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The stoichiometry of the chloroplast ATP synthase oligomer III in *Chlamydomonas reinhardtii* is not affected by the metabolic state

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Abstract

The chloroplast H^+-ATP synthase is a key component for the energy supply of higher plants and green algae. An oligomer of identical protein subunits III is responsible for the conversion of an electrochemical proton gradient into rotational motion. It is highly controversial if the oligomer III stoichiometry is affected by the metabolic state of any organism. Here, the intact oligomer III of the ATP synthase from *Chlamydomonas reinhardtii* has been isolated for the first time. Due to the importance of the subunit III stoichiometry for energy conversion, a gradient gel system was established to distinguish oligomers with different stoichiometries. With this methodology, a possible alterability of the stoichiometry in respect to the metabolic state of the cells was examined. Several growth parameters, i.e., light intensity, pH value, carbon source, and CO_2 concentration, were varied to determine their effects on the stoichiometry. Contrary to previous suggestions for *E. coli*, the oligomer III of the chloroplast H^+-ATP synthase always consists of a constant number of monomers over a wide range of metabolic states. Furthermore, mass spectrometry indicates that subunit III from *C. reinhardtii* is not modified posttranslationally. Data suggest a subunit III stoichiometry of the algae ATP synthase divergent from higher plants.

Keywords: ATP synthase; Oligomer III; Stoichiometry; Chloroplast; Metabolic state

1. Introduction

Most of the ATP in the cell is generated by proton translocating (F_OF_1) ATP synthases which utilize a proton motive force across thylakoid membranes, inner mitochondrial membranes, and bacterial membranes. This enzyme consists of a hydrophilic F_1 subcomplex with three catalytic sites [1–3] and a hydrophobic F_O domain which is embedded in the membrane and acts as a proton turbine. Vectorial proton translocation is probably directed along the transmembrane interface of subunit IV and a cylinder of subunits III. It is assumed that the binding and translocation of one proton per monomer III results in complete rotation of oligomer III, which in turn triggers conformational changes in F_1 and thus leads to the ejection of 3 newly formed ATP [4,5].

The H^+/ATP ratio and the efficiency of the enzyme can be determined, if the number of subunits III is known. In yeast, the cylinder consists of 10 c monomers [6]. The oligomer III of spinach is made up of 14 subunits [7–9] and the c cylinder of the sodium-driven *Ilyobacter tartaricus* ATP synthase consists of 11 subunits [8,10,11]. In all three systems, the theoretically calculated H^+/ATP ratios based on structurally determined oligomer stoichiometries are non-

Abbreviations: AA, amino acid; CF_OF_1, proton translocating ATP synthase of chloroplasts; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DDM, *n*-dodecyl-*β*-D-maltoside; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; *m*/*z*, mass/charge; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 0.1% (v/v) Tween-20; TOF, time of flight

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integer numbers, i.e., 3.3 in yeast, 3.7 in *I. tartaricus* and 4.7 in spinach, but not 3 [12,13] or 4 [14,15] as previously determined in experiments aiming at proton transfer and ATP yield. This seemingly discrepancy has been elucidated for the thermophilic *Bacillus* PS3 [16].

As in the only three phylogenetically far separated organisms studied to date different stoichiometries have been found, it is speculated that in the same organism the number of subunits III/c may vary at different metabolic states. For *E. coli*, data were presented indicating that different carbon sources during cultivation lead to different ratios (subunit c compared to subunit b) assembled into the ATP synthase [17,18].

Our intention was to answer the question if *Chlamydomonas reinhardtii*, a unicellular green alga, is able to vary the stoichiometry of subunit III in the hydrophobic domain of the proton translocating ATP synthase of chloroplasts (CF_OF_1), in response to the metabolic state. The green algae usually grow phototrophically, but can also grow heterotrophically with acetate as a second carbon source. This makes it a suitable model organism apart from bacteria and fungi for the study of the influence of different growth conditions on the composition of the proton turbine F_O.

We could demonstrate unequivocally that the stoichiometry of subunit III does not depend on the metabolic state, and present evidence indicating a stoichiometry unlike that of the plant spinach.

2. Materials and methods

2.1. Cultivation of C. reinhardtii

C. reinhardtii strain 83–81 (cw-15 mutant) (Sammlung für Algenkulturen, Göttingen, Germany) was grown synchronously in 5-l flasks under a 12 h light/12 h dark rhythm at 30 °C in Tris-acetate-phosphate medium (TAP, pH 7.0) [19] or in high-salt minimal medium (HS) [20]. Light (250 μmol photons m^{-2} s^{-1}) was provided by fluorescent lamps (Osram L18 W/25, Universal White, Germany). Cultures were bubbled with CO_2-enriched air (4%, 90 l/h) and stirred to ensure uniform illumination and to prevent settling. Depending on the number of cells initially added, the cultures reached maximal densities of 3×10^7 cells/ml, 60 h after inoculation. This corresponds to time zero in Fig. 5. To investigate the effect of pH, light intensity, and acetate on the stoichiometry of the III oligomer of the ATP synthase, each parameter was varied compared to the standard growth conditions described above.

2.2. Determination of starch, chlorophyll, and cell titer

For determination of the amount of starch in the cells, 0.5–1.0-ml culture was centrifuged (20,000×*g*, 10 min) and 1 ml of 80% (v/v) acetone was added to the pellet. After 3-h incubation at 4 °C the sample was centrifuged at 20,000×*g* for 10 min and the supernatant was used for chlorophyll determination. The pellet was resuspended in 0.2-ml H_2O. After addition of 0.5-ml 1 M KOH, the sample was incubated for 30 min at 95 °C. Subsequently, 0.5-ml acetic acid (1 M; final concentration: 333 mM) and 0.3-ml sodium acetate buffer (50 mM, pH 5.0) containing 1 U amyloglucosidase and 1.7 U amylase (Boehringer-Mannheim, Germany; all enzyme units as defined by the supplier) were added and the sample was incubated for 12 h at 37 °C. Glucose was determined by mixing 150-μl aliquots of the sample with 176-μl incubation buffer (750 mM triethanolamine–HCl, pH 7.6; 10 mM $MgSO_4$) in the wells of a microtiter plate. NAD (final concentration: 0.2 mM), ATP (final concentration: 1.4 mM) and glucose-6-phosphate dehydrogenase (0.5 U; from *Leuconostoc mesenteroides*, Sigma Chemicals, Steinheim, Germany) in a total volume of 18 μl were added. The reaction was started by the addition of 6-μl enzyme solution containing 1 U hexokinase (Sigma). Formation of NADH was followed photometrically at 340 nm with an ELISA-photometer Spectra III (SLT Labinstruments, Austria) [21].

The chlorophyll content of the cell suspensions was measured according to Arnon [22]. Cells were counted with a Coulter Counter (Industrial D, Coulter Electronics, Luton, UK).

2.3. Harvesting of cells and preparation of cell extracts

Cultures (5 l) were harvested after 84 h, i.e., corresponding to 24 h in Fig. 5, spun down at 4 °C for 10 min (3500×*g*) and washed with growth medium. The pellet was resuspended in 100-ml ice cold buffer (1 mM $MnCl_2$, 5 mM $MgCl_2$, 35 mM HEPES, pH 7.8, 2 mM Na_2EDTA, 250 mM sorbitol). Cells were disrupted by passing a french press two times at 180 bar. After spinning down the cell extract (3 min, 3000×*g*), the resulting pellet was homogenized in 5 mM $MgCl_2$ solution to disrupt intact chloroplasts and obtain the thylakoid membrane. Subsequently, a chlorophyll concentration of 2 mg/ml was adjusted with solubilization buffer (200 mM sucrose, 10 mM Tricine/NaOH, pH 8.0, 10 mM $MgCl_2$, 11.6 mM Na-cholate (Sigma), 30 mM *n*-octyl-β-D-glucopyranoside (Calbiochem, La Jolla, USA). After stirring for 30 min in the dark at 4 °C, non-solubilized material was spun down for 1 h at 120,000×*g*.

2.4. Chromatographic purification of ATP synthase

Dye–ligand columns were equilibrated with a buffer containing 4 mM *n*-dodecyl-β-D-maltoside (DDM) (Calbiochem), 20 mM Tris/HCl, pH 8.0, 20% (w/v) glycerol, and 5 mM $MgSO_4$. To purify the ATP synthase, a solution of solubilized thylakoid membrane proteins was mixed 1:1 (v/v) with equilibration buffer and then loaded on a Reactive Red 120 column (40-ml resin volume; Sigma) [23]. Fractions containing the ATP synthase were eluted with the same buffer. Following this chromatography, the ATP

synthase solution was injected into a Cibacron Blue 3GA column (12-ml resin volume; Sigma) [23]. Thereafter, the column was washed with elution buffer (equilibration buffer including 1.5 M NaCl). Finally, the ATP synthase was eluted with a modified equilibration buffer in which DDM was substituted by 8 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Calbiochem). Fractions containing CF_OF_1 were pooled and concentrated (Centricon 50 concentrators, Millipore, Bedford, USA). All chromatographic steps were carried out with 3 column volumes of buffer at 4 °C and a flow rate of 3 ml/min (Reactive Red 120) and 1 ml/min (Cibacron Blue 3GA).

2.5. Lipase assay

The c-oligomer from *I. tartaricus* (1.2 μg) was incubated with 5 U phospholipase C (Sigma) up to 3 h and analyzed subsequently on a SDS-PA gel.

2.6. Electrophoresis and Western blot analysis

SDS-PAGE was performed according to Ref. [24] on 15% acrylamide gels or 7–15% acrylamide gradient gels (8.0×7.5×0.15 cm). Protein samples were incubated in SDS sample buffer before electrophoretic separation at room temperature, in order to maintain the integrity of the 100-kDa oligomer III of CF_O [25]. Proteins were transferred (at 1 mA/cm^2 gel) to PVDF membranes (Bio-Rad, Hercules, USA) in buffer containing glycine (192 mM), 0.1% (w/v) SDS and Tris (25 mM, pH 8.5). Membranes were incubated for 1 h in 1% BSA in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 7.9 mM Na_2HPO_4, pH 8.5) containing 0.1% (v/v) Tween-20 (PBST). After that, the membranes were incubated overnight with polyclonal antibodies (1:2000 dilution) raised against monomer III from *Spinacea oleracea* L. Membranes were washed five times with PBST before anti-rabbit secondary antibodies (1:2000 diluted with PBS) (Molecular Probes, Eugene, USA) linked to alkaline phosphatase were applied for 1 h. Membranes were washed again five times with PBST. Antibodies were detected using Sigma fast BCIP/NBT solution (Sigma).

2.7. AA sequencing

For sequencing, the monomer of subunit III immobilized on the PVDF membrane was deformylated [26]. Membranes were stained with 0.1% (w/v) Coomassie R-250 in 50% (v/v) methanol and bands cut out with a scalpel. N-terminal sequencing was carried out by automated Edman degradation.

2.8. MALDI-TOF-MS

Protein and peptide samples were analyzed on a Voyager-DE PRO (Applied Biosystems, Foster City, USA) MALDI time-of-flight mass spectrometer [27].

For molecular mass determination of the III-subunit, the homooligomer III solubilized in DDM was diluted to 0.15 mg/ml. A saturated solution of 2-(4-hydroxyphenylazo)-benzoic acid in 50% acetonitrile/0.1% trifluoroacetic acid was used as matrix for dried droplet target preparation. Mass spectra were obtained using the linear, delayed extraction mode.

3. Results

Prerequisites for this study are the purification and identification of the III oligomer from *C. reinhardtii*, as well as the demonstration that the growth parameters chosen, i.e., CO_2 concentration, light intensity, addition of acetate, and different pH values in the culture medium, have a significant influence on the metabolic state of the green algae. Additionally, a suitable tool to distinguish different oligomers easily had to be established.

3.1. Isolation and characterization of oligomer III

To purify the ATP synthase from *C. reinhardtii*, dye–ligand chromatography, previously established for purifying the homologue enzyme from spinach [23], was successfully modified. Two chromatographic procedures were combined, a negative chromatography with the dye Reactive Red 120 and a positive chromatography with Cibacron Blue 3GA. The latter chromatography replaced the density gradient centrifugation step [23]. The isolated and solubilized membrane protein samples (see Materials and methods) were applied to the columns. The fractions collected were analyzed by SDS-PAGE. Fractions of isolated ATP synthase from *C. reinhardtii* include LHC and some minor impurities. Comparison with CF_OF_1 from spinach (Fig. 1) reveals that no complete enzyme was obtained. Subunits α, β, γ, δ and ε were identified by MALDI-TOF as well as subunits I and III whereas subunits II and IV seem to dissociate during preparation. Therefore, no ATP synthase activity could be observed. However, the "core" enzyme with the αβ-hexamer, the central stalk γ and, important for this study, the III oligomer was preserved.

Isolation and purification of the ATP synthase from the unicellular green algae *C. reinhardtii* have been previously described [28,29], however, oligomer III was always dissociated into its monomers. In these studies monomer III had an apparent molecular mass of 5.5 [29] or 7.0 kDa [28] in SDS-PA-gels. We were able to preserve the intact oligomer during solubilization, purification, and gel electrophoresis and have detected an oligomer band with an apparent molecular mass of ~ 100 kDa in SDS gels (Fig. 1, lane 1). Hence, for the first time, the oligomer III of *C. reinhardtii* is available for structural and functional investigations, e.g., for determination of the stoichiometry of subunit III.

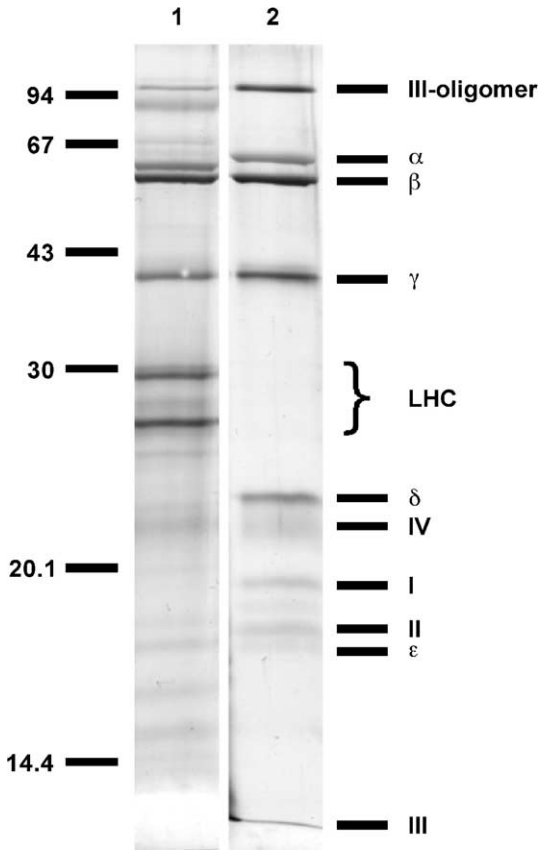

Fig. 1. Comparison of the purified ATP synthases from *C. reinhardtii* (lane 1) and from spinach (lane 2) after dye–ligand chromatography on a silver-stained SDS-PAGE (15%). To purify spinach ATP synthase, a Reactive Red 120 column was used. ATP synthase subunits are assigned as well as the LHC (light harvesting complex). Molecular mass standards are indicated in kDa.

Since the oligomer is stable (no III monomer occurred) and easy to visualize under the conditions used, additional purification steps or an intact enzyme are not required for the present study. To unambiguously identify the III oligomer bands of *C. reinhardtii* and spinach, the sequence of the first six N-terminal amino acids of that protein band, which was expected to be the III oligomer from spinach chloroplasts, was determined (MNPLIA) and identified as III. More evidence for the identity of the studied III oligomers was gained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of monomers III from both organisms. Treating oligomer III with heat or acetone led to dissociation into monomer III, subsequently analyzed by MALDI-TOF-MS. The resulting peak at 8125 mass/charge (*m*/*z*) for the *C. reinhardtii* subunit III corresponds well with the calculated mass of 8120 Da from the amino acid sequence, including N-formylmethionine in position one (Fig. 2a). A peak at 8003 *m*/*z* was detected for the dissociated oligomer III from spinach, compared to the expected 8002 *m*/*z* (Fig. 2b). The determined difference in the molecular mass of these monomers is based on different peptide chain lengths of 81 AA (spinach) and 82 AA (*C. reinhardtii*), respectively. Therefore, posttranslational modifications of subunit III can be excluded for both organisms, spinach and *C. reinhardtii*.

For a more detailed characterization of the III oligomer of *C. reinhardtii*, SDS-PA gels were optimized to gain high resolution for proteins with masses of about 100 kDa. Suitable gradient gels with total acrylamide concentrations from 7% to 15% are able to distinguish III oligomers with different stoichiometries by their migration rate. As internal molecular mass references, III oligomers from spinach with 14 subunits and from *I. tartaricus* with 11 subunits were employed. Their distinct migration behavior in the gradient gel chosen is shown in Figs. 3 and 4. In addition, an inhomogeneity of the c-oligomer from *I. tartaricus*, expressed as a double band, is revealed. The c cylinder of *I. tartaricus* often contains lipids within the central hole, which can be removed by incubation with lipase [30]. To elucidate the cause for the observed double band within the gradient gel (Fig. 3, lane 4; Fig. 4, lane 5), the sample was incubated with phospholipase C up to 3 h. During this

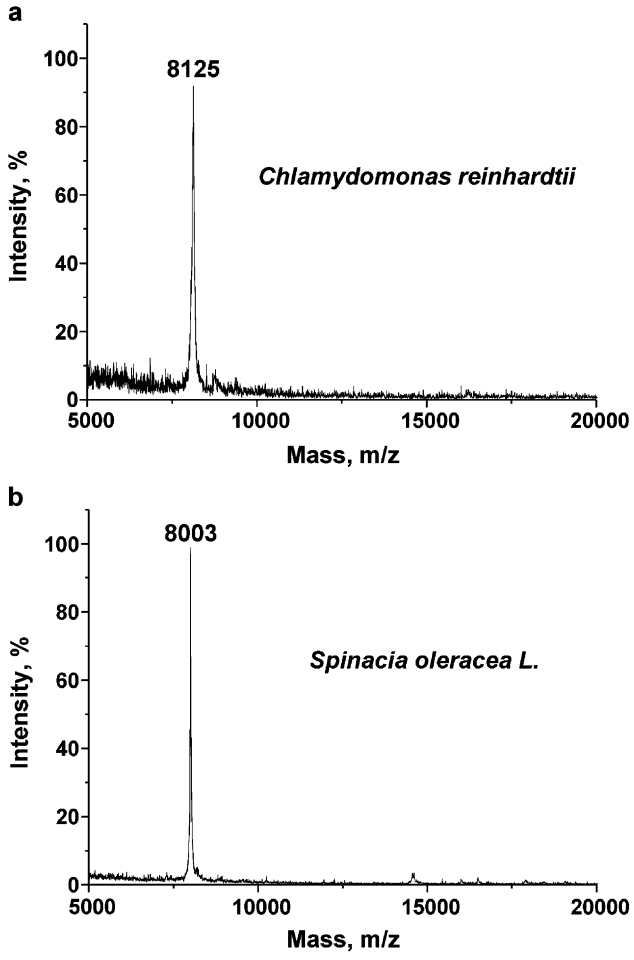

Fig. 2. Determination of the molecular mass of monomer III from *C. reinhardtii* (a) and from spinach (b) by MALDI-TOF-MS analysis. Both masses correlate well with the calculated masses of 8120 Da (*C. reinhardtii*) and 8002.4 Da (spinach) from the amino acid sequences. Therefore, in both cases posttranslational modifications can be excluded.

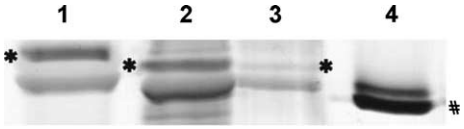

Fig. 3. Effect of acetate as a second carbon source on the stoichiometry of oligomer III from *C. reinhardtii*. Growth with acetate at pH 6.8 (lane 2) or growth at pH 6.0 without acetate (lane 3). In both lanes (2, 3) protein from an identical number of cells was applied. The amount of ATP synthase per cell in the culture with acetate as an additional carbon source is significantly larger. For comparison, in lane 4 the oligomer c of *I. tartaricus* lacking additional lipids, marked by #, and in lane 1 spinach ATP synthase were applied. The III oligomer bands are marked by *. Protein bands on the SDS-gradient-PAGE (7–15%) are silver-stained.

incubation the upper band disappears (data not shown). This confirms previous results [30], and clearly demonstrates the resolution power of the gradient gel employed. It is noteworthy that the oligomer III of *C. reinhardtii* migrates slightly faster than that of spinach.

3.2. Effects of the growth conditions on the oligomer III stoichiometry

3.2.1. Illumination

To examine if changes in light intensity do have any influence on the stoichiometry of subunit III, *C reinhardtii* was cultivated under high light (250 μmol photons m^{-2} s^{-1}) and low light (~50 μmol photons m^{-2} s^{-1}) conditions. As expected, cells under low light conditions reached the desired cell density of $3–5\times10^6$ cells/ml ~24 h later than under standard conditions. Nevertheless, the oligomer III isolated from both cultures displayed the same apparent molecular mass on a gradient SDS-gel (data not shown). Evidently the stoichiometry was not affected.

3.2.2. CO_2 concentration

Similar results were obtained by the decrease of CO_2 content in the air from 4% to 0.04%, reducing culture growth, as inferred from the decreased concentration of chlorophyll and protein, as well as from cell density. No influence on the stoichiometry of oligomer III was detected (data not shown).

3.2.3. Acetate as a second carbon source

We have grown *C. reinhardtii* in the absence of acetate, a second carbon source other than CO_2, which is present in standard growth medium. *C. reinhardtii* can utilize both carbon sources and, as a consequence, the algae grow photomixotrophically. Previous studies have reported that acetate transiently inhibits photosynthesis [31].

To visualize the effects of a switch from photoautotrophic to photomixotrophic growth, the subproteome of the inner thylakoid membrane, isolated from identical numbers of cells, was compared by 2D-PAGE [27]. Characteristic protein complexes such as the ATP synthase, PSI, PSII, and LHCII have been identified unambiguously by MALDI-TOF-MS analysis. In the presence of acetate, both the amount of protein per cell increases and the composition of photosynthetic supercomplexes change.

The pronounced changes within the protein pattern clearly illustrate the different metabolic states of the cells. A detailed description of the variation in the subproteome is given elsewhere [27]. On the other hand, comparison of the isolated oligomers III of these two cultures does not reveal any differences in the apparent molecular mass on a SDS gradient gel (Fig. 3) and therefore in the stoichiometry.

3.2.4. External pH

For activating the ATP synthase, the chloroplast membrane has to be energized by a ΔpH. Previous studies determined ΔpH values between 1.6 [32] and 3.4 [33].

We tested different pH values (pH 6.0, 6.8, and 8.0) of the culture medium and their influence on the metabolic state as well as on the stoichiometry of oligomer III. Due to CO_2 supply during cultivation, pH values changed to 5.9, 6.9, and 7.5 before harvesting, respectively. An optimal growth rate was expected at pH 6.8 [34]. Nevertheless, the cultures grew with nearly identical growth rates. For that reason, the chlorophyll concentration and the starch to chlorophyll ratio were measured, which are established parameters [21] to describe the growth of *C. reinhardtii* (Fig. 5). For these parameters a significant pH dependence was revealed. As illustrated in Fig. 5a, the chlorophyll concentration increases during the last 24 h before harvesting. This effect is stronger during the last light phase (time 0–12 h) and is less pronounced during darkness (time 12–24 h). Overall, there is a tendency to increase the chlorophyll concentration with pH becoming more alkaline; 2.3 μg chlorophyll/ml at pH 6.0, 2.3 μg/ml at pH 6.8 and 2.7 μg/ml at pH 8.0 were determined immediately before harvesting. In spite of the same cell densities, differences of up to 18% in the chlorophyll concentration occur. Fig. 5b shows the glucose to chlorophyll ratio during cultivation at different pH values, reflecting the ability of cells to use light efficiently for the photosynthetic CO_2 fixation. The amount of starch, measured as glucose to chlorophyll ratio, increases during light exposure in all cultures, but the values which were reached before entering the last dark phase differ up to 60% with pH. The more acidic the medium, the higher the

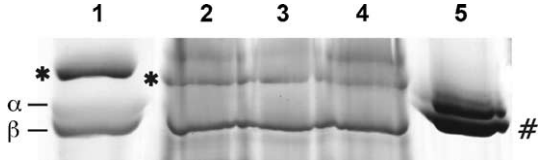

Fig. 4. Apparent molecular mass of oligomer III from *C. reinhardtii* grown with acetate and at high light intensity at different pH (lane 2: pH 8.0; lane 3: pH 6.8; lane 4: pH 6.0). Spinach oligomer III (lane 1) and the c cylinder of *I. tartaricus* with its characteristic double band (lane 5) are shown for comparison. The oligomer III bands are marked by * and the oligomer c of *I. tartaricus* is marked by #. Protein bands on the SDS-gradient-PAGE (7–15%) are silver-stained.

ratios of starch to chlorophyll are. During the last dark phase the starch was metabolized. It is worth mentioning that this reduction in starch content does not occur at pH 8.0. To summarize the data obtained, increasing pH values led to higher chlorophyll concentrations but lower starch to chlorophyll ratios, and remarkable deviations in the chlorophyll and chlorophyll to starch content of the cells were detected. The reason for this unexpected observation is still unknown but starch accumulation in cultures grown at pH 8.0 may indicate a reduced cell growth [21]. Although the external pH of the culture influences the metabolic state of the algae cells, the apparent molecular masses of the oligomer III isolated at these three pH conditions were identical (Fig. 4).

3.3. A homogeneous oligomer III stoichiometry

After demonstrating the dominance of one distinct oligomer III stoichiometry over a wide range of energetic conditions within *C. reinhardtii*, the existence of alternative stoichiometries in minor quantities had to be excluded. This was done by Western blot analysis with antibodies raised against the chloroform extracted monomer III from *S. oleracea* L. (Fig. 6). All samples analyzed contained just one oligomer III protein band and one of the monomeric subunit III. It is unlikely that the parameters chosen are unsuitable to solubilize different oligomer III cylinders or lead to the dissociation of them except for one defined stoichiometry. Hence, the existence of different stoichiometries of oligomer III even in minor quantities within *C. reinhardtii* can be excluded for the growth conditions tested.

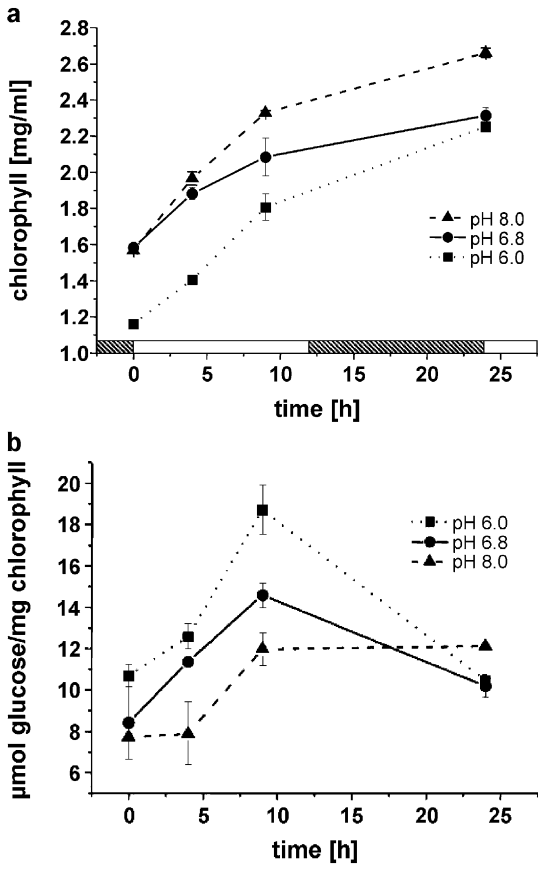

Fig. 5. Variation in the concentration of chlorophyll (a) and of starch, measured as glucose to chlorophyll ratio (b) during the last 24 h before harvesting in response to the different pH values of the culture medium. Mean and standard deviation of three measurements. The horizontal bar indicates the dark (hatched) and light (white) cycle during cultivation.

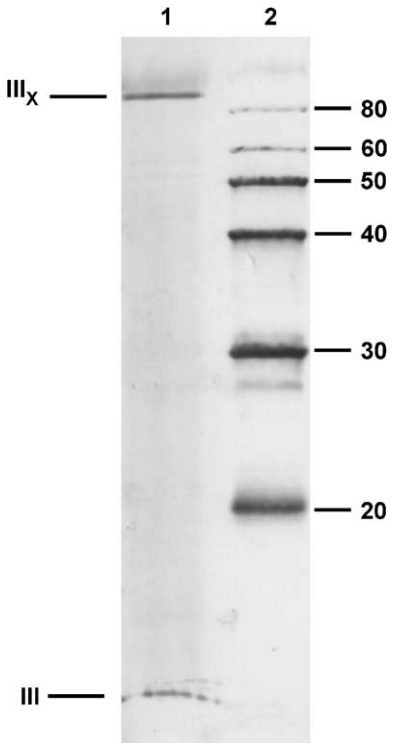

Fig. 6. Immunoblot of the chloroplast ATP synthase oligomer III from *C. reinhardtii*. Solubilized ATP synthase (lane 1) and a molecular weight marker (kDa) (lane 2) were transferred to a PVDF membrane and then incubated with polyclonal antibodies raised against the chloroform extracted monomer III from spinach.

4. Discussion

The aim of the present study was to elucidate if, and to which extent, the metabolic state of a plant cell affects the number of subunits III in the proton turbine of the chloroplast ATP synthase, and thereby the efficiency of energy conversion. For that purpose, the enzyme was isolated from algae cells grown under a variety of different culture conditions. As depicted above, the different growth conditions significantly altered the metabolic state of the cells in which the ATP synthase was assembled. The III oligomers, obtained from *C. reinhardtii* at different metabolic states, were compared concerning their migration behavior in gradient SDS gels to reveal any possible alteration in the oligomer stoichiometry. Since any alteration leads to changes in the molecular mass and the diameter of the III cylinder, it will cause a significant change of the RF

value of the oligomer band within the gradient gel system used.

The observed absence of variability of the subunit III stoichiometry in response to the different cell energetics cannot be attributed to the performance of the gel system used. As demonstrated, it provides sufficient resolution power to distinguish oligomers consisting of different numbers of subunits (see Figs. 3 and 4). The gradient gel system not only clearly separates the spinach oligomer III, with a protein mass of 112 kDa (14×8002.4 Da), from the *I. tartaricus* oligomer c, with a protein mass of 96.7 kDa (11×8790.71 Da) [10], but also distinguishes the pure oligomer c, corresponding to the lower band in lane 4, Fig. 3, from an oligomer c fraction including an internal lipid monolayer [30] (upper band in lane 4, Fig. 3).

The results described above unambiguously demonstrate that the stoichiometry of the III cylinder within the ATP synthase of the green algae *C. reinhardtii* remains constant even though the culture conditions, and therefore the metabolic state of the algae, have been altered drastically.

In *E. coli* it was shown after quantification of the c stoichiometry that the ratio of subunits c/b in F_1F_O depends on the given carbon source. This ratio increases when glucose is included within the medium, as opposed to succinate as carbon source. The conclusion drawn is the existence of a mechanism in *E. coli* to regulate the composition of F_1F_O ATPase in response to the metabolic state [18]. Furthermore, in *E. coli* cross-linking experiments of genetically constructed c_3 and c_4 subcomplexes resulted in F_OF_1 complexes with different c stoichiometries but with ATP hydrolysis activity [35].

In contrast to the data obtained for the bacterium *E. coli* based on artificially constructed c subcomplexes [35] or densitometric analysis of the relative proportion of subunits c to b [18], our investigation clearly demonstrates that the stoichiometry of the III cylinder of *C. reinhardtii*, a model organism for higher plants, is not mutable under the growth conditions applied. Our results are solely based on differences in the migration behavior and are independent of the absolute and relative amount of respective subunits. Different pH values, carbon sources, or light intensities do not lead to any variation in the stoichiometry. Thus, no structural adaptation of the enzyme to metabolic states does occur. The stoichiometry remains constant over a wide range of energetic conditions. This fact is in line with observations made by AFM on defect III/c cylinders from spinach and *I. tartaricus* [8]. The diameter of the rotor was found to be independent of the number of subunits missing and equal to that of an intact oligomer. Each transmembrane monomer contains the information on the cylinder size and the stoichiometry. This conclusion was drawn for *E. coli*, too [36].

At present, the divergent migration behavior of oligomer III from *C. reinhardtii* within high resolution SDS gradient gels, when compared to the corresponding oligomer III from spinach, remains puzzling. As the calculated hydropathies of both homologue subunits III are quite similar, and since posttranslational modifications are excluded for both species (Fig. 2), the possibility of a III cylinder consisting of 13 subunits, which fits the migration behavior in SDS gels, has to be considered for *C. reinhardtii*. It is worth mentioning that for the archaebacterium *Methanopyrus kandleri* 13 repeats of the hairpin domain have been found [37].

Atomic force and electron microscopic studies on 2D crystallized oligomers and F_O complexes shall determine the exact stoichiometry in the future.

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