synthase.* Panel A) Time course of incubation (in minutes) at 36 °C of the dimeric form of the enzyme (50 μg of protein in each lane). Panel B) Time course of incubation (in minutes) at 36 °C of the monomeric form of the enzyme (50 μg of protein per lane). Dimer (V_2), monomer (V); F_1 sector (F_1); free subunits (FS). V_x denotes oligomeric forms of the enzyme, probably tetramers

(Fig. 6A). In contrast, the monomeric enzyme released F_1 moieties at barely 200 bar, and completely dissociated into free subunits at 1,200 bar (Fig. 6B).

The dimeric and monomeric forms of the enzyme were also subjected to proteolytic degradation with chymotrypsin. The time course of cleavage of the dimeric and monomeric enzymes was followed by BN-PAGE (Fig. 7A, B). The dimeric enzyme was resistant to proteolytic degradation for as long as 1 h. In contrast, the monomeric form of the enzyme was degraded much faster, and F_1 moieties were released. The time course of cleavage was also followed by SDS-PAGE (Fig. 7C, D). When the pattern of digestion of individual subunits was examined, it was found that in the dimeric enzyme, the only proteolitically cleaved subunit was ASA3, although there were minor cleavages of the α and β subunits. When the monomeric enzyme was incubated in the presence of the protease, the cleavage of additional subunits was evident. Several of the small, membrane-bound subunits ASA6, ASA8 and ASA9, were rapidly degraded. In addition, the largest subunit, ASA1, was also rapidly cleaved. Subunit ASA5 exhibited a time course of cleavage similar to the one of ASA1. Therefore, it would appear that in the monomer, the accessibility of the external protease at various defined subunits is much higher than in the dimer.

Discussion

ATPase activity of Polytomella mitochondrial ATP synthase Our preparations of *Polytomella* mitochondrial ATP synthase exhibit a very low ATPase activity that readily increases when incubated at relatively high temperatures. The enzyme can also be activated by non-ionic detergents. LM in the activity assay medium increased the ATPase activity of the enzyme. This is noteworthy, since LM was in contact with the enzyme throughout the purification procedure. The isolated enzyme contained detergent at a 0.1 (*w*/*w*) detergent/protein ratio. In this regard it may be mechanistically relevant that the 200-fold activation was achieved when LM was added to the ATPase activity assay cuvette at a 5.0 (*w*/*w*) detergent/protein ratio. This 50-fold increase in the detergent/protein ratio may explain the strong activation of the ATPase. The excess detergent may either induce a conformational change in the enzyme, or alternatively, it may release a yet undetermined inhibitory factor, analogous to the regulatory protein IF_1 in the bovine enzyme (Gledhill et al. 2007). Activation of the ATP hydrolytic activity of other ATP synthases has been described. The detergent LDAO stimulated 5 to 6-fold *Escherichia coli* ECF_1 and ECF_1-Fo ATPase activities in a concentration-dependent manner. The detergent effect was attributed to a release of the inhibitory action of subunit ε on subunit β (Lötscher et al. 1984). LDAO also activated ATP hydrolysis of the mitochondrial F_1Fo-ATPsynthase, probably due to the release of the inhibitor protein IF_1 (Vázquez-Laslop and Dreyfus 1986). The mechanism by which LM stimulates the ATP hydrolytic activity of the algal mitochondrial ATP synthase remains to be ascertained, since an orthodox inhibitor protein does not seem to be present in the isolated enzyme. Also, a homolog of the gene encoding the bovine ATPase inhibitor protein IF_1

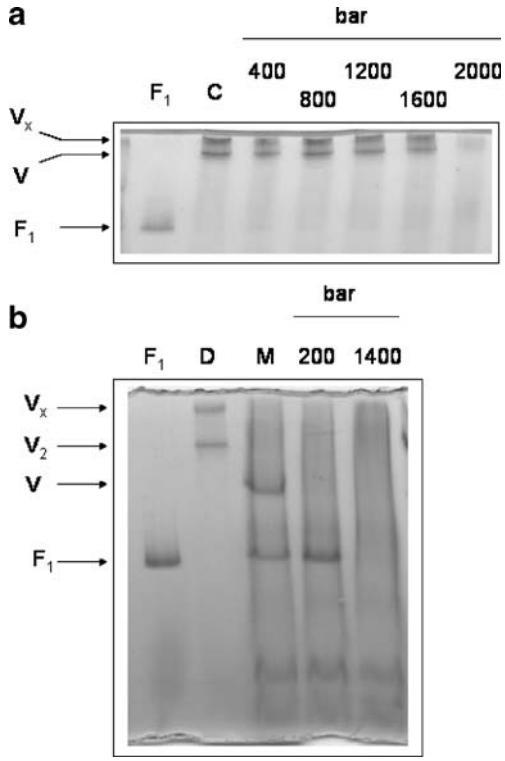


Fig. 6 *Effect of high hydrostatic pressures upon the dimeric and monomeric forms of the algal ATP synthase.* Panel A) Incubation of the dimeric algal ATP synthase at high hydrostatic pressures. Lane F_1 shows the F_1 sector generated by heating the ATP synthase for 2 min at 60 °C; lane C, uncompressed, intact, dimeric ATP synthase. The five following lanes show the enzyme subjected for 30 min to increasing high hydrostatic pressures (in bars). Panel B) Incubation of the monomeric algal ATP synthase at high hydrostatic pressures. Lane F_1 shows the F_1 sector generated by heating the ATP synthase for 2 min at 60 °C; lane D, intact, dimeric ATP synthase; Lane M, monomeric form of the enzyme. The two following lanes show the monomeric enzyme subjected for 15 min to increasing high hydrostatic pressures (in bars). The oligomeric (V_x), dimeric (V_2), and monomeric (V) forms of the enzyme are indicated, as well as the F_1 sector. 50 μg of protein were loaded in each lane

could not be identified in the genome of the closely-related green alga *Chlamydomonas reinhardtii* (Cardol et al. 2005). LM activates the hydrolytic activity of the ATP synthase of *Polytomella* sp. possibly exerting a conformational change upon subunits α, β, γ, δ, or ε,. It was previously proposed that the C-terminal extensions of the chlorophycean ATP synthase β subunits, originally described by Franzén and Falk (1992), could function similarly to the inhibitor protein IF_1 (Atteia et al. 1997). The possible regulatory role of these extensions remains to be explored.

ATPase activity of Polytomella mitochondrial ATP synthase and oligomycin sensitivity Oligomycin is a classical inhibitor of proton translocation in F_1Fo-ATPase (Slater 1967; Tzagoloff 1970). Sensitivity to oligomycin is related to the structural integrity of the enzyme. If F_1 is detached from Fo, although ATP hydrolysis may take place, its sensitivity to oligomycin is lost. The inhibitor interacts with subunits *a* and *c* of the Fo sector (John and Nagley 1986; Galanis et al. 1989). Oligomycin was found to be a potent inhibitor of the mitochondrial ATP synthase of *Polytomella*, when it was preincubated with the enzyme and also added in the assay medium. In these conditions, oligomycin inhibited more than 98% of the detergent-stimulated ATPase activity of the complex. The high sensitivity of the algal ATP synthase to oligomycin, suggests that the increase in ATP activity by non-ionic detergents does not involve dissociation of the dimer, indeed, sensitivity to oligomycin was lost when dissociation of the enzyme was induced by heat treatment. This process was accompanied by monomerization and eventual disruption of the ATP synthase into F_1 sectors and free subunits (Fig. 1A). Oligomycin also seems to partially prevent the heat-induced dissociation of the dimeric algal ATP synthase, providing some ligand-induced thermostability to the complex (Fig. 1B). In this regard, it is noted that binding of ligands may confer thermostability to ATP synthases, i.e., the binding of Mg^{2+}-ADP to the F_1Fo-type ATP synthase from the thermophilic *Bacillus* PS3 (Villaverde et al. 1997).

On the dimeric nature of mitochondrial ATP synthase of chlorophycean algae In vivo, mitochondrial ATP synthases are thought to form oligomeric structures that determine the shaping of the mitochondrial cristae (Arnold et al. 1998; Arselin et al. 2004; Minauro-Sanmiguel et al. 2005; Strauss et al. 2008; Zick et al. 2009). These F_1Fo-ATP synthase supracomplexes microdomains may ensure mitochondria with an optimal bioenergetic function (Bornhövd et al. 2006). When solubilized, the oligomeric associations are disrupted and the ATP synthase usually monomerizes. This monomerization seems to be accompanied by the loss of small polypeptides (Meyer et al. 2007). In fact, LM-solubilized complex V from different mitochondrial sources, usually migrates in BN-PAGE as monomers of 550–600 kDa (Schägger 1994; Jänsch et al. 1996; Arnold et al. 1998; Horvath et al. 2000; Eubel et al. 2004). Milder solubilization conditions, i.e. in the presence of digitonin, are required to observe dimeric complex V (Schägger 2001; Paumard et al. 2002; Minauro-Sanmiguel et al. 2005), and higher oligomeric forms, such as trimers and tetramers (Krause et al. 2005) in BN-PAGE. In the presence of LM, complex V from chlorophycean algae (i.e., *Chlamydomonas reinhardtii*, *Polytomella* sp., *Scenedesmus obliquus*, etc.) migrates with an apparent molecular mass of 1,600 kDa in BN-PAGE (van Lis et al. 2003; van Lis et al. 2005; Vázquez-Acevedo et al. 2006). The migration properties of algal ATP synthase in BN-PAGE already suggested that it is a remarkable stable dimer. In certain preparations, the presence of oligomeric forms of the enzyme (V_x), possibly tetrameric forms, were

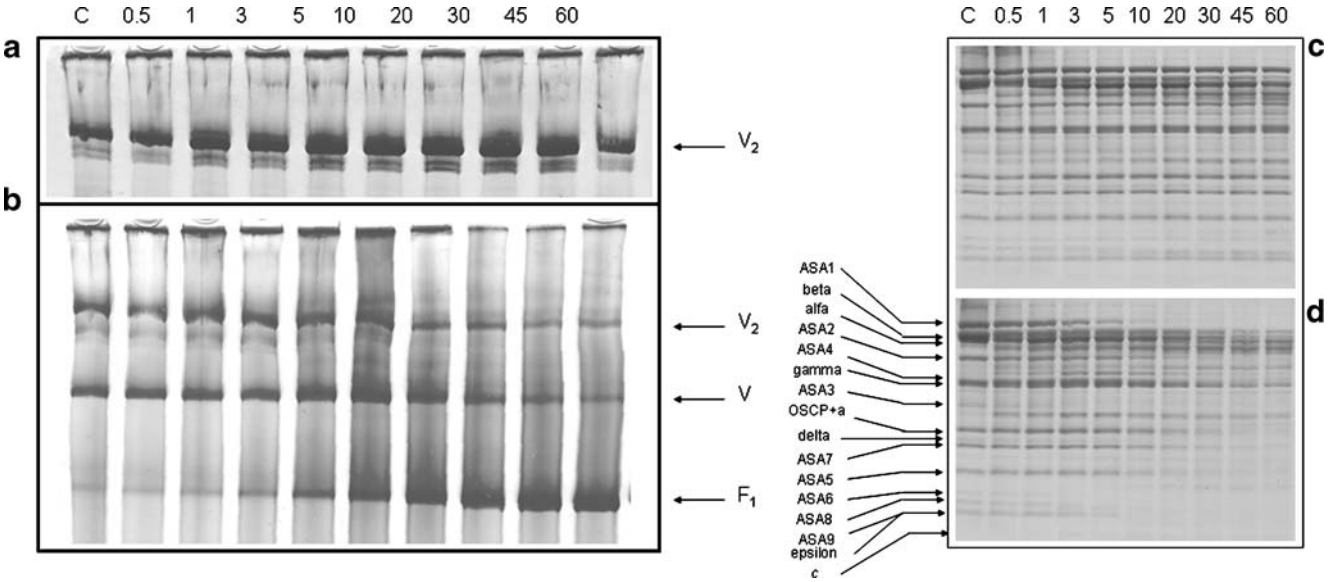


Fig. 7 *Effect of a time course of incubation with chymotrypsin on the dimeric and monomeric forms of the algal ATP synthase.* Panels A and B) BN-PAGE of a time course of incubation (in minutes) of the dimeric (Panel A) and monomeric (Panel B) forms of the enzyme with chymotrypsin (1:100 *w*/*w* ratio protease/enzyme). Panel C and D) SDS-Tricine-PAGE electrophoretic patterns of a time course of incubation (in minutes) of the dimeric (Panel C) and monomeric (Panel D) forms of the enzyme with chymotrypsin (1:100 *w*/*w* ratio protease/enzyme). Protease digestions were carried out at room temperature for the indicated times (in minutes). 100 μg of protein were loaded in each lane

also evident (see for example, Fig. 6). In this work, we found that treatment of the algal complex V with 3% TDOC allowed the formation of stable monomers. The monomeric form of the enzyme and the dimer exhibited the same polypeptide composition as judged by SDS-Tricine-PAGE, although some times monomerization was accompanied by a partial loss of the small, membrane bound polypeptides ASA6 and ASA9. van Lis et al. (2007) also observed that incubation of *Polytomella* sp. mitochondria at 55 °C brought about monomerization of the mitochondrial ATP synthase in BN-PAGE. In their conditions, monomerization was accompanied by the loss of subunits ASA6 and ASA9, which suggested that these subunits participate in enzyme dimerization. Here, the participation of ASA6 in the dimerization of the enzyme was also demonstrated by the cross-link experiments. Cross-linking by bifunctional reagents has been traditionally used to explore close-vicinity of polypeptides in oligomeric complexes (González-Halphen 1988). While an ASA6-ASA6 cross-link product readily formed when the dimeric ATP synthase was incubated with DSP, no cross-link product was obtained with the monomeric form of the enzyme. Thus, subunit ASA6 seems to be instrumental in maintaining the dimeric conformation of the algal ATP synthase. It is important to note that subunit ASA6 shows no sequence similarity to the orthodox subunits *e* and *g*, which are thought to mediate dimerization of the yeast ATP synthase (Arnold et al. 1998; Brunner et al. 2002). The sequence of ASA6 also lacks a GXXXG motif, involved in the dimerization of the yeast *e* subunits (Yao et al. 2008).

The dimeric and monomeric forms of the algal ATP synthase were subjected to different treatments in order to gain insight into their stability. The dimer was more resistant than the monomeric form to heat treatment, high hydrostatic pressures, and protease degradation.

The effects of high hydrostatic pressure on the mammalian mitochondrial ATP synthase have been studied before (Penniston 1971; Dreyfus et al. 1988). While the F_1 portion lost activity upon compression-decompression (unless protected by 30% glycerol), the beef heart F_1Fo-ATP synthase complex was resistant to pressures up to 1,700 bar, and retained activity upon decompression (Dreyfus et al. 1988). The algal F_1Fo-ATP synthase retained its dimeric conformation after compression at 1,600 bar and decompression, whereas the monomeric form of the enzyme readily dissociated when subjected to the relatively low pressures of 200 bar.

When the dimer was subjected to chymotrypsin degradation, it was found that the ASA3 subunit was cleaved, along with some of the α and β subunits. ASA3 subunit is known to be susceptible to protease cleavage; in the purified enzyme it usually exhibits a degraded N-terminus (Vázquez-Acevedo et al. 2006). In contrast, the monomeric form of the enzyme has more exposed subunits to protease degradation, such as the small membrane-bound polypeptides

ASA6 to ASA9, and other subunits like ASA1 and ASA5. Altogether, the data suggest that the algal ATP synthase forms stable dimers, less susceptible to be monomerized by detergents than the mitochondrial ATP synthases from various species, including mammals, yeast and plants. It is likely that the robust dimeric nature of the algal complex may be relevant *in vivo*, and could be related to the tightly regulated ATPase activity of the enzyme or/and to maintain the highly structured tubular cristae typical of *Polytomella* mitochondria (Dudkina et al. 2006). Therefore, the data indicate that the fully-active form of the algal ATPase is the structurally stable dimer, and that the monomeric form of the enzyme is a less active, unstable complex.

Acknowledgments We thank Prof. Armando Gómez-Puyou for his critical review of the paper. This research was supported by grants 56619 from CONACyT (Mexico) IN217108 from DGAPA, UNAM (Mexico), CNPq and FAPERJ (Brazil), and by grants from the Belgian FRFC (2.4587.04, 2.4582.05 and F.4735.06). PC is posdoctoral fellow of the FRS-FNRS (Belgium).

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